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CENTRAL AMYGDALA CART MODULATES ETHANOL WITHDRAWAL-INDUCED ANXIETY

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CENTRAL AMYGDALA CART MODULATES ETHANOL WITHDRAWAL-INDUCED ANXIETY

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CENTRAL AMYGDALA CART MODULATES ETHANOL WITHDRAWAL-INDUCED ANXIETY

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Cocaine- and amphetamine-regulated transcript (CART), as its name implies, was initially identified as an upregulated transcript in response to psychostimulant administration. Consequently, it has been posited to play a role in psychostimulant abuse and dependence. Spurred on by the finding that a polymorphism in the CART gene was associated with alcoholism, we initiated studies designed to elucidate the role of CART peptide in alcohol dependence. We first investigated the functional significance of CART peptide in alcohol dependence *in vivo* using a CART KO mouse. We found that CART KO mice had a significant decrease in ethanol consumption that could not be attributed to differences in total intake, taste perception, metabolism, or sensitivity to ethanol. *In vitro* we found that CART peptide facilitated NMDA receptor-mediated currents in central amygdala neurons. Given the emerging role of CART peptide in anxiety and stress, we decided to examine basal and stress-induced anxiety behaviors in CART KO mice. Under basal and acute stress conditions, CART KO mice did not differ in anxiety-like behaviors from WT mice; however, in response to a stressor, CART KO mice exhibited a potentiated corticosterone response. Using chronic intermittent ethanol exposure (CIE), we tested CART KO and WT mice for common signs of ethanol dependence including an escalation of volitional consumption and the presence of

withdrawal-induced anxiety. We further investigated glutamatergic neuroadaptations within the central amygdala of CART KO and WT mice following CIE exposure and early withdrawal. CIE increased ethanol consumption and anxiety-like behaviors in mice of both genotypes but to a lower extent in CART KO mice. Electrophysiologically, CIE enhanced spontaneous excitatory postsynaptic currents in both genotypes and decreased the probability of presynaptic release in WT mice only. We believe that these electrophysiological neuroadaptations contribute to the development of ethanol dependence and may mediate withdrawal-induced anxiety behaviors. Overall, these studies indicate a role for CART peptide in alcohol dependence and specifically in modulating ethanol withdrawal-induced anxiety.

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List of Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ARC	arcuate nucleus of the hypothalamus
ACSF	artificial cerebrospinal fluid
aEPSCs	asynchronous excitatory postsynaptic current
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
BEC	blood ethanol concentration
CeA	central amygdala
CET	chronic ethanol treatment
CIE	chronic intermittent ethanol
CART	cocaine- and amphetamine-regulated transcript
CPP	conditioned place preference
CRE	cAMP response element
CREB	cAMP response element binding protein
DOPAC	3,4-dihydroxyphenylacetic acid
DL-AP5	DL-2-Amino-5-phosphonopentanoic acid
DNQX	6,7-dinitroquinoxaline-2,3-dione
DMS	dorsal medial striatum
EPSC	excitatory postsynaptic current
ERK	extracellular regulated kinase
GABA	gamma-aminobutyric acid
HVA	homovanillic acid
HPA	hypothalamic-pituitary-adrenal
icv	intracerebroventricular
ip	intraperitoneal
KO	knockout
LTD	long term depression
LTP	long term potentiation
LORR	loss of righting reflex
mEPSC	miniature excitatory postsynaptic current
NMDA	N-methyl-D-aspartate
NAc	nucleus accumbens
PPR	paired pulse ratio
PVN	paraventricular nucleus of the hypothalamus
PVT	paraventricular nucleus of the thalamus
PKA	protein kinase A
PKC	protein kinase C
sEPSC	spontaneous excitatory postsynaptic current
TTX	tetrodotoxin
VTA	ventral tegmental area
WT	wild type

Chapter 1:
Background

ALCOHOL DEPENDENCE

Definition

"Addiction" is a loosely used term in the popular vernacular for any behavior or vice that is indulged often. Clinically, the term "dependence" is preferred over "addiction." According to the American Psychiatric Association's fourth edition of the Diagnostic and Statistical Manual of Mental Disorders, alcohol dependence can be summarized as:

> A maladaptive pattern of alcohol use, leading to clinically significant impairment or distress, as manifested by three or more of the following seven criteria, occurring at any time in the same 12-month period: 1) tolerance, 2) a withdrawal syndrome, 3) unintended escalations in consumption, 4) inability to abstain, 5) increased time spent preoccupied with or seeking alcohol, 6) neglecting social or professional obligations to use alcohol, and 7) continued use in spite of adverse consequences caused by the alcohol use.

With this definition in mind, the terms addict and alcoholic may be used in this chapter to mean any person that is dependent on alcohol. Alcohol dependence can arise from persistent alcohol use alone (rarely), but more often than not other factors are involved. It is estimated that up to 60% of addiction liability or predisposition to

addiction is inherited (Hiroi and Agatsuma, 2005). Indeed, a number of heritable characteristics have been found to positively correlate with or predispose someone to the development alcohol dependence. These characteristics can include novelty seeking, impulsivity, aggression, early onset of drinking alcohol, and poly drug use (Basiaux et al., 2001; Bohman, 1996; Sigvardsson et al., 1996). In fact, these latter characteristics are often used to define a subset of alcoholics referred to as Type II alcoholics. Type I alcoholics are characterized as having few antisocial behaviors, low impulsivity, and a late onset of drug use (Hiroi and Agatsuma, 2005). It has been shown that over 51% of Americans (18 years and older) reported using alcohol regularly (CDC, 2012). Further, of those in the 18-24 age group, approximately one-third meet the criteria for alcohol dependence (NIAAA, 2005). These numbers are staggering and reflect a growing problem in our society.

Societal impact

Alcohol abuse and dependence, as well as, complications from alcohol abuse and dependence make up the third leading cause of death in the United States; in fact, recent estimates from the CDC found that an average of 79,000 deaths and over two million years of potential life in the United States were lost every year (CDC, 2004). When confronted with such great numbers, it is easy to overlook that each one of those deaths was someone's child, sibling, or parent. It is also easy to overlook the personal impact that a loved one's death can have on his or her surviving family and friends. While the enormity of these numbers seems unfathomable, they serve to illustrate the enormity of the problem that is alcohol dependence, but fall short of illustrating the personal impact that alcohol abuse and dependence can have on society.

There are additional health issues besides loss of life. Heavy alcohol use can lead to physical impairment and increased engagement of risky behaviors. This combination often leads to physical injury. Furthermore, long-term alcohol use has been associated with a number of serious health issues including alcoholic hepatitis and liver cirrhosis (Schiff, 1997), various cancers (notably, mouth, throat, and esophagus) (Baan et al., 2007), psychiatric issues (Castaneda et al., 1996), and coronary heart disease and stroke (Rehm et al., 2003). Taken altogether, it is clear that alcohol abuse and dependence have significant human, personal, health, and societal impacts that warrant greater resource allocation to finding effective treatments.

Current treatment strategies

The current FDA-approved pharmacological treatment options for alcohol dependence are few and include acamprosate, disulfiram, and naltrexone. Given the limited effectiveness of these compounds, additional treatments are needed. Off-label use of FDA-approved drugs is the common practice or prescribing a medication for an unapproved indication. Off-label use of other drugs (e.g. baclofen, topiramate, vareniclene) to treat alcohol dependence has yielded some promising results but these drugs are largely not yet proven to be effective in clinical trials specifically aimed at treating alcohol dependence.

The mechanism of action for each of the FDA-approved compounds varies widely. Acamprosate (Campral), for example, is a GABA analogue that was approved by the FDA in 2004. It has been found to decrease drinking frequency and relapse episodes in alcoholics (Paille et al., 1995). Disulfiram (Antabuse), approved in 1951, is an inhibitor of acetaldehyde dehydrogenase. Under normal conditions, disulfiram is without

any significant adverse physiological effects; however, when alcohol is consumed while disulfiram is present, the inhibition of acetaldehyde dehydrogenase results in the accumulation of acetaldehyde and severe illness ensues. The treatment strategy for disulfiram is based on the idea of punishment. Naltrexone (Revia, Vivitrol), an opioid receptor antagonist, was approved by the FDA in 1994 for the treatment of alcohol dependence (Brunton et al., 2006). It is has been shown to reduce craving for alcohol and though an exact mechanism is still not known, it is thought to participate in the modulation of the mesolimbic dopamine system (Anton et al., 2006).

The most commonly used off-label medications have mechanisms of action just as diverse as the FDA-approved drugs. Baclofen (Lioresal, Kemstro), a GABA-B receptor agonist, was initially used neurologically for the treatment of spastic movement disorders & amyotrophic lateral sclerosis symptoms (Brunton et al., 2006). Research in rodents and anecdotal case reports (including a popular autobiography by a baclofen-assisted, recovering alcoholic and cardiologist, Olivier Ameisen, M.D., *The End of My Addiction*) prompted wider study of the effectiveness of baclofen in Europe (Ameisen, 2008). A one year, open study that followed over 100 patients found that baclofen was effective in suppressing cravings for alcohol (Rigal et al., 2012). These results led to the provisional approval of baclofen by the French Agency for the Safety of Health Products for the treatment of alcoholism on a case-by-case basis in 2012. A follow-up study one year later found that baclofen was effective in craving suppression (de Beaurepaire, 2013). Topiramate (Topamax) is an anticonvulsant drug approved for the treatment of epilepsy, migraines, and weight loss. It has many actions including inhibition of some voltage dependent ion channels, enhancement of GABA-A receptor-mediated currents, and antagonism of some glutamate receptors (Sommer et al. 2013); unfortunately, which of these effects (if any) is responsible for the abstinence enhancing effect of topiramate is

not known. Vareniclene (Chantix) was approved in 2006 by the FDA for smoking cessation. Its mechanism of action is partial agonism of nicotinic acetylcholine receptors. It was recently found to reduce ethanol consumption in a preliminary clinical trial (Litten et al., 2013). The results are promising, but further trials with more subjects are needed to examine the long term use and effectiveness.

In summary there are several FDA approved and off-label pharmacological options currently available, but their relatively limited efficacy in treating addicts is a significant hurdle. Thus investigation into novel mechanisms of alcohol dependence are required to uncover potential new drug targets for the treatment of alcoholism.

Models for research

Given the great societal cost, research aimed at treating ethanol dependence is essential. To that end, numerous models of ethanol dependence have been developed for basic research scientists studying ethanol dependence. Two of the most popular are chronic ethanol treatment (CET) and chronic intermittent ethanol (CIE). Both models utilize a vapor inhalation method as the route of delivery.

CET was developed in and has been used since the late 1970s by a number of research groups. Until very recently, CET was considered the standard for ethanol dependence induction models. CET consists of continuous ethanol vapor exposure selected to yield a specific blood ethanol concentration. The exposure period can vary in length, but is generally a minimum of two weeks. This model has been found to stably increase voluntary ethanol consumption following withdrawal.

Despite the success of the CET model, a continuous exposure does not really parallel consumption in humans. To address this, Becker and Lopez (2004) began

interspersing withdrawal periods into the CET model. Eventually they came across a combination of ethanol vapor exposure and withdrawal that resulted in an even greater increase in ethanol consumption in dependent mice in a shorter time than that obtained with the CET model (Becker and Lopez, 2004; Lopez and Becker, 2005). The CIE model has since become the standard, not only because it produces a more drastic effect in one-half of the time, but also because it is thought to more closely mirror human patterns of ethanol consumption by mimicking "binge" drinking.

Anxiety, stress, and alcoholism

The progression of alcohol dependence is often summarized with the phrase: use, abuse, and dependence. The "use" phase is characterized by drug seeking for its pleasurable effects. The "abuse" phase reflects heavy usage of the drug that eventually leads to dependence. Abstinence from drug use after dependence is made difficult by a number of factors including withdrawal symptoms. In particular, withdrawal-induced anxiety is thought to play a role in mediating relapse behaviors after periods of abstinence.

Alcohol dependence and anxiety are postulated to be interconnected; in fact, anxiety and stress are often listed as contributors to relapse in treatment seeking addicts (Brown et al., 1995; Sinha et al., 2011). Furthermore, several studies have correlated a predisposition to anxiety disorders and subsequent substance abuse/dependence to drugs of abuse, including alcohol (Bekman et al., 2013; Cowley, 1992; Cox et al., 1990; Kushner et al., 2000a; Kushner et al., 2000b; Quitkin et al., 1972).

One of the neurobiological effectors of anxiety (including withdrawal-induced anxiety) is thought to be the central amygdala (CeA). Numerous studies have indicated

the importance of this brain region in alcohol dependence and withdrawal-induced anxiety (Dandekar et al., 2008a; Le et al., 2001; Moller et al., 1997; Pandey et al., 2003; Roberto et al., 2004a; Roberto et al., 2004b). One of the most convincing of these studies found that lesions of the CeA, but not the basolateral amygdala, resulted in reduced anxiety behaviors and voluntary ethanol consumption (Moller et al., 1997).

THE CENTRAL AMYGDALA

Anatomy, connectivity, and role in anxiety

The central amygdala is one of five nuclei (medial, lateral, basal and basolateral being the other four) comprising the amygdaloid complex (Best, 1990; LeDoux, 1992). The neurocircuitry of the amygdala is such that inputs from thalamus and sensory systems (olfactory, visual, auditory, nociceptive, etc.) innervate primarily the lateral and basolateral amygdala (BLA), but also the CeA (Price and Amaral, 1981). The environmental information is processed and associated with somatic responses (e.g. pain) or reinforcing events (i.e. pavlovian conditioning stimuli). The primary output of the amygdala nuclei is the CeA, which as mentioned previously receives afferent inputs from within the amygdala (basolateral & medial nuclei) and somatosensory systems (Price and Amaral, 1981). A simplified diagramitization can be found in illustration 1. Not surprisingly, this arrangement places the central amygdala in a key role for the emotional processing of events, as well as, a mediator or relay for a number of physiological processes (Best, 1990; Davis, 1992b; LeDoux, 1992).

"Negative" emotions like anxiety, stress, and fear have profound effects on animals with easily observed/studied stereotypic responses; hence, most research in the

CeA has focused on the behaviors in the "negative" emotional spectrum. The CeA projects to several brain regions involved in emotional behaviors (including anxiety, stress, and fear). The following list includes some of the CeA projections with the associated physiological functions in parentheticals: the lateral hypothalamus (tachycardia, pupil dilation), the motor nucleus of the vagus (urination, defecation), the midbrain central gray (freezing behaviors, social interaction, hypoalgesia), the paraventricular nucleus of the hypothalamus (corticosteroid release, stress responses), the locus coeruleus (arousal), and the substantia nigra (attention allocation) (Hopkins and Holstege, 1978; Davis, 1992a; LeDoux et al., 1988; Price and Amaral, 1981; Smith et al., 2013).

The central amygdala also has a major output pathway to the bed nucleus of the stria terminalis (BNST) and the nucleus accumbens (NAc) shell (Hopkins and Holstege, 1978). Collectively, these three regions are sometimes referred to as the extended amygdala complex because of their shared neuronal morphology and circuitry similarities (Heimer and Alheid, 1991; Hopkins and Holstege, 1978). The extended amygdala complex, as a functional unit, has been implicated not only in emotional processing of stimuli but in drug addiction as well (Koob et al., 2013).

Given its neuroanatomical connectivity, the CeA is poised to mediate negative emotional states, including anxiety, and stress responses (Davis, 1992b). In fact, it has been shown that lesions of the CeA, but not the BLA, result in reduced anxiety behaviors (Moller et al., 1997). Furthermore, site specific injections of the neuropeptide cocaine- and amphetamine-regulated transcript (CART) peptide into the CeA, but not the BNST or NAc, also increased anxiety-like behaviors (Dandekar et al., 2008a). This finding is not unique to rodents. Studies in primates (rhesus monkeys) have also found that specific

lesioning of the CeA results in a reduction of anxiety-related behaviors (Kalin et al., 2004).

Neuroadaptations in alcohol dependence

Within the CeA, numerous neuroadaptations take place over the course of dependence induction. These include changes in glutamatergic and GABAergic neurotransmission (Roberto et al., 2006; Roberto et al., 2004a; Roberto et al., 2004b). GABAergic neurotransmission in the CeA was found to be increased in ethanol dependent rats relative to control rats (Roberto et al., 2004a). Interestingly, (Roberto et al., 2004a) glutamatergic neurotransmission and NMDA receptor expression were also found to be increased in rats exposed to the same chronic ethanol treatment paradigm (Roberto et al., 2006; Roberto et al., 2004b). In ethanol naïve rats, ethanol was found to enhance GABA neurotransmission (Roberto et al., 2003). This finding, when taken together with the ability of ethanol to inhibit NMDA receptors (Lovinger et al., 1989; Lovinger et al., 1990), supports the allostasis hypothesis that the enhancement of glutamatergic neurotransmission and NMDA receptors is a responsive adaptation to the chronic inhibitory effects of ethanol at NMDA receptors (Lovinger et al., 1989) and ethanol facilitated GABA neurotransmission in alcoholics. However, upon removal of ethanol inhibition of NMDA receptors and ethanol-facilitated GABAergic neurotransmission (during abstinent periods), there is a net increase in excitatory glutamatergic transmission. This increase in glutamatergic neurotransmission may contribute to the expression of ethanol withdrawal-induced anxiety. A possible compensatory mechanism for this increased excitatory signaling might be the increased GABAergic inhibitory signaling observed over the same period of time (Roberto et al.,

2004a). This effect included increased basal release of GABA, as well as, enhanced inhibitory synaptic currents (Roberto et al., 2004a).

COCAINE- AND AMPHETAMINE-REGULATED TRANSCRIPT (CART)

Discovery, regulation, and anatomical distribution

Cocaine- and amphetamine-regulated transcript (CART) was initially identified by PCR differential display as an mRNA species that was upregulated in the striatum of rats in response to treatment with cocaine or amphetamine (Douglass et al., 1995). Though somewhat controversial (Marie-Claire et al., 2003; Vrang et al., 2002), this general finding that psychostimulant treatment increases CART mRNA & peptide is widely accepted and has been repeated a number of times and under varying conditions (Brenz Verca et al., 2001; Fagergren and Hurd, 1999; Hunter et al., 2005). Work by (Thim et al., 1999) extracted and sequenced several CART peptides from several brain and endocrine regions; they speculated that several of these species were intermediate processing products and that there were in fact only two bioactive forms of CART: CART 42-89 and 49-89. These fragments are more commonly known as CART 62-102 and 55-102. Interestingly, only the former, shorter CART peptide is found in humans. Though this study is widely considered the first to discover CART peptides, a study by (Spiess et al., 1981) identified a "somatostatin-like polypeptide" from ovine hypothalamus that would later be identified as a proCART species.

CART is highly conserved across multiple vertebrate species including a number of fish, porcine, bovine, rodent, amphibian, avian, and canine species. The human (and rodent) CART gene encodes for three exons (with two introns) and is localized to

chromosome 5q13-14 in humans (Douglass and Daoud, 1996) and chromosome 13 in mice (Adams et al., 1999). There is a greater than 90% homology between the human and rat CART genes at the nucleotide and amino acid sequences (Douglass and Daoud, 1996). There are a number of known transcription factor binding sites located in the promoter region of the CART gene including elements for CRE, estrogen receptors, STAT, and the AP-1 complex (Dominguez, 2006; Dominguez et al., 2002). Notably, the CRE sequence form the CART gene was found to bind CREB and is thought to be the effector of psychostimulant induced increases in CART transcription (Barrett et al., 2002; Rogge et al., 2009; Xu et al., 2006).

CART peptides are widely distributed throughout the brain. They are generally expressed most prominently, however, in brain regions associated with feeding and energy homeostasis. CART was first identified in the striatum of psychostimulant treated rats (Douglass et al., 1995). Subsequent studies of its anatomical distribution using immunohistochemistry found the distribution of CART to be much more widespread and included several hypothalamic nuclei, the pituitary gland, and the adrenal medulla (Elias et al., 2001; Koylu et al., 1997). A subsequent study from the same group using *in situ* hybridization found the distribution of CART to be even more wide spread than previously thought and demonstrated the presence of CART in the retina, spinal cord, olfactory bulbs and somatosensory cortex (Couceyro et al., 1997). These findings were confirmed and extended by a third study emphasizing the presence of CART in forebrain structures including the nucleus accumbens, thalamus, hippocampus, and the central, medial, and basolateral amygdala (Koylu et al., 1998). Subsequent studies have also localized CART peptides in the midbrain (Dallvechia-Adams et al., 2002) and sympathetic ganglia (Dun et al., 2000). The widespread distribution of CART peptide

throughout the brain foreshadows the many functions that CART peptides have been found play physiologically.

Evidence for CART as a neurotransmitter and the putative CART receptor

The basic requirements for a protein to be classified as a neurotransmitter include the following: the identification of its synthetic pathway, its presence in presynaptic vesicles, a postsynaptic effect, and the identification of receptor (Cooper et al., 2003). The synthetic pathway for CART peptides has been well characterized and begun with the identification of the CART gene (Dominguez, 2006; Douglass and Daoud, 1996). After transcription and translation, the nascent preproCART is then designated for the secretory pathway by a 27 amino acid signal on the N-terminus (Douglass and Daoud, 1996; Douglass et al., 1995). Several studies examining the ultrastructural localization of CART peptides using scanning electron microscopy have demonstrated that CART peptides are indeed present in presynaptic terminals and in large dense-core vesicles (Blanco et al., 2013; Smith et al., 1999; Smith et al., 1997). CART peptides have also been shown to have varied postsynaptic effects. Among the first studies to demonstrate a specific cellular effect of CART peptides was (Yermolaieva et al., 2001), who demonstrated that CART peptide could modulate the function of L-type voltage-gated calcium channels in the hippocampus. Subsequent studies have found that CART peptides enhance the phosphorylation state of MAP kinase proteins (ERK 1 and6 2, specifically) (Lakatos et al., 2005), as well as, serine residues 896 and 897 of the NR1 subunit of the NMDA receptor (Chiu et al., 2009; Chiu et al., 2010). The final criterion, however, has proven more difficult. Several research groups and studies have attempted to identify the CART receptor (see Vicentic et al. (2006) for review) without success;

however, there is some evidence to suggest the existence of a specific CART receptor. For example, specific CART peptide binding has been demonstrated in AtT20 cells (Vicentic et al., 2005), PC-12 cells (Maletinska et al., 2007), acutely prepared brain slices containing the hypothalamus (Keller et al., 2006), and in primary cultures of nucleus accumbens neurons (Jones and Kuhar, 2008). Further studies have found that the putative CART receptor is likely a G-protein coupled receptor of the Gi/o family, as demonstrated by the pertussis toxin-sensitivity of CART peptide effects including CART peptide-induced phosphorylation of ERK (Lakatos et al., 2005), CART peptide modulation of L-type voltage-gated calcium channels (Yermolaieva et al., 2001), and CART peptide-mediated inhibition of FSH effects in granulosa cells (Sen et al., 2007). Some studies have postulated the existence of more than one CART receptor. This idea is based on the different binding and relative potencies of the CART peptides in rodents, as well as, some minor variation in the distribution of the two bioactive CART peptides (Thim et al., 1999). Evidence supporting this idea includes the finding that CART 55-102 & 62-102 differentially modulate several behaviors including the acoustic startle response, inhibition of feeding, and prepulse inhibition (Bannon et al., 2001). Taken all together, these studies strongly support the notion that CART peptides are indeed unique neurotransmitters and the existence of at least one putative CART receptor.

Interactions with anxiety and stress

CART has been repeatedly demonstrated to have anxiogenic properties (for review see Stanek (2006)). In rodents, CART peptides were found to dose-dependently decrease open arm times in an elevated plus maze paradigm (Asakawa et al., 2001; Kask et al., 2000). CART peptides administered icv have also been shown to increase anxiety

as measured by a decrease in social interaction time in rodents (Chaki et al., 2003). These results demonstrate the direct anxiogenic properties of CART peptides. A more recent study, however, has demonstrated that site-specific injections of CART peptide into the central amygdala, but not the NAc or bed nucleus of the stria terminalis (BNST), produced increases in anxiety-like behaviors of rats (Dandekar et al., 2008a). This study further correlated an increase in CART peptide expression in the central amygdala during acute withdrawal with increases in anxiety-like behaviors. Further studies of CART peptides and anxiety found that icv injection of CART peptides could increase plasma levels of corticosterone (Smith et al., 2004).

Acute, but not chronic, restraint stress treatment was shown to increase CART mRNA in the central amygdala of male rats. Interestingly, this study also found that chronic restraint stress did increase CART mRNA in the dentate gyrus (Hunter et al., 2007). A related study found that female rats exposed to a forced swim test had a greater number of CART peptide containing cells in the PVN than non-stressed control rats (Gozen et al., 2007).

CART, nociception, and the glutamate system

Given its high level of expression in the spinal cord and specifically in the dorsal horn laminae (Dun et al., 2000; Koylu et al., 1998; Kozsurek et al., 2007; Ohsawa et al., 2000), a putative role for CART in the mediation of pain was postulated. Indeed, (Bannon et al., 2001) found that icv administration of CART peptides dose-dependently increased the paw lick latencies of mice in the hot-plate assay, implying an antinociceptive effect of CART peptides. This basic antinociceptive effect of icv CART peptide was replicated in mice using the formalin paw lick assay (Damaj et al., 2003). It

was further shown that intrathecal CART peptide administration could potentiate the antinociceptive actions of morphine in a tail flick latency test, which measures the latency of rodents to flick their tail away from a heat source (Damaj et al., 2004). This same group extended their previous findings by demonstrating that intrathecal CART peptide administration attenuated hyperalgesia and allodynia (the reversal of pain due to a stimulus that would normally not provoke pain) via an opioid-independent mechanism in a mouse model of neuropathic of pain (Damaj et al., 2006).

Despite all of the evidence indicating an anti-nociceptive role for CART peptide, there exists literature indicating a nociceptive/hyperalgesic role for CART. It was reported that intrathecal administration of CART peptide decreased the latency of hind paw withdrawal from a heat source, suggesting that CART peptide increases sensitivity to heat or pain from the heat source (Ohsawa et al., 2000).

Given the important role that NMDA receptors play in spinal cord nociceptive transmission, it was hypothesized that CART peptide may be interacting with NMDA receptors to regulate nociceptive transmission (Hsun Lin et al., 2005). Indeed, this group found that administration of CART peptide potentiated the ability of intrathecal NMDA, but not AMPA, to increase pain sensitivity; furthermore, when they examined the electrophysiological effect of CART peptide on NMDA- or AMPA-induced depolarizations in substantia gelatinosa neurons, they found that CART peptide specifically potentiated NMDA-induced depolarizations while having no effect on AMPA-induced depolarizations (Hsun Lin et al., 2005). Following up on this report, another research group confirmed the NMDA receptor potentiating effect of CART peptide on sympathetic preganglionic neurons in spinal cord slices; briefly, they iontophoretically applied NMDA in a whole-cell voltage clamp configuration before and after bath application of CART peptide and found that NMDA receptor-mediated

currents were enhanced after application of CART peptide (Dun et al., 2006). This finding was extended by subsequent studies showing that the mechanism of CART peptide enhancement of NMDA receptor-mediated transmission was attributable to increases in the phosphorylation of serine residues 896 and 897 of the perquisite NR1 subunit of NMDA receptors (Chiu et al., 2009). They further showed that this effect was age-dependent (i.e. CART peptide could not enhance NMDA receptor mediated currents or the phosphorylation of NR1 residues ser896 and ser897 in spinal cord slices prepared from mice less than three weeks of age) and that pretreatment of slices with inhibitors of PKA or PKC (targeting residues ser897 and ser896, respectively) attenuated these effects (Chiu et al., 2010).

CART peptides and dopamine

CART peptides have been shown to have substantial interactions with psychostimulants and the brain dopamine systems. Given its initial discovery as an upregulated transcript in response to cocaine or amphetamine (Douglass et al., 1995) and its altered gene expression in human cocaine abusers (Albertson et al., 2004) and overdose victims (Tang et al., 2003), CART has been the focus of several studies investigating psychostimulants and addiction (see Hubert et al. (2008) for review).

Anatomically, CART peptides are present throughout the mesolimbic dopamine system. In the VTA, CART peptide containing synaptic terminals have been observed synapsing onto dopamine neurons and GABA containing interneurons (Dallvechia-Adams et al., 2002; Koylu et al., 1998). In medium spiny neurons of the NAc, CART peptides have been observed to colocalize with prodynorphin and dopamine D1 receptors, both markers for the direct pathway (Hubert and Kuhar, 2006). Additional

studies on CART peptide localization in the NAc have shown that CART peptides are expressed in substance P-expressing GABAergic neurons that receive a dopamine input (Dallvechia-Adams et al., 2002; Hubert and Kuhar, 2005; Koylu et al., 1998; Smith et al., 1997; Yang et al., 2005). CART peptides have also been observed in non-mesolimbic dopamine systems including the arcuate nucleus of the hypothalamus (ARC) and the paraventricular nucleus of the thalamus (PVT) (Elias et al., 2001; Parsons et al., 2006).

Given the common propensity of cocaine and amphetamine to increase dopamine release in the nucleus accumbens (Di Chiara and Imperato, 1988; Weiss et al., 1993), it was postulated that the psychostimulant-induced increases of CART peptides may be dopamine receptor-dependent. Indeed, modulation of dopamine D3 receptors with either antisense oligonucleotides, dopamine D3 receptor knockout mice, or antagonists were found to modulate CART expression in the NAc (Beaudry et al., 2004; Hunter et al., 2006). The interactions of dopamine and CART, however, are not one-sided. CART peptide administration (icv) has been found to increase the levels of DOPAC & HVA (dopamine metabolites) in the shell of the NAc (Shieh, 2003; Yang et al., 2004). Intra-VTA administration of CART peptide was also found to increase dopamine efflux in the NAc (Kuhar et al., 2005). Furthermore, intra-VTA injections of CART peptide were found to produce an increase in locomotor activity that was blocked by pretreatment with the dopamine D2 receptor antagonist, haloperidol (Kimmel et al., 2000). Additionally, intra-VTA injections of CART peptide could also produce a conditioned place preference (Kimmel et al., 2000). Administration of psychostimulants typically results in increases in locomotor activity, however, when CART peptide is microinjected into the NAc before systemic psychostimulant administration, psychostimulant-induced increases in locomotor activity are significantly attenuated (Jaworski et al., 2003; Kim et al., 2003). These studies suggest a role for CART in modulating aberrant increases in dopamine

signaling in the NAc in response to drugs of abuse (Hubert et al., 2008; Jaworski and Jones, 2006; Jaworski et al., 2003; Kim et al., 2003).

Other studies examining extra-mesolimbic dopamine systems have posited a role for CART peptides. Briefly, it is has been shown that CART containing neurons of the ARC project to and innervate neurons of the PVT (Kirouac et al., 2006). These neurons subsequently project to the NAc shell (Parsons et al., 2006). Stimulation of glutamatergic afferents originating in the PVT have been shown to increase dopamine release in the NAc, presumably by stimulating dopaminergic presynaptic terminals (Parsons et al., 2007). It is therefore believed that increased CART peptide expression and release in response to drug-induced aberrant dopamine signaling might inhibit PVT glutamatergic afferents to the NAc. Supporting this hypothesis, a study by James et al. (2010) found that microinjections of CART peptide into the PVT significantly decreased the number of active lever presses for cocaine in a drug-primed model of reinstatement.

All together, these lines of evidence strongly suggest that CART peptides interact with and regulate brain dopamine systems in a meaningful way and that aberrations in CART peptide function in the brain may contribute to addiction processes.

CART knockout mice

Two separate mouse strains have been developed and used for studies of addiction (see Moffett et al. (2006) for review). The consensus is that CART KO mice have a reduced propensity to consume and respond for drugs of abuse including ethanol. One group, however, found no genotype differences in cocaine-induced locomotor activity or self administration (Steiner et al., 2006). This finding is in direct contrast to a substantial study by Couceyro et al. (2005) which found that CART KO mice self administered less

cocaine than WT mice. This study further found reductions in a variety of psychostimulant related behaviors including rearing, grooming responses, locomotor sensitization, and conditioned place preference in CART KO mice. A third study (Moffett et al., 2006) found that CART KO mice had a reduction in cocaine-, but not amphetamine-induced locomotor activity relative to WT mice. Similarly, Salinas et al. (2012) found that CART KO mice displayed a reduction in ethanol consumption and preference that could not be attributed differences in total intake, taste preference, ethanol metabolism, or sensitivity.

Overall, the consensus is that CART KO mice display a reduction in the reinforcing properties of ethanol and psychostimulants. When taken together with the rest of the CART literature apropos drugs of abuse and addiction, one can presume that CART is critically involved in mediating the reinforcing properties of drugs of abuse.

CART peptides and alcoholism

The number of studies examining CART peptides and alcoholism are far fewer than those for psychostimulants. This is surprising, given that a direct relationship between a CART polymorphism in intron 1 of the CART gene was strongly associated with alcoholism in a population of Korean males (Jung et al., 2004). Interestingly, this association was unique to alcoholism and did not correlate with bipolar or schizophrenia, two disorders with high comorbidity with alcohol abuse. Spurred by this finding, our lab examined the effect of ethanol administration on CART mRNA and peptide levels in the NAc. Similar to findings with psychostimulants, we found that ethanol dose-dependently increased CART mRNA and peptide levels (Salinas et al., 2006). We further found that pretreatment with either a dopamine D1 or D2/D3 receptor antagonist attenuated the

ethanol-induced enhancement of CART mRNA. These findings were largely in accord with the literature examining CART and psychostimulants. Another group found that exposing rats to stimuli previously associated with access to ethanol activated CART peptide-containing neurons in the ARC (Dayas et al., 2008). Dandekar et al. (2008a) established a role for CART in ethanol withdrawal-induced anxiety with their pivotal study. Briefly, they injected CART peptide into the NAc, bed nucleus of the stria terminalis, and the CeA and found that only injections into the CeA resulted in increased anxiety. They then exposed rats to an ethanol liquid diet for two weeks to induce dependence and proceeded to examine ethanol withdrawal-induced anxiety. Their principal finding was that central amygdala CART peptide levels positively correlated with ethanol withdrawal-induced anxiety. They further found that immunoneutralization of CART in the central amygdala attenuated ethanol withdrawal-induced anxiety. In a related study, this group also found that CART peptide levels were increased in the hypothalamus during withdrawal from an ethanol liquid diet (Dandekar et al., 2008b). Finally, a group from Australia found that icv CART peptide administration blocked context-induced reinstatement behaviors (King et al., 2010). These studies, though few, largely support a role for CART peptides in alcoholism.

HYPOTHESIS AND AIMS

Overall project rationale

It is our contention that CART peptides play a significant role in ethanol dependence. Specifically, we believe that CART peptides mediate, at least in part, ethanol withdrawal-induced anxiety which may contribute to relapse behaviors. Several

lines of evidence to that end include: 1) icv and central amygdala injections of CART peptide were found to be anxiogenic (Chaki et al., 2003; Dandekar et al., 2008a), 2) CART peptide is strongly expressed in the CeA (a key region for anxiety behaviors), 3) CART peptide levels in the CeA are increased during withdrawal from chronic ethanol and correlate positively with withdrawal-induced anxiety (Dandekar et al., 2008a), and 4) immunoneutralization of CeA CART peptide blocked withdrawal-induced anxiety behaviors (Dandekar et al., 2008a). We further believe that CART peptide exerts its anxiogenic effects by altering glutamatergic neurotransmission in the CeA. Chronic ethanol has been shown to result in increased NMDA receptor expression and function in several brain regions, including the CeA (Roberto et al., 2004b; Roberto et al., 2006; Roberto et al., 2004a). This altered function in the CeA is accompanied by a compensatory increase in GABA neurotransmission (Roberto et al., 2004a). The increase in NMDA receptor expression is likely an adaptation to the persistent inhibitory effect of ethanol on NMDA receptors. Upon relief from ethanol (during withdrawal), the GABA system works to inhibit excessive NMDA neurotransmission and produce an anxiolytic effect (Koob and Le Moal, 2001). Concurrent with these actions, CART peptide expression has been found to be increased in the CeA. Given the finding that CART peptide potentiates NMDA receptor-mediated currents in a spinal cord preparation (Dun et al., 2006; Hsun Lin et al., 2005), we believe that by potentiating NMDA receptor-mediated currents in the CeA, CART peptide opposes the anxiolytic effects of GABA and contributes to ethanol withdrawal-induced anxiety and dependence.

Specific aims

Specific aim 1: To investigate the effect of removal of the CART gene on ethanol consummatory behaviors

Given the previous findings that CART KO mice have a reduced propensity to administer cocaine (Couceyro et al., 2005) and reductions in psychostimulant-induced behaviors (e.g. locomotor activity, sensitization, and conditioned place preference) (Couceyro et al., 2005; Moffett et al., 2006), we believe that removal of the CART gene results in altered responses to normally reinforcing substances. We further believe that this change will result in a reduced consumption or preference for ethanol.

Aim 1.1: To determine if CART KO mice will consume or prefer ethanol less than WT mice. We will assess ethanol consumption and preference in CART KO and WT mice at a number of ethanol concentrations using a two bottle choice, unlimited access paradigm.

Aim 1.2: To compare factors known to contribute to ethanol consummatory behaviors between CART KO and WT mice. If a genotype difference in ethanol consumption or preference is observed, we will examine bitter/sweet taste perception, ethanol metabolism and sensitivity to ethanol (using a loss or righting reflex assay). Genotype differences in these factors could account for any observed changes in ethanol consumption.

Specific aim 2: To investigate the effects of CART peptide on glutamatergic neurotransmission in the central amygdala of ethanol naïve mice.

CART has been found to potentiate NMDA receptor mediated currents in spinal cord preparations (Dun et al., 2006; Hsun Lin et al., 2005). I therefore believe that CART will have the same effect on neurons in the CeA. Further, the reported increase in

CART expression in the CeA during ethanol withdrawal (Dandekar et al., 2008a) is likely to have a significant impact on local glutamatergic currents.

Aim 2.1: To determine the effects of CART peptide on AMPA receptor-mediated spontaneous events. Utilizing whole-cell voltage clamp recordings of mEPSCs, we will determine if CART peptide modulates AMPA receptor-mediated currents in a pre- or postsynaptic manner.

Aim 2.2: To determine the effects of CART peptide on AMPA/NMDA ratios. Utilizing whole-cell voltage clamp recordings of paired eEPSCs at -80 mV and +40 mV, we will determine if CART peptide alters evoked AMPA or NMDA receptor-mediated currents and AMPA/NMDA ratios.

Specific aim 3: To investigate the effects of chronic intermittent ethanol on ethanol consumption, ethanol withdrawal-induced anxiety, and glutamatergic neurotransmission in CART KO and WT mice.

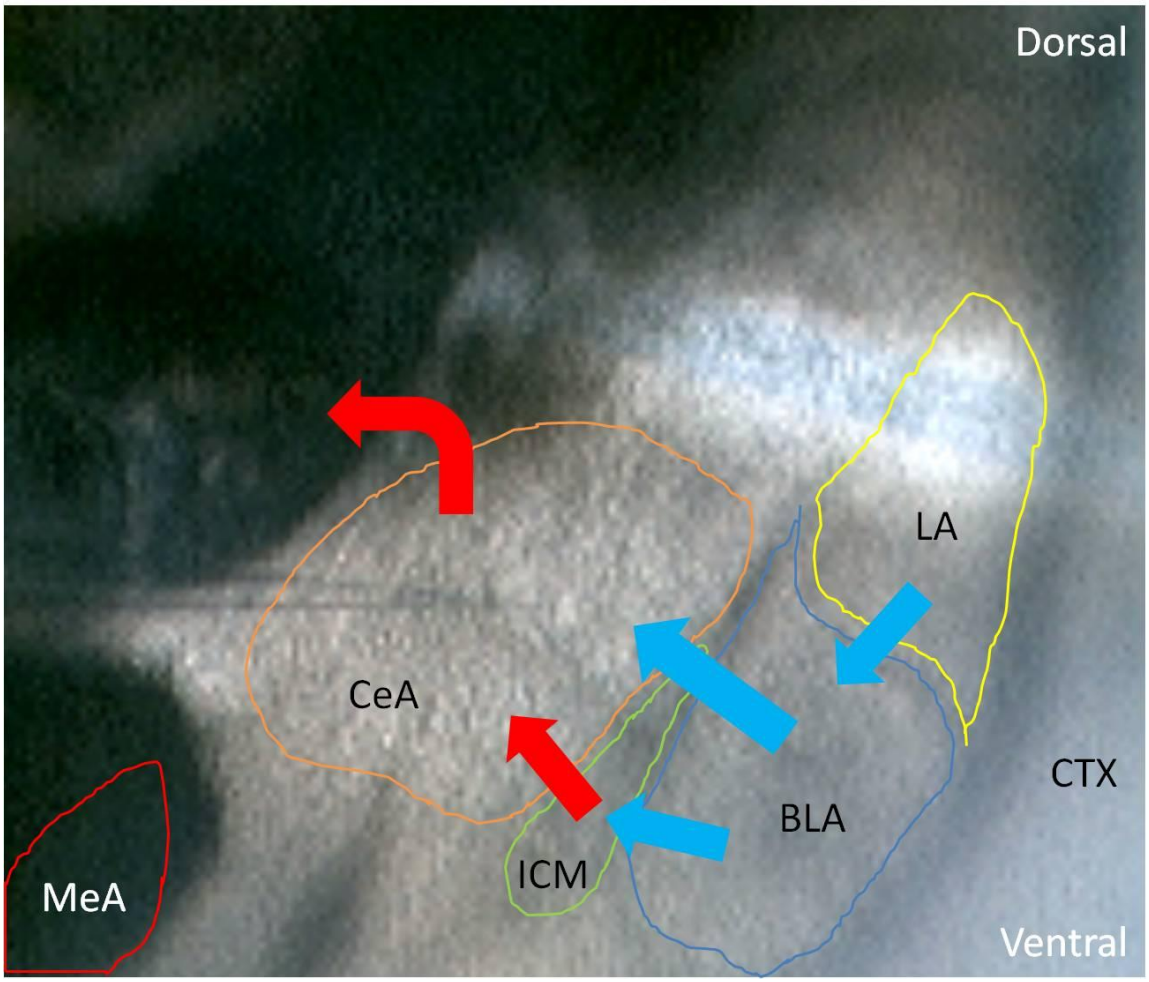
CART has been implicated in ethanol withdrawal-induced anxiety (Dandekar et al., 2008a). That study, however, failed to examine any drinking behavior associated with dependence. To amend this and to expand upon their findings, chronic intermittent ethanol vapor exposure will be used to induce dependence. Ethanol consumption and withdrawal anxiety will be assessed in CART KO and WT mice. Finally, mice from both groups will be used in electrophysiological experiments examining glutamatergic neurotransmission in the central amygdala.

Aim 3.1: To assess the efficacy of chronic intermittent ethanol vapor exposure to enhance ethanol consumption in CART KO and WT mice. The chronic intermittent ethanol vapor treatment model has been well established and is known to induce ethanol dependence (Becker and Hale, 1993; Becker and Lopez, 2004; Lopez and Becker, 2005) including an increase in volitional ethanol consumption in a two-bottle choice paradigm.

Aim 3.2: To assess the effects of withdrawal from chronic intermittent ethanol on anxiety-like behaviors and HPA axis activation in CART KO and WT mice. Chronic intermittent ethanol treated CART KO and WT mice will be tested in the light/dark box assay for anxiety-like behaviors after one and two bouts of chronic intermittent ethanol. Following the second bout, mice will be sacrificed and trunk bloods collected for determining plasma corticosterone content as a second, physiological, measure of anxiety.

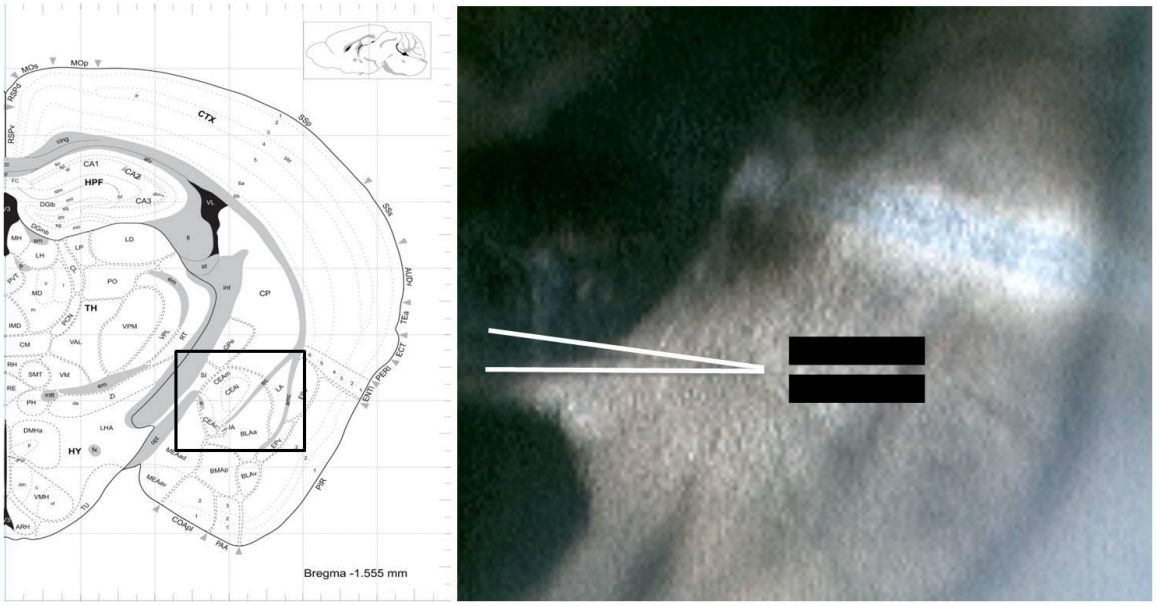
Aim 3.3: To assess the effects of withdrawal from chronic intermittent ethanol on glutamatergic neurotransmission in CART KO and WT mice. Using whole-cell voltage clamp recordings of sEPSCs, AMPA/NMDA ratios, and paired pulse ratios, we will investigate the effects of chronic intermittent ethanol on glutamatergic neurotransmission in CART KO and WT mice.

Illustration 1. Amygdala nuclei and connections



Schematic of basic amygdala microanatomy and connectivity. The blue arrows represent glutamatergic projections. The red arrows represent GABAergic projections. BLA – basolateral nucleus of the amygdala, CeA – central nucleus of the amygdala, CTX – cortex, ICM – intercalated cell mass, LA – lateral nucleus of the amygdala, MeA – medial nucleus of the amygdala. Basal nucleus of the amygdala is not shown.

Illustration 2. Coronal central amygdala section illustrating the typical configuration used in recordings



The white lines and black bars demonstrate the typical placement of the recording and stimulating electrodes, respectively. The black box on the atlas image outlines the approximate are shown in the photograph.

Chapter 2:

Reduced ethanol consumption and preference in cocaine- and amphetamine-regulated transcript knockout mice

ABSTRACT

Cocaine- and amphetamine-regulated transcript (CART) is a neuropeptide implicated in addiction to drugs of abuse. Several studies have characterized the role of CART in psychostimulant addiction, but few have examined the role of CART in alcohol use disorders including alcoholism. The current study utilized a CART knockout (KO) mouse model to investigate the role of CART in ethanol appetitive behaviors. A two-bottle choice, unlimited-access paradigm was used to compare ethanol appetitive behaviors between CART wild type (WT) and KO mice. The mice were presented with an ethanol solution (3%-21%) and water, each concentration for four days, and their consumption was measured daily. Consumption of quinine (bitter) and saccharin (sweet) solutions were measured following the ethanol preference tests. In addition, ethanol metabolism rates and ethanol sensitivity were compared between genotypes. CART KO mice consumed and preferred ethanol less than their WT counterparts in both sexes. This genotype effect could not be attributed to differences in bitter or sweet taste perception or ethanol metabolism rates. There was also no difference in ethanol sensitivity in male mice; however, CART KO female mice showed a greater ethanol sensitivity than the WT females. Taken together, these data demonstrate a role for CART in ethanol appetitive behaviors and as a possible therapeutic drug target for alcoholism and abstinence enhancement.

INTRODUCTION

Cocaine- and amphetamine-regulated transcript is a putative neuropeptide transmitter found in the brain and gut. Initially identified as an up regulated striatal mRNA transcript in response to systemic administration of cocaine or amphetamine (Douglass et al., 1995), CART has been shown to modulate a number of biological processes including appetite regulation, metabolism, nociception, anxiety/depression, and ion channel modulation (see Rogge et al. (2008) for review).

The role of CART in drug reinforcement, while first suggested by its responsivity to psychostimulant administration, is further implicated by its localization to and interactions with numerous brain regions known to be critically important in drug responses (Couceyro et al., 1997; Dallvechia-Adams et al., 2002; Koylu et al., 1997). Furthermore, changes in CART gene expression in human cocaine abusers (Albertson et al., 2004) and overdose victims have been repeatedly observed (Tang et al., 2003). CART prominently interacts with the mesolimbic dopamine system (for review, see Hubert et al. (2008); for instance, icv infusions of CART appear to increase DOPAC and HVA levels in the shell of the nucleus accumbens (NAc) (Shieh, 2003; Yang et al., 2004). Conversely, the expression of CART mRNA in the NAc is directly modulated by dopamine receptor agonists and antagonists (Hunter et al., 2006; Salinas et al., 2006), and behavioral studies suggest that intracranial injections of CART into the ventral tegmental area produce psychostimulant-like increases in locomotor activity (Kimmel et al., 2000) and a conditioned place preference (CPP) (Kuhar et al., 2005). Injections of CART into the NAc following systemic cocaine or amphetamine also blunt the drug-induced increases of locomotor activity (Jaworski et al., 2003; Kim et al., 2003). Taken together, these studies strongly suggest that CART may function to significantly modulate the

mesolimbic dopamine system and thereby contribute to the development or expression of aberrant drug-seeking behaviors.

Two CART KO mice lines have been generated and used to further investigate the role of CART in psychostimulant drug related behaviors. The first line, generated by Asnicar et al. (2001), removed the first two exons of the CART gene and utilized a ES-129/SvJ bacterial artificial chromosome clone injected into 129 SvJ mouse ES cells. The subsequent cells were injected into C57BL6 blastocysts. The resultant male offspring were crossed with C57BL6 wild type females and backcrossed to a C57BL6J background. These mice appeared normal and had no gross physical abnormalities except that at approximately twenty weeks of age their body weights were greater than their wild type counterparts. CART KO mice from the second line generated by Wierup et al. (2005) also displayed an increase in body weight when fed a regular diet except that the differences were not apparent until 40 weeks of age. The Wierup CART KO mouse line was generated by removing all three of the CART gene exons and was maintained on an outbred, black swiss x 129SvJ background. Both of these mouse strains have been used in studies examining responses to psychostimulant drugs of abuse (see Moffett et al. (2006) for review). The results are mixed; one study (Steiner et al., 2006) showed no genotype differences in cocaine-induced locomotor sensitization or cocaine self administration. However, a review by Moffett et al. (2006) which contained new data found a reduction in cocaine-induced, but not amphetamine-induced, locomotor activity in CART KO mice relative to their WT counterparts. A third study comparing CART KO and WT mouse psychostimulant-related behaviors found that CART KO mice had diminished acute amphetamine-induced vertical activity and grooming responses relative to their WT counterparts (Couceyro et al., 2005). The CART KO mice did, however, display an increase in locomotor activity in response to acute amphetamine. With

repeated amphetamine administration, dose-dependent increases in locomotor activity (sensitization) were observed in CART WT, but not CART KO mice. The authors also tested two doses (0.3 and 1.0 mg/kg) of amphetamine in a conditioned place preference (CPP) paradigm and found that CART KO mice failed to acquire a CPP response to the low dose of amphetamine. Finally, Couceyro et al. (2005) also found that cocaine self-administration was reduced in CART KO mice relative to their WT counterparts; WT mice had greater intake of and responding for cocaine at the majority of doses tested. Overall, it appears that the results from the CART KO mouse studies reflect a decrease in the reinforcing/rewarding properties of psychostimulant drugs in CART KO mice. This conclusion is somewhat controversial, though when taken together with the CART literature in WT mice mentioned previously, one can generally conclude that CART is involved in the reinforcing properties of psychostimulant drugs of abuse.

In contrast to the numerous studies which document the role of CART in psychostimulant drug reinforcement, our knowledge of CART in alcohol-seeking behaviors and alcoholism is limited. (Jung et al., 2004) first provided strong evidence of a role for CART in human alcoholism when they documented a correlation between a polymorphism in intron 1 of the CART gene with alcoholism in a population of Korean men. Subsequent work from our lab demonstrated a direct link between ethanol and CART expression. We found that high doses of acute ethanol increase CART mRNA and protein in the NAc in a dose-dependent manner. Furthermore, we demonstrated that this increase is dependent on both D1 and D2/D3 dopamine receptors (Salinas et al., 2006). Work by Dayas et al. (2008) found that exposure to stimuli previously linked with ethanol availability activates CART-containing neurons in the arcuate nucleus as measured by c-fos immunoreactivity. A role for CART in ethanol withdrawal has also been shown in two studies: the first demonstrated that ethanol withdrawal induced

anxiety was partially mediated by increases in central amygdala CART levels (Dandekar et al., 2008a); whereas the second study found that CART levels in specific hypothalamic nuclei were increased during acute withdrawal from 15 days of a liquid ethanol diet (Dandekar et al., 2008b). Furthermore, King et al. (2010) demonstrated that icv infusions of CART inhibit context-induced reinstatement of alcohol seeking behaviors; thereby strongly suggesting a role for CART in mediating the rewarding or motivational properties of ethanol.

The present study sought to continue to directly examine the role of CART in ethanol preference and consumption. Specifically, we examined ethanol appetitive behaviors in a two bottle choice, unlimited ethanol access paradigm in a CART KO mouse model generated by Asnicar et al. (2001). We then examined several factors involved in mediating ethanol appetitive behaviors including bitter/sweet taste perception, ethanol metabolism, and ethanol sensitivity.

MATERIALS AND METHODS

CART KO mice

Three breeding trios of CART KO mice were obtained from Eli Lilly (Indianapolis, IN). Their generation has been previously described (Asnicar et al., 2001). The progeny of these trios were bred with C57BL6J wild type mice to produce CART heterozygotes. The resulting heterozygotes were further crossed to a C57BL6J WT background for three generations. Heterozygotes were then crossed to produce litters with CART WT, heterozygote, and KO mice. At weaning age, mice were separated by sex with 2-5 mice per cage. Within one week of weaning, mice were ear labeled and tail biopsies collected for genotyping. Genomic DNA was isolated from the tail biopsies

using the Promega Wizard gDNA kit (Madison, WI). The purified gDNA was then used to genotype the mice with primers previously described by Asnicar et al. (2001). Briefly, three CART primers were used; one targeting the WT allele (5'-AAG GTA GCA GTA GCA GCA GG-3'), one targeting a region in the mutant/CART KO allele (5'-GAA AAT GGC CGC TTT TCT GG-3'), and the third targeting a common fragment (5'-TAT GTG TAC ACG AGT GCA GG -3') in both alleles. The predicted product sizes obtained were 880 bp and 658 bp, respectively, for the WT and mutant/CART KO alleles. A second set of actin primers (5'-GCT CGT CGT CGA CAA CGG CTC-3' and 5'-CAA ACA TGA TCT GGGT CAT CTT CTC-3'; 353 bp expected product) was included in each reaction to serve as an internal control. All of primers used were synthesized by Integrated DNA Technology (Coralville, IA). The JumpStart REDTaq Readymix (Sigma-Aldrich, St. Louis, MO) was used according to the manufacturer's protocol. To minimize non-specific products in the multiplex reaction, a touchdown PCR protocol was used. The cycling parameters consisted of an initial 95°C denaturation cycle for 4 minutes. Then 30 cycles of a 30 second 95°C denaturation step, a 60°C 30 second hybridization step (decreasing 0.5°C every subsequent cycle), and a one minute extension step at 72°C. An additional 30 cycles consisting of a 30 second 95°C denaturation step, a 30 second 45°C hybridization step, and one minute 72°C extension step. A final 72°C extension step for seven minutes ended the protocol. The resultant products were run on 2% agarose/TBE – ethidium bromide gels. The PCR was repeated for every sample to ensure the genotypes were correct. Only WT and KO littermates were used in all experiments. At 2-3 months of age, the mice were individually housed under a reversed 12:12 hour light schedule (lights on at 8 p.m.) for 3 weeks before beginning any experiments. Food and water were available ad libitum. All of the experiments were approved by the Institutional Animal Care and Use Committee.

Ethanol consumption & preference

A continuous two-bottle choice paradigm was used to compare ethanol preference between WT and KO mice of both sexes. During the acclimation period to the reversed light schedule and individual housing, the mice were presented with two bottles containing only water. At the onset of the ethanol preference test, one of the water bottles was replaced with a 3% ethanol solution for four days. After the fourth day, the ethanol solution was replaced with a 6% ethanol solution for four days, followed by 9, 12, 15, 18, and 21% ethanol solutions, each following the same paradigm. The bottles were removed only briefly for daily measurements of the total amounts of ethanol and water. In addition, the position of the ethanol and water bottles in each cage were alternated daily to avoid a bottle position preference. Mouse body weights were measured every other day.

The amount of daily ethanol consumption was determined (in g/kg of body weight) for each animal and averaged over the four day period for each ethanol concentration. Two control tubes (one with water and the other with the corresponding ethanol solution) were used to estimate evaporation and spillage and the consumption amounts obtained for experimental animals were adjusted accordingly. Preference was determined by dividing the volume of ethanol solution consumed by the total volume of ethanol and water consumed. Total intake of ethanol and water consumption was also calculated for each subject.

Bitter/Sweet tastant preference

To control for any differences in bitter or sweet tastant preferences, consumption of quinine and saccharin solutions was assessed following the ethanol preference tests. After the final day of ethanol consumption, the mice were given two water tubes for two weeks. The mice were then once again offered water and a non-alcohol tastant tube containing either a bitter (quinine) or sweet (saccharin) tastant as before (four days, escalating concentrations). Quinine hemisulfate (Sigma-Aldrich) was prepared as 0.03 and 0.06 mM solutions. Saccharin was prepared as 0.033 and 0.066% solutions. As before, the mice were weighed every other day, the consumption of each solution was determined daily, and the bottle position was alternated daily to control for any possible bottle position preference. There was a two-week water only period between the quinine and saccharin tastants.

Ethanol metabolism

Ethanol naïve mice were given an i.p. injection of ethanol (2.5 g/kg, 20% solution in sterile PBS) between 1 p.m. and 2 p.m. Three blood samples were collected from each mouse at one of the 30, 60, 120, 180, 240, or 300 minute post injection time points. The blood samples were collected in heparinized capillary tubes, centrifuged at 5K rpm for two to three minutes (to clear the serum), and immediately used to determine blood ethanol content with the Pointe Scientific (Canton, MI) enzyme assay.

Ethanol Sensitivity/Loss or Righting Reflex

Ethanol naïve male and female mice of both genotypes were tested for their sensitivity to ethanol with a loss of righting reflex (LORR) test. Briefly, mice were

injected (i.p.) with either 3.4 or 3.8 g/kg ethanol (20% solution in sterile PBS). The doses were selected because they were high enough to induce a loss of the righting reflex without inducing an excessively long sleep time (Blednov et al., 2006). Fifteen to twenty minutes after the injection all mice were tested to ensure the righting reflex (defined here as the ability of the mouse to right itself three times in a thirty second period when placed in the supine position) was lost. Every ten minutes after the initial loss of the righting reflex, the animals were tested until they had fully recovered. One mouse failed to lose the righting reflex (most likely due to a misplaced injection) and was excluded from this study. The experimenter testing the mice was not aware of the genotypes of the mice throughout the study. All of the experiments were carried out at room temperature with the ethanol injection taking place between 1 p.m. and 2 p.m.

Immunohistochemistry

At the conclusion of the experiment, the mice were intracardially perfused and processed for CART-immunoreactivity (CART-ir) to confirm, on the protein level, the completion of the knockout. Briefly, the mice were anesthetized with chloral hydrate, the thoracic cavity was opened and the heart freed from the pericardium, the descending aorta was clamped and 0.5 mL of 5,000 U/mL Heparin (Sigma-Aldrich, St. Louis, MO) in phosphate buffered saline (PBS) was injected into the left ventricle to prevent clot formation in the vasculature. To prevent recirculation, the right atrium was cut and a small incision into the left ventricle allowed the insertion of the perfusion catheter. Saline (0.9% NaCl) containing 0.2% $NaNO_2$ as a vasodilator was perfused for two minutes to clear the vasculature of any remaining blood. This step was followed by 4% paraformaldehyde for 12 minutes. The brains were then quickly excised and post fixed in

4% paraformaldehyde for two hours before being transferred to a 30% sucrose/PBS solution for at least two days. Following this step, the brains were sectioned on a rotary microtome (Leica Microsystems, Bannockburn, IL) equipped with a freezing stage (Physitemp Instruments, Clifton, NJ). The brains were sectioned at 40 μms and collected sequentially in three series. The tissue was then stored at -20°C in a cryoprotectant solution (Watson et al., 1986) to maintain peptide immunoreactivity and tissue morphology until immunohistochemistry could be performed.

Free floating tissue sections were rinsed six times for ten minutes with tris buffered saline (TBS) to remove the cryoprotectant. To remove residual aldehydes, the sections were pretreated with a 1% sodium borohydrite/TBS solution for ten minutes. This step was followed by a one hour preincubation step in a 20% Normal Goat Serum (NGS), 1% hydrogen peroxide, 0.3% Triton-X 100 solution (to block nonspecific labeling, quench endogenous peroxide activity, and permeabilize, respectively) at room temperature. The sections were then incubated in a 1/4,000 dilution of Rabbit anti CART (Phoenix Pharmaceuticals, Burlingame, CA) in 2% NGS, 0.3% Triton-X 100 for 48 hours at 4°C with gentle agitation. After primary antibody incubation, the sections were rinsed with TBS four times for five minutes and then incubated in a biotinylated secondary antibody raised in goat directed against rabbit antibodies at a 1/200 dilution in 2% NGS, 0.3% Triton-X 100 solution for two hours at room temperature with agitation. After washing as described before, a one hour tertiary incubation with the Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA) was performed according to manufacturer's protocol. The sections were washed again before labeling with a 3,3'-diaminobenzidine (DAB; 0.8 mg/mL), 0.05% hydrogen peroxide, TBS solution for five minutes. The labeling reaction was halted by rinsing the sections with TBS as described before. The labeled sections were mounted onto subbed slides and allowed to dry

overnight. The slides were then dehydrated in an increasing gradient of ethanols, cleared with xylenes, and coverslipped with permount. The slides were allowed to dry for at least three days. The slides were then coded before image acquisition. DAB stained images were captured using an Olympus IX-70 microscope with a Hamamatsu camera and Simple PCI 6 image acquisition software (Leeds Instruments, Irving, TX).

Statistics

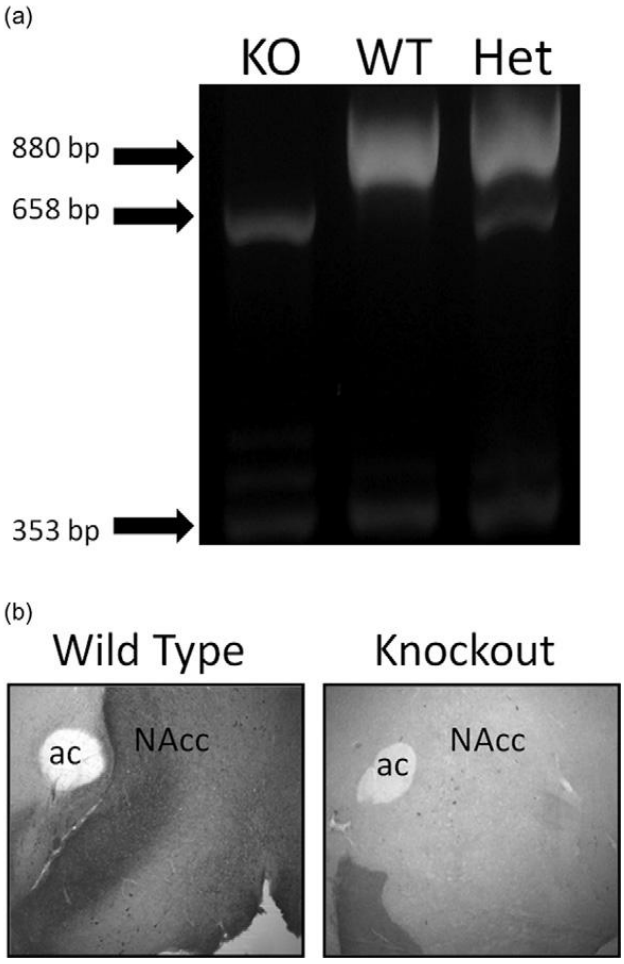
All data are reported as the mean ± SEM. All data were compiled into excel and exported to R 2.11.1 for statistical analysis. A repeated measures, two-factor analysis of variance (ANOVA) was conducted to examine group differences in the ethanol drinking data. Two-factor ANOVAs were conducted to examine group differences in the remaining experiments.

RESULTS

Genotyping & Immunohistochemistry

Since this study is the first by our lab utilizing the CART KO mice, and given that the genetic knockout of the CART gene is incomplete (see (Asnicar et al., 2001) for details), we chose to verify the complete knockout of the CART protein using immunohistochemistry with an antibody directed against the full length (55-102) CART protein. Our PCR genotyping results were verified in a random sample of mice used in the study. We observed no CART protein expression in any brain region in the CART KO mice (NAc is shown in Fig. 1). The WT mice displayed a CART-ir distribution similar to that previously described (Koylu et al., 1998) (Fig. 1B).

Figure 1. Polymerase Chain Reaction (PCR) genotyping results and immunohistochemistry confirmation

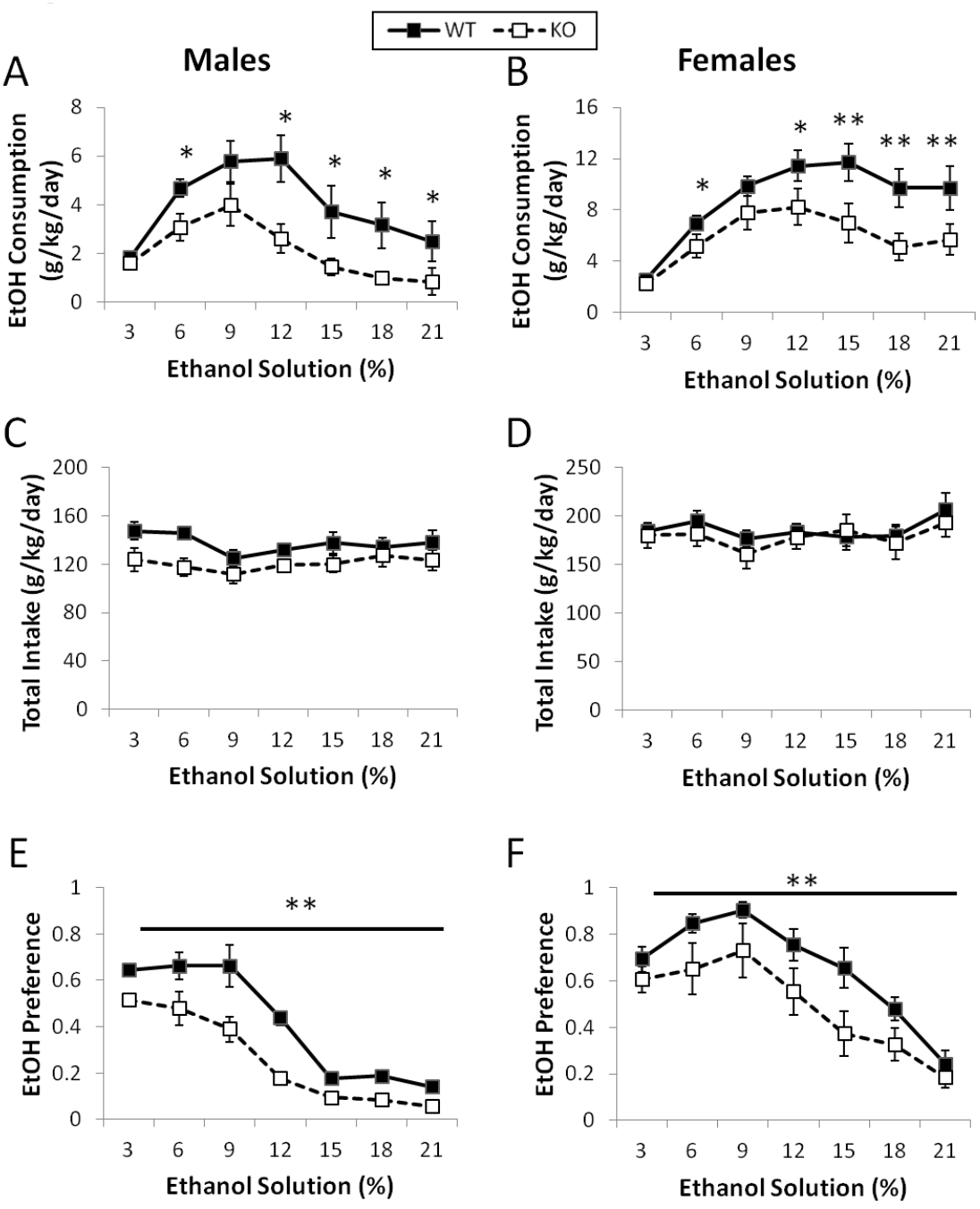


(a) Typical example of PCR genotyping results indicate the presence (or absence) of the wild type (WT) and cocaine- and amphetamine-regulated transcript (CART) knockout (KO) alleles, as well as the actin internal control bands (880, 658 and 353 bps, respectively). Periodically, the PCR genotyping results were verified with immunohistochemistry in brain tissues using a CART antibody. (b) The nucleus Accumbens (NAc) region of the brain expresses CART in the WT sample, but not in the CART KO sample. ac = anterior commissure; Het = heterozygote

Ethanol consumption, preference, and total intake

We observed a main effect of genotype on ethanol consumption ($F(1,21)=8.4$, $p<0.01$ and $F(1,27)=11.7$, $p<0.01$, for males & females, respectively) such that, CART KO mice (of both sexes) consumed less ethanol than their WT counterparts (Figure 2A & B). Total intake did not differ between genotypes (Figure 2C & D). Ethanol preference was altered in the CART KO mice such that they also preferred ethanol less than their WT counterparts ($F(1,21)=6.2$, $p<0.01$ and $F(1,27)=4.4$, $p<0.01$, for males and females, respectively; Figure 2E & F).

Figure 2. CART KO mice display reduced consumption and preference of ethanol

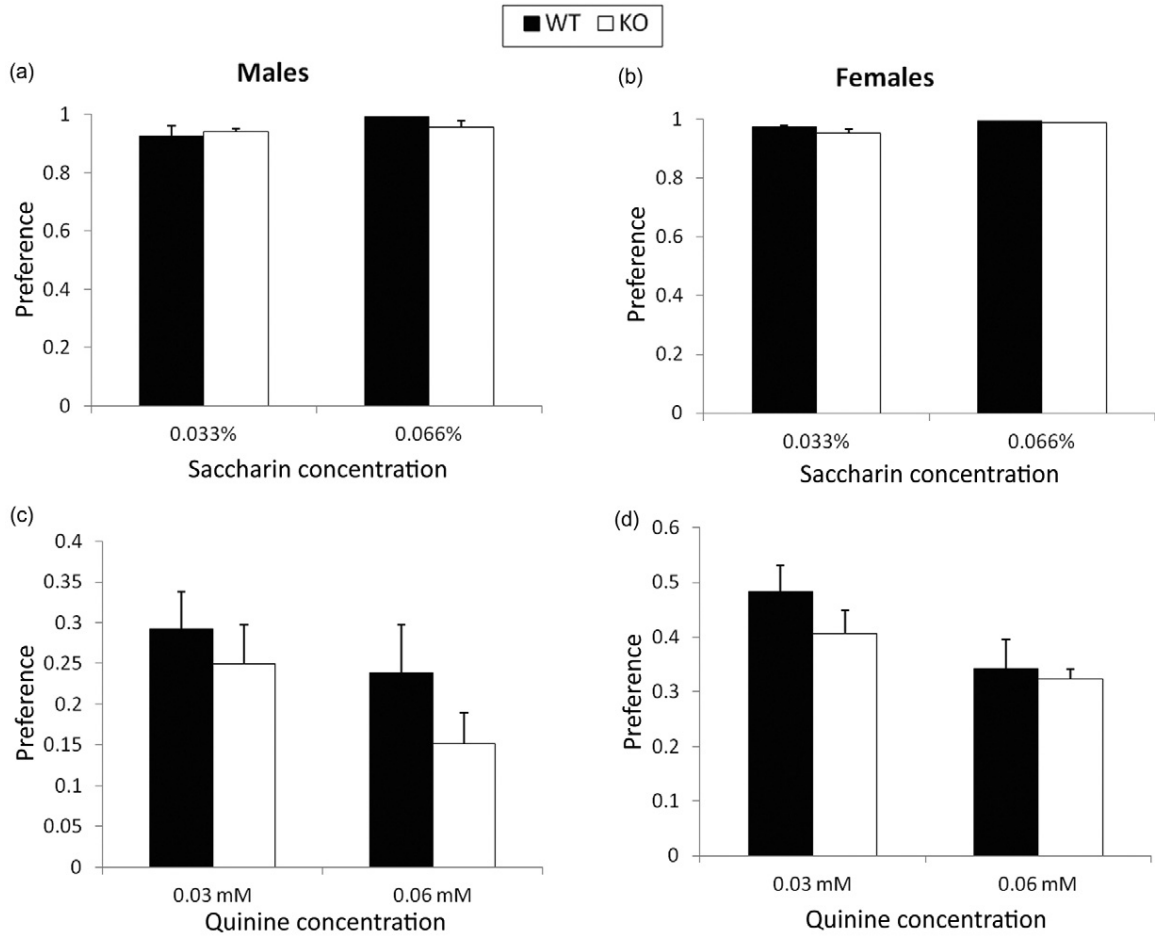


Male CART KO mice consumed less EtOH than their wild type (WT) counterparts (A & E, respectively), but did not differ in their total liquid intake (C). Female CART KO mice also consumed and preferred less EtOH than their WT counterparts (B & F, respectively) and had similar levels of total liquid intake to the WT mice (D). n = 11 per genotype for males and n = 14 per genotype for females. *P<0.05; **P<0.01.

Bitter/Sweet tastant preference, ethanol metabolism, and sensitivity

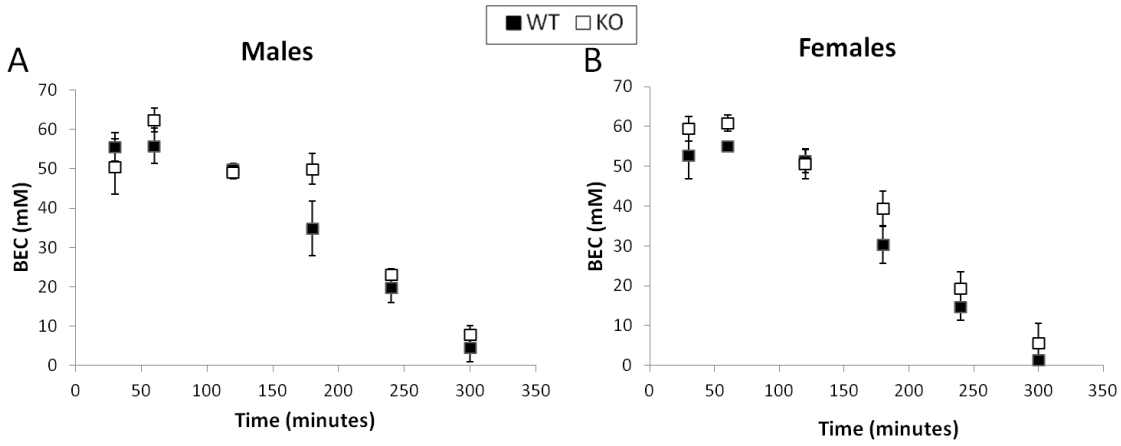
There was no effect of concentration or genotype on saccharin (Figure 3A and B) or quinine consumption (Figure 3C and D). There was no effect of sex or genotype on ethanol metabolism. Both male (Figure 4A) and female (Figure 4B) mice reached a peak BEC of approximately 60 mM within the first hour after the injection. Five hours after the injection, the BEC of the mice returned to basal levels. There was no main effect of ethanol dose on ethanol sensitivity, as measured by the duration of the LORR. There was also no genotype effect in male mice (Figure 5A); however, in female mice, a genotype effect was observed such that CART KO mice had a longer duration of the LORR at both of the ethanol doses ($F(1,27)=2.9$, $p<0.01$; Figure 5B).

Figure 3. Removal of the CART gene does not alter taste preference for bitter or sweet tastants



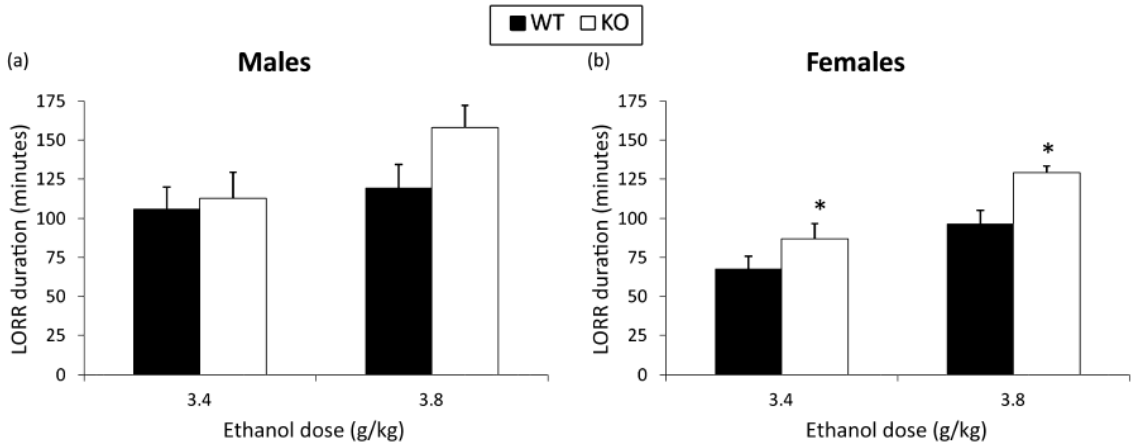
Removal of the cocaine- and amphetamine-regulated transcript gene does not alter taste preference for saccharin (a & b, respectively, for males and females) or quinine (c & d, respectively for males and females) tastants. n = 11 per genotype for males and n = 14 per genotype for females. WT = wild type; KO = knockout

Figure 4. The metabolism of ethanol did not differ between CART KO and WT mice.



The metabolism of ethanol did not differ between cocaine- and amphetamine-regulated transcript knockout (KO) and wild type (WT) mice in male (A) or female (B) mice. n = 7 per genotype for males and n = 8 per genotype for females. BEC = blood ethanol content

Figure 5. Sensitivity to ethanol does not differ between genotypes in male mice



There was no difference in ethanol sensitivity between cocaine- and amphetamine-regulated transcript (CART) knockout (KO) and wild type (WT) male mice (a). In females, however, the CART KO mice had an increased loss of righting reflex (LORR) duration compared with their WT counterparts (b). n = 6 per genotype for males and n = 7 per genotype for females. *P<0.05

DISCUSSION

This constitutes the first report that removal of CART protein is accompanied with a decrease in ethanol consumption and preference behaviors that cannot be attributed to differences in taste perception, ethanol metabolism, or ethanol sensitivity. Most notably, these data are in direct accord with previous work that reduction of CART expression reduces cocaine self-administration, but not of sweet tastants (Couceyro et al., 2005). In light of the fact that non-drug rewards are not affected by removal of the CART gene, this consistent response to both ethanol and psychostimulants is strongly suggestive that CART expression is functionally significant in a variety of drug-seeking states. Behavioral and pharmacological studies examining the role of CART in drug reinforcement indicate that CART itself is reinforcing. Kimmel et al. (2000) and Kuhar et al. (2005) found that intra-VTA injections of CART produce psychostimulant-like behavioral effects (including dose-dependent increases in locomotor activity and conditioned place preference) and increase dopamine release in the NAc. It is therefore possible that without this agonist-like activity of CART in the VTA (which would be increased in response to drug administration), the reinforcing properties of drugs of abuse are mitigated or occluded, resulting in the observed decrease of drug and ethanol consumption in CART KO mice reported here and previously (Couceyro PR, 2005; Couceyro et al., 2005).

The reduced consumption and preference for ethanol observed in CART KO mice of both sexes supports the aforementioned theory. The genotype difference in preference and consumption cannot be attributed to differences in total liquid intake between genotypes. Therefore, other factors known to contribute to ethanol appetitive behaviors were examined. It is possible that the palatability or taste preference of ethanol differs between CART KO and wild type mice; however, in our studies, we observed no

differences in the consumption of saccharin or quinine solutions, suggesting that there were no differences in taste preference between genotypes. We next compared the metabolism rates in CART KO and WT mice to assess whether or not the differences in ethanol consumption and preference could be accounted for by a decrease in ethanol metabolism rate in CART KO mice. Mice of both sexes and genotypes received a 2.5 g/kg i.p. dose of ethanol and blood samples were collected and assayed for blood ethanol content across a number of time points. There was no difference in the time-matched blood ethanol concentrations between genotypes, suggesting no differences in ethanol metabolism rates. Given the known role of CART in feeding, metabolism, and energy homeostasis, the lack of a CART KO effect on the ethanol metabolism rate was surprising (see Rogge et al. (2008) for review). It is also noteworthy that contrary to previous reports, no significant differences in body weight were observed between genotypes in any of the experiments. The lack of a body weight difference could be attributed to the fact that these studies were all conducted on mice that were at most 25 weeks old. Previous studies of genetically modified mice have shown a negative relationship between the latency to recover the righting reflex and ethanol consumption (Thiele et al., 1998). We therefore examined the ethanol sensitivity of CART KO and wild type mice using the loss-of-righting-reflex assay. The reduced consumption of ethanol could be attributed to a relative increase in ethanol sensitivity; indeed, in female mice we observed a genotype effect such that CART KO mice had an increased sensitivity to the doses of ethanol tested here. However, we observed no such difference in ethanol sensitivity between male CART KO and wild type mice.

Another CART-rich brain region shown to be important for ethanol consumption, the Edinger-Westphal nucleus (Bachtell et al., 2004), was recently shown to have a significantly reduced expression of CART in the low alcohol-drinking DBA/2J mouse

strain relative to the high alcohol-drinking C57BL6J mouse strain (Giardino et al., 2012). These results further support a role for CART in ethanol consumption and strengthen the main finding of the current study that null (or low) levels of CART are associated with reduced ethanol consumption.

It should be noted that the use of global CART knockout mice leaves open the possibility for unforeseen compensatory effects in CART-related neurotransmitter systems that could account for the differences in ethanol consumption and preference observed. Throughout this study, no gross abnormal physiology or behaviors were observed in any of the CART KO mice. Additionally, a preliminary study (Couceyro PR, 2005) using a second CART KO mouse reported results similar to those described in the current study with two exceptions, the CART KO mice in that study were on a different background strain than the mice in this study and had a greater total fluid intake than their wild type counterparts. Nonetheless, the overall finding that CART KO mice consumed and preferred ethanol less than their WT counterparts is consistent with the findings in our study.

An alternative (or additional) role for CART suggested by the literature posits that CART acts as a homeostatic mechanism to curtail excessive dopamine signaling (in response to psychostimulant administration) in the NAc (Hubert et al., 2008; Jaworski and Jones, 2006; Jaworski et al., 2003; Kim et al., 2003). These studies have focused on the mesolimbic dopamine system; however, other studies have investigated the role of thalamic and hypothalamic CART in regulating NAc dopamine signaling and drug related behaviors. The arcuate nucleus (ARC) is a CART-neuron rich region that projects to the neurons of the paraventricular thalamus (PVT) (Kirouac et al., 2006) which in turn project to the NAc shell (Parsons et al., 2006). Stimulation of the PVT (and its glutamatergic afferents) has been shown to increase NAc shell dopamine levels

independent of VTA activity, presumably by activating presynaptic dopamine terminals in the NAc (Parsons et al., 2007). It has been previously reported that in a reinstatement model of relapse, stimuli previously linked to ethanol activated CART neurons in the ARC (Dayas et al., 2008). Given that CART neurons in the ARC project to and appose PVT neurons, and that PVT neurons subsequently project to and modulate dopamine levels in the NAc, it is not unexpected that CART in the PVT could modulate drug-related behaviors. Indeed, James et al. (2010) found that injections of CART into the PVT dose-dependently decreased active lever pressing for cocaine in a drug-primed reinstatement model. Taken together, a common mechanism for CART as an inhibitor of aberrant dopamine signaling in response to drugs of abuse begins to form. Thus, one would expect that CART KO mice may readily engage in uncontrolled drug intake (without a negative or inhibitory feedback signaling system in response to high or binge doses of drugs). However, this is not what we observed in this study. It is likely that the role of CART in mediating the reinforcing/rewarding effects of drugs of abuse is more complex than behavioral studies in rodents would seem to indicate and warrants further investigation.

In summary, the present study demonstrates a role for CART in ethanol consummatory behaviors; moreover, we demonstrated that removal of the CART gene reduced volitional ethanol consumption in mice in a manner that cannot be attributed to altered intake levels, taste preference, ethanol metabolism, or ethanol sensitivity (at least in males). Further research aimed at exploring and understanding exactly how the CART system is involved in addiction (and alcoholism) and how the removal of CART was able to reduce ethanol consumption is necessary. Future behavioral, biochemical, and electrophysiological studies will elucidate the mechanism(s) by which CART exerts its effects on ethanol consumption and its potential role in mediating the reinforcing effects

of drugs of abuse. The findings of this study imply a role for CART in drug reinforcement and as a potential therapeutic drug target that may reduce volitional ethanol intake or enhance abstinence.

Author Contributions

Armando Salinas designed the studies, conducted all of the animal experiments, analyzed the data, and prepared the manuscript. Chinh TQ Nguyen and Dara Ahmadi-Tehrani assisted with the genotyping and immunohistochemistry experiments. Richard A. Morrisett contributed to the study design and preparation of the manuscript.

Salinas AG, Nguyen TQ, Ahmadi-Tehrani D, Morrisett RA. Reduced ethanol consumption and preference in cocaine- and amphetamine-regulated transcript (CART) knockout mice. Addiction Biology 2012 doi:10.1111/j.1369-1600.2012.00475.x

Chapter 3:

Central amygdala CART modulates ethanol withdrawal induced anxiety

ABSTRACT

Ethanol withdrawal-induced anxiety is a common contributing factor to relapse behaviors. A key locus of ethanol withdrawal-induced anxiety is the central nucleus of the amygdala, which in addition to its role in stress and anxiety, has also been shown to be critically involved in ethanol consumption and dependence. The central amygdala neuropeptide, cocaine- and amphetamine-regulated transcript (CART), is anxiogenic and has recently been implicated in mediating ethanol withdrawal-induced anxiety, which is among the most common contributing factors to relapse in ethanol dependence. We recently acquired a CART KO mouse line and found a reduction in ethanol preference and consumption. Given the role of CART peptide in ethanol consumption and in mediating ethanol withdrawal-induced anxiety, the current study sought to characterize the effect of chronic intermittent ethanol vapor exposure (CIE) on ethanol consumption, withdrawal-induced anxiety and stress, and glutamatergic neurotransmission in the central amygdala of CART KO mice. We additionally sought to determine the cellular mechanism through which CART peptide may exert its anxiogenic effects. To that end, we studied the effect of CART peptide on glutamatergic transmission in central amygdala neurons and found a reduction in AMPA/NMDA ratios with no change in the AMPA component, suggesting that CART peptide enhances NMDA receptor-mediated neurotransmission. We then characterized basal and acute stress-induced anxiety-like behaviors with the light/dark box assay and found no difference between CART KO and

WT mice under basal or acute stress-induced conditions. We did, however, observe a potentiated corticosterone response to an acute stressor in CART KO mice. We then proceeded to induce ethanol dependence using a chronic intermittent ethanol vapor model (CIE) and found that a single bout of CIE treatment resulted in escalated ethanol consumption and anxiety-like behaviors in both CART KO and WT mice with statistical trends towards a reduction in ethanol consumption in CART KO mice. We further found that following two bouts of CIE treatment, anxiety-like behaviors were differentially altered such that CART KO mice displayed a reduction in anxiety-like behaviors relative to WT mice; however, the plasma corticosterone levels were similar between genotypes. Electrophysiologically, we found an enhancement of AMPA receptor-mediated sEPSCs in CIE treated mice of both genotypes. Moreover, we found no CIE or genotype induced differences in AMPA/NMDA ratios. Finally, we found an increase in paired pulse ratios, suggesting a decrease in presynaptic release probability, of CIE-treated WT mice. These combined findings support the importance of CART peptide in ethanol withdrawal-induced anxiety behaviors and ethanol dependence and posit a mechanism for the anxiogenic effects of CART in the central amygdala. Furthermore, they implicate distinct pre- and postsynaptic glutamatergic neuroadaptations in ethanol dependence.

INTRODUCTION

Cocaine and amphetamine regulated transcript (CART) has been implicated in a number of biological processes including addiction and anxiety/stress. As its name suggests, CART was initially identified as an upregulated striatal transcript in response to psychostimulant administration (Douglass et al., 1995). Subsequent study of CART has

found that it may play a role in mediating the reinforcing properties of drugs of abuse as evidenced by its ability to produce a conditioned place preference (CPP) and increased locomotor activity when injected into the ventral tegmental area (VTA) (Kimmel et al., 2000; Kuhar et al., 2005). Furthermore, intracerebroventricular (icv) administration of CART peptide has been shown to increase dopamine turnover in the nucleus accumbens (NAc) (Shieh, 2003; Yang et al., 2004), suggesting an increase in dopaminergic activity. Further still, studies using CART knockout mouse models have shown: a reduction in cocaine-induced locomotor activity (Moffett et al., 2006), a reduction in amphetamine-induced CPP and locomotor sensitization (Couceyro et al., 2005), a reduction in consumption and preference for ethanol (Salinas et al., 2012), and reduced self administration of cocaine (Couceyro et al., 2005). Taken together with studies demonstrating dopamine receptor modulation of CART expression and protein levels (Hunter et al., 2006; Salinas et al., 2006), as well as, reports of altered CART expression in the VTA and NAc of overdose victims and cocaine abusers, respectively, a role for CART peptide in addiction can be assumed (Albertson et al., 2004; Tang et al., 2003).

A role for CART in anxiety and stress has also been shown. An acute restraint stress was shown to increase CART mRNA in the central amygdala of adult male rats and in the dentate gyrus of chronically restrained rats (Hunter et al., 2007). Female rats had a greater number of CART peptide positive cells in the paraventricular nucleus of the hypothalamus following a forced swim test than non-stressed rats (Gozen et al., 2007). Further lines of evidence indicating a direct role for CART peptide in anxiety and stress include icv injection studies of CART peptide demonstrating: 1) increased plasma adrenocorticotropin hormone and corticosterone (Smith et al., 2004), 2) decreased time spent in the open arms of the elevated plus maze (Kask et al., 2000), and 3) decreased social interaction time in rats (Chaki et al., 2003).

Despite all of the research centered on CART peptide and anxiety and stress, an exact mechanism of how CART peptide produces its anxiogenic effects is not known. One basic research study found increased CART peptide levels in the central amygdala of rats undergoing withdrawal from chronic ethanol and correlated the increased CART with elevated ethanol withdrawal-induced anxiety (Dandekar et al., 2008a). The same study found that reducing the amount of CART peptide in the central amygdala, specifically, attenuated alcohol withdrawal-induced anxiety. This finding was the first to implicate CART peptide in the central amygdala (a region well known to be involved in addiction and anxiety and stress) as a contributor to alcohol withdrawal-induced anxiety. Like previous studies, however, this report did not investigate the mechanism for how CART peptide may modulate anxiety. To that end, this study sought to determine a mechanism for how CART peptide administration could be effecting increases in anxiety-like behaviors. A review of the literature reveals that CART peptide enhances NMDA receptor-mediated transmission by increasing phosphorylation at the ser896 and ser897 residues of the perquisite NR1 subunit of NMDA receptors (Chiu et al., 2009; Chiu et al., 2010; Dun et al., 2006; Hsun Lin et al., 2005). We posit that this enhancement of NMDA receptor-mediated transmission may be the mechanism through which CART peptide exerts its anxiogenic effects; therefore, we examined the effect of CART peptide on glutamatergic transmission in the central amygdala.

Interestingly, addiction and anxiety are thought to be interconnected; indeed, anxiety and stress are often listed as contributors to relapse in treatment seeking addicts (Brown et al., 1995; Sinha et al., 2011). Also, a number of studies have positively correlated a person's predisposition to anxiety disorders and subsequent addiction to drugs of abuse, including alcohol (Bekman et al., 2013; Cowley, 1992; Cox et al., 1990; Kushner et al., 2000a; Kushner et al., 2000b; Quitkin et al., 1972).

Given the relationships between CART peptide, anxiety, and ethanol dependence, we hypothesized that CART KO mice would be resistant to ethanol withdrawal-induced anxiety and escalations in ethanol consumption induced by chronic intermittent ethanol (CIE) exposure (which has been shown to result in an ethanol withdrawal phenotype and enhance ethanol consumption (Becker and Hale, 1993; Becker and Lopez, 2004; Lopez and Becker, 2005)). We further hypothesized that alterations in glutamatergic signaling in the central amygdala would accompany ethanol dependence and be absent or attenuated in CART KO mice. Therefore the present study sought to (1) compare basal anxiety states between CART KO and WT mice, (2) examine stress responsivity in CART KO mice, (3) determine the effects of acute CART peptide on central amygdala neurons, (4) determine if CART peptides are necessary for CIE treatment to increase ethanol consumption, (5) examine anxiety and stress levels during early withdrawal from CIE, and (6) determine the electrophysiological consequences of CIE and early withdrawal on glutamatergic synaptic transmission in the central amygdala.

MATERIALS & METHODS

Subjects

The mice used for the acute restraint stress studies were eight to ten week old, male C57BL6J mice from the Jackson Laboratory (Jackson Labs, Bar Harbor, ME). Upon arrival at the University of Texas Animal Resources Center, the mice were housed up to four per cage and allowed to acclimate for one week prior to transport to the laboratory vivarium. Once at the laboratory vivarium, the mice were individually housed

and allowed to acclimate to the reversed light cycle for a three week period before the initiation of any studies.

The mice used for the electrophysiological studies of acute CART peptide effects on central amygdala glutamatergic activity were also ordered from the Jackson Laboratory. These mice were also males and ordered to be three to four weeks old at the time of arrival at the Animal Resources Center. The mice were housed up to four per cage and allowed to acclimate for at least three days before being moved to the laboratory vivarium. Once there, the mice were allowed to acclimate for one week before being used in experiments.

The generation of the CART KO mice used in these studies has been previously described (Asnicar et al., 2001; Salinas et al., 2012). Briefly, CART KO mice were obtained from Eli Lilly (Indianapolis, IN) and bred with C57BL6J wild type mice to produce CART heterozygotes. These CART heterozygotes were further back crossed with C57BL6J mice for three generations. Heterozygote crosses were used to produce CART KO and WT littermates for experiments and heterozygote pups were used as breeders. At 21-23 days of age, mice were weaned and separated by sex with no more than five mice per cage. At the time of weaning, mice were ear labeled and a tail biopsy was collected for genotyping. Genomic DNA was isolated using the Promega Wizard gDNA kit (Madison, WI) and used to PCR genotype the mice as described by Asnicar et al. (2001) with the addition of an internal control described in Salinas et al. (2012). Three CART primers were used: one targeting the WT allele (5'-AAG GTA GCA GTA GCA GCA GG-3'), a second targeting a region in the KO allele (5'-GAA AAT GGC CGC TTT TCT GG-3'), and the third targeting a fragment (5'-TAT GTG TAC ACG AGT GCA GG -3') common to both alleles. The product sizes for each of the primer sets were 880 bp for the WT allele and 658 bp for the CART KO allele. A set of actin

primers (5'-GCT CGT CGT CGA CAA CGG CTC-3' and 5'-CAA ACA TGA TCT GGGT CAT CTT CTC-3'; 353 bp expected product) was included in each reaction to serve as an internal control. The primers were synthesized by Integrated DNA Technology (Coralville, IA). The JumpStart REDTaq Readymix (Sigma-Aldrich, St. Louis, MO) was used according to the manufacturer's protocol. A touchdown PCR protocol that consisted of an initial 95°C denaturation cycle for 4 minutes. Then 30 cycles of a 30 second 95°C denaturation step, a 60°C 30 second hybridization step (decreasing 0.5°C every subsequent cycle), and a one minute extension step at 72°C. Thirty additional cycles consisting of a 30 second 95°C denaturation step, a 30 second 45°C hybridization step, and a one minute 72°C extension step. A final 72°C extension step for seven minutes followed. The products were separated on 2% agarose/TBE – ethidium bromide gels. Each sample was run in duplicate reactions to ensure the genotypes obtained were correct. At 10-12 weeks of age, male mice to be used experimentally were moved to a laboratory vivarium and individually housed under a reversed 12:12 hour light schedule (lights off at 12 p.m.) for 3 weeks before beginning any experiments.

Food and water were available ad libitum to all of the mice in all of the experiments. All of the experiments were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin and are consistent with the guidelines of the NIH guide for the Care and Use of Laboratory Animals.

Experimental design

The overall study design involved five main experiments intended to elucidate the effects of CART peptide on central amygdala neurons, examine anxiety-like behaviors in

CART KO and WT mice, and to examine the effects of chronic intermittent ethanol exposure on anxiety-like behaviors and electrophysiological properties of central amygdala neurons in CART KO and WT mice.

The first experiment aimed to determine the effects of CART peptide on glutamatergic activity in central amygdala neurons of C57BL6J mice with the hope of demonstrating in our hands that application of CART peptide would enhance glutamatergic activity in the central amygdala and serve as a potential cellular mechanism for the anxiogenic effects of CART peptide reported by others (Chaki et al., 2003; Kask et al., 2000; Dandekar et al., 2008a). This was achieved by electrophysiologically examining excitatory post synaptic currents (EPSCs) and AMPA/NMDA receptor mediated ratios of central amygdala neurons.

The second experiment aimed to validate the light/dark box assay as a suitable method for assessing anxiety-like behaviors in our hands. To this end, adult male, C57BL6J mice were exposed to an acute restraint stress or a clean novel cage as a control and then tested in the light/dark box. Following the behavioral trial in the light/dark box, the mice were sacrificed and plasma corticosterone content assessed with an ELISA as a secondary, physiological measure of anxiety.

The third experiment aimed to determine if removal of the CART gene would result in a reduction of basal anxiety-like behaviors in CART KO mice relative to CART WT mice using the light/dark box.

The fourth experiment aimed to determine if stress responsiveness was altered in CART KO mice relative to CART WT mice. This was determined by acutely restraining adult male mice of both CART genotypes and then assessing anxiety-like behavior using the light/dark box. Immediately following the behavioral trial in the light/dark box, these mice were sacrificed and their plasma corticosterone content was assessed as before with

an ELISA. Non-stressed control groups were run concurrently with the acute restraint stress groups for each genotype.

The fifth experiment was a comprehensive study examining the effects of early withdrawal from chronic intermittent ethanol on ethanol consumption, anxiety-like behavior, plasma corticosterone, and basic electrophysiological properties of glutamatergic signaling in the central amygdala in CART WT and KO mice. These aims were accomplished by measuring ethanol consumption before and after a bout of chronic intermittent ethanol vapor, measuring anxiety-like behaviors using the light/dark box and plasma corticosterone with an ELISA, and electrophysiological measures of spontaneous glutamatergic activity, AMPA/NMDA receptor mediated ratios, and paired pulse ratios. Air vapor control mice of both genotypes were run in parallel with the chronic intermittent ethanol treated mice and examined for the same parameters as the experimental mice.

Acute restraint stress

For the acute restraint stress studies, individually housed mice were restrained with a DecapiCone™ (Braintree Scientific, Braintree, MA), a triangular, heavy plastic cone with an open tip for the mice to breathe. A binder clip was used to secure the open end and ensure that the mouse remained in the DecapiCone™. Each restraint stress session lasted ten minutes and was carried out in ambient room light on a 3D rotator rotating at 10 rpm. Fecal boli deposited in the DecapiCone™ were counted.

Light/dark box behavioral assay

Anxiety-like behaviors were assessed using the light/dark box assay (for review see Bourin and Hascoet (2003)) which is designed to exploit rodents' natural aversion to brightly lit areas. The light/dark box used in this study (Stoelting Co., Wood Dale, IL) consists of two plexi glass compartments – one clear and the other darkened – measuring 20.5 x 40 x 35 cm (W x D x H) and 19 x 40 x 35 cm, respectively, with an opening for mouse movement between the two compartments. The light/dark box was contained (and behavioral experiments run) in a custom-built sound attenuation cubicle with an LED ceiling lamp centered over the "light" side of the light/dark box, a ventilation port, a fan for air circulation, and a camera port in the ceiling for video recording of the behaviors.

For the acute restraint stress studies, each behavioral trial was carried out immediately following the end of the restraint stress period. For the chronic intermittent ethanol studies, each behavioral trial was carried out approximately six and a half hours into the withdrawal periods.

Each behavioral trial was ten minutes in duration and began with the mouse being placed in the center of the light compartment of the light/dark box. The behavioral trial was video recorded with a Sony digital video camera and analyzed offline by two reviewers blind to the treatment conditions of each mouse. Several parameters were scored including: the latency time for the first entry into the dark compartment, the number of transitions between the two compartments, the amount of time spent in the light and dark compartments, the number of rears, and the number of fecal boli. The scores from the two reviewers were averaged and used for statistical analysis. In the rare circumstance that the scorers differed by more than 10%, a third reviewer, also blind to the treatment conditions, was asked to review the video in question and the two scores closest in value to each other were used for statistical analysis. Immediately following

each trial the light/dark box was cleaned with a 5% bleach solution and then with a soapy water solution. For the acute restraint stress tests, the chambers were allowed to dry after cleaning for at least 25 minutes between mice/trials. Only one mouse per day was tested for the chronic intermittent ethanol study so the chambers were allowed to dry overnight after cleaning. Four mice that displayed little to no locomotion, consequently spending the entire behavioral trial in the light compartment, were excluded from their respective studies. One mouse in the chronic intermittent ethanol study displayed seizure-like activity and was removed from the study.

Corticosterone ELISA

In the acute restraint stress experiments, mice were sacrificed by rapid decapitation under isoflurane anesthesia immediately following the light/dark box assay. The trunk bloods were collected and centrifuged at 14,000 rpm at 4°C for 6 minutes. The plasma was then isolated with a glass Pasteur pipette, divided into four equal aliquots of at least 50 µLs each, and stored at -20°C until the corticosterone ELISA could be run. For the chronic intermittent ethanol study, following the light/dark box assay, mice were rapidly decapitated under isoflurane anesthesia and the trunk bloods collected and kept on ice until brain tissues could be harvested and sliced for electrophysiology experiments (approximately 25 minutes). The trunk bloods were then processed in a similar fashion to the trunk bloods from the acute restraint stress study.

Plasma corticosterone levels were assayed in control and experimental mice using the Corticosterone ELISA kit (Enzo Life Sciences, Plymouth Meeting, PA). For the assay, plasma samples were thawed and mixed with a steroid displacement reagent (39:1 ratio, sample to reagent) provided in the kit to free all corticosterone in the sample from

any binding proteins in the plasma. The samples were then diluted in a 39:1 ratio with assay buffer before running the ELISAs. This sample dilution ratio was determined empirically to yield the greatest recovery of corticosterone while still falling within the dynamic range of the assay. The corticosterone ELISA was then carried out according to the manufacture's recommended protocol. Once the assay was completed, the microplate was scanned at 405 nm using a VictorX microplate reader (PerkinElmer, Waltham, MA) and the results were exported and saved to an Excel spreadsheet.

The corticosterone ELISA results were then analyzed online with the immunoassay software package, MyAssays (www.myassays.com), and the specific module for the Enzo Life Sciences Corticosterone kit. This module utilizes a four parameter logistic curve fit for the standard curve generation and sample concentration determination (expressed as pg/mL). The average intraassay variability was 5.5% and the interassay variability was 13.1%.

Two bottle choice ethanol drinking

During the acclimation period to the reversed light schedule and individual housing in the laboratory vivarium, the mice were presented with two bottles containing only water. Then a limited access, two-bottle choice paradigm was used to determine daily ethanol consumption for a three week period. At 11:30 (30 minutes before the beginning of the dark cycle), the normal water bottles were replaced with two bottles, one containing a 15% ethanol solution and the second containing water, for two hours. At the end of the two hour period, the new ethanol and water bottles were removed and the normal water bottles were replaced. Beginning on the fifteenth day (the start of the third week), after the two hour drinking period, the ethanol and water bottles were weighed

and ethanol consumption was calculated for each mouse. The amount of daily ethanol consumption was determined (grams of ethanol per kilogram of body weight) for each animal and averaged over the final five day period to establish the baseline ethanol consumption levels.

Following three days of withdrawal from chronic intermittent ethanol or control vapor exposure (see below), the mice were once again allowed to consume ethanol daily for a two hour period as before for five consecutive days to determine if the CIE vapor exposure would affect ethanol consumption.

The position of the ethanol and water bottles in each cage was alternated daily to avoid the development of a bottle position preference. Mouse body weights were measured every other day throughout the drinking periods of the experiment at approximately two hours into the dark cycle.

Chronic intermittent ethanol exposure

Chronic intermittent ethanol vapor exposure models have been previously shown to lead to ethanol dependence as characterized by an escalation in volitional ethanol intake and the presence of ethanol withdrawal seizures (Becker and Hale, 1993; Becker and Lopez, 2004; Lopez and Becker, 2005). A variation of this basic procedure was employed in the current studies. The ethanol vapor set up consisted of air-tight biocontainment mouse cages (Allentown Inc., Allentown, NJ) that resembled typical polycarbonate mouse cages with the following exceptions – the tops for each cage were specifically designed to form an air-tight seal, as well as, inlet and outlet ports built in to the cage tops and bottoms, respectively. Three hundred milliliters of 95% ethanol in a side arm flask were volatilized by bubbling with air at a flow rate between 200-400

mLs/minute. The resulting ethanol vapor was combined with air at a flow rate of 3.75 L/minute (for a total flow rate of ~4L/minute). The diluted ethanol vapor was channeled into the ethanol vapor cages containing up to four mice. The 95% ethanol in the flask was refilled daily to 300 mLs. The ethanol flow rate was adjusted daily to yield empirically determined blood ethanol concentrations (BECs) between 175-225 mg/dL (~38-48 mM). Air control cages were run in parallel to the ethanol vapor cages except the ethanol vapor stream was not combined with the normal air stream.

Following the final day of baseline ethanol consumption, mice were divided into chronic intermittent ethanol (CIE) and control (Air) groups with matched levels of baseline ethanol consumption. Immediately before beginning the ethanol vapor or air control exposure (at 1700 hours) mice received an ip injection with either an ethanol/pyrazole/PBS or a pyrazole/PBS solution, for CIE or Air control mice, respectively. The ethanol/pyrazole/PBS solution contained 20% ethanol (v/v) and 1 mM pyrazole in sterile PBS and was intended to act as a loading ethanol dose (1.5 g/kg) to reach the target blood ethanol concentration. The pyrazole was included to help maintain steady BECs on account of its inhibition of alcohol dehydrogenase. The pyrazole/PBS solution was identical with the omission of the ethanol. After sixteen hours of ethanol or air vapor exposure (0900 hours), mice were removed from their respective chambers, lightly anesthetized with isoflurane, and tail blood samples collected for BEC determination (described below). The ethanol exposure procedure was repeated for four consecutive days and followed by a three day withdrawal period at which time limited access two bottle choice ethanol consumption was again measured for five days. The day after this five day drinking period, mice underwent a second bout of chronic intermittent ethanol or air vapor exposure as before.

Gas chromatography

Tail blood samples were collected for every mouse immediately after the end of each ethanol vapor exposure period and assayed to determine the blood ethanol concentration. Two, ten microliter samples of whole blood were collected and added to 10 mL vials containing 90 µLs of saturated saline (~5.5 M) and sealed with a septum until samples from all of the mice could be obtained.

Once all of the samples for the day had been collected, they were prepared for gas chromatography with a Bruker 430-GC (Bruker Corporation, Fremont, CA) equipped with a flame ionization detector and Combi PAL autosampler. Briefly, the samples were loaded onto the autosampler and the program was started. Each sample was warmed to 65°C for three minutes before the solid-phase microextraction fiber (SPME; 75 µm CAR/PDMS, fused silica; Supelco, Bellefonte, PA) absorbed the ethanol vapor for three minutes. The SPME fiber then desorbed the sample into the GC injection port for one minute at 220°C. Helium, at a flow rate of 8.5 mL/minute was used as the carrier gas and a capillary column (30 m x 0.53 mm x 1 µm film thickness; Agilent Technologies, Santa Clara, CA) was used for separation.

The resultant chromatograms were analyzed using CompassCDS Workstation software (Bruker Corporation, Fremont, CA). Known ethanol standards were included in every GC run and the peak heights for ethanol (~1.9-2.1 minutes retention time) were used to construct a standard curve. The peak heights of all samples were then compared against their respective standard curve and used to determine BEC. If necessary, ethanol vapor flow levels were adjusted to ensure that BEC remained in the target range of 175-225 mg/dL (~38-48 mM) for each experimental mouse.

Electrophysiology

Whole-cell voltage clamp recordings were obtained on visually identified central amygdala neurons using an Olympus BX-50WI microscope with IR-DIC optics (Leeds Instruments, Irving, TX) and a Dage MTI VE 1000 video camera and controller (Dage-MTI, Michigan City, IN) mounted on a vibration isolation table. Recording electrodes were pulled from borosilicate glass capillary tubes with a filament (WPI Inc., Sarasota, FL) using a Brown-Flaming model P-88 electrode puller (Sutter Instruments, San Rafael, CA). Only electrodes with resistances between 4-7 MΩ were used for experiments. The recording electrodes for all experiments were filled with an intracellular solution containing (in mM): 120 $CsMeSO_4$, 15 CsCl, 8 NaCl, 10 HEPES, 0.2 EGTA, 10 $TEA{\bullet}Cl^-$, 4 $ATP{\bullet}Mg^{2+}$, 0.3 $ATP{\bullet}Na^+$, 0.1 Spermine, and 5 $QX\text{-}314{\bullet}Cl^-$ (Alomone Labs, Jerusalem, Israel), osmolarity 280-290 mOsm, pH 7.3 with CsOH. Excitatory post synaptic currents (EPSCs) were electrically evoked with a stainless steel bipolar stimulating electrode (FHC, Inc., Bowdoin, ME) and an Isostim A320 stimulator (WPI Inc., Sarasota, FL). Stimulation intensities ranged from 10-75 μA. Data were acquired with either a PC-501A (Warner Instrument Co., Hamden, CT) or a PC-ONE (Dagan Corporation, Minneapolis, MN) amplifier, filtered at 2 kHz, and digitized at 10 kHz with a Digidata 1200 Series digitizer and interface board using pClamp 8.2 (Axon Instruments, Foster City, CA). Access resistance was monitored throughout all experiments by application of a 100 ms, -10 mV step and cells with changes >20% were excluded from the study.

For the experiments examining the effects of CART peptide (55-102 fragment; American Peptide Company, Sunnyvale, CA) on central amygdala neurons, 4-5 week old, male C57BL6J mice were anesthetized with isoflurane and rapidly decapitated. The brains were rapidly extracted and placed in ice-cold (4°C) cutting artificial cerebrospinal

fluid (ACSF) that had been bubbled with 95% O_2/5% CO_2 and contained (in mM): 210 sucrose, 26.2 $NaHCO_3$, 1 NaH_2PO_4, 2.5 KCl, 11 dextrose, 2.5 $CaCl_2$, and 6 $MgSO_4$ for three to five minutes. The brain was then mounted onto the cutting stage of a VT1000S vibratome (Leica, Nussloch, Germany) with a cyanoacrylate adhesive and 230-250 μm thick coronal slices containing the central amygdala (approximately -1.3 to -1.9 Bregma) were prepared in the same cutting ACSF used before. Immediately after being cut, the slices were bisected along the midline and incubated at 32°C in a recovery ACSF continuously bubbled with 95% O_2/5% CO_2 and containing (in mM): 124 NaCl, 26 $NaHCO_3$, 10 dextrose, 4.4 KCl, 1 NaH_2PO_4, 1.8 $CaCl_2$, and 2.4 $MgSO_4$. The slices were incubated for at least one hour before electrophysiological recordings were performed.

For electrophysiological recording, slices were transferred to a recording chamber and perfused at a flow rate of ~2 mLs/minute with room temperature (21-23°C) recording ACSF continuously bubbled with 95% O_2/5% CO_2 and containing (in mM): 124 NaCl, 26 $NaHCO_3$, 10 dextrose, 4.4 KCl, 1 NaH_2PO_4, 2 $CaCl_2$, and 1.2 $MgSO_4$. Picrotoxin (50 μM) was added to the recording solution for all experiments to inhibit any GABAA receptor-mediated inhibitory synaptic currents. Miniature EPSCs (mEPSCs) were recorded in the presence of 500 nM tetrodotoxin (TTX; Alomone Labs, Jerusalem, Israel) to block any action potential-mediated events; this concentration eliminated all electrically evoked EPSCs in pilot experiments (data not shown). For AMPA/NMDA ratio determinations, the recording ACSF $MgSO_4$ concentration was reduced to 0.5 mM to facilitate NMDA receptor-mediated responses. In every neuron, after achieving a whole-cell configuration (with neurons clamped at -80 mV), a five minute wait/equilibration period was taken to allow the intracellular solution to equilibrate with the cell cytoplasm. During this wait period, spontaneous EPSC activity was monitored. Three neurons displayed no spontaneous activity during this period and those recordings

were terminated. For recordings involving electrically evoked EPSCs (eEPSCs), the stimulation intensity was determined immediately following the equilibration period to yield consistent sub-maximal currents.

For mEPSC recordings, recording ACSF containing TTX was washed on after the equilibration period for at least five minutes before proceeding with the recording. Five minutes (30 sweeps, 10 seconds/sweep) of mEPSC activity was then recorded to determine the baseline activity level. Then CART peptide (500 nM) was bath applied with the recording ACSF for a fifteen minute period with mEPSC activity recorded for the last five minutes of the fifteen minute period. After a ten minute wash out period, miniature activity was recorded for an additional five minutes. For mEPSC recordings, quantal events were analyzed using the event detection module in Clampfit 10.3 (Axon Instruments, Foster City, CA). Briefly, a template mEPSC was constructed by averaging 200 quantal events and then using this template to scan sweeps for spontaneous quantal events. These events were manually inspected to ensure the template was suitable and that no false events were detected. Quantal events with a peak amplitude of less than five picoamps were omitted from the analysis because they could not be reliably differentiated from background noise levels.

For the determination of AMPA/NMDA ratios, eEPSCs were collected at -80 mV and at +40 mV in the presence of reduced $MgSO_4$. Evoked EPSCs collected at -80 mV were insensitive to application of the NMDA receptor antagonist CPP (20 μM; data not shown) in pilot experiments thus confirming that these eEPSCs were strictly AMPA receptor-mediated. EPSCs collected at +40 mV were compound AMPA and NMDA receptor-mediated currents. Application of a NMDA receptor antagonist, DL-AP5 (100 μM; Tocris Bioscience, Minneapolis, MN), was used to pharmacologically isolate the AMPA receptor-mediated portion of the compound current collected at +40 mV and to

determine the time at which the AMPA receptor-mediated portion of the current was attenuated. This time (65 ms after the stimulus artifact) was then used to determine the peak of the NMDA receptor-mediated currents for AMPA/NMDA ratio calculations. Twelve, one minute trials consisting of an eEPSC at -80 mV followed by a +40 mV eEPSC ten seconds later were conducted. The AMPA and NMDA peaks were analyzed, averaged, and compiled to yield the AMPA/NMDA ratio. This procedure was carried out before the application of CART peptide (500 nM), after ten minutes in CART peptide, and after a 10 minute wash out period.

For mice from the chronic intermittent ethanol vapor exposure study, spontaneous EPSCs (sEPSCs) were collected in a manner similar to mEPSCs except that no TTX was present in the recording solution and the $MgSO_4$ concentration was reduced to 0.5 mM to allow for determination of AMPA/NMDA ratios following collection of the sEPSC activity. The AMPA receptor antagonist, DNQX (20 μM; Tocris Bioscience, Minneapolis, MN), was used periodically after recordings were gathered to confirm that -80 mV EPSCs were AMPA receptor-mediated. Tetrodotoxin was excluded from these studies because it was found to have no significant effects on sEPSCs (average peak amplitude and frequency) in central amygdala neurons (data not shown). As before, after a five minute equilibration period, sEPSC activity was collected for a five minute period. A separate template was constructed, using the same process as for the mEPSC template, for the analysis of sEPSCs from mice in the CIE experiment. Following the collection of sEPSC activity, the stimulation intensity was established and the AMPA/NMDA ratio was determined as before.

Paired pulse ratios were determined in mice from the CIE experiment to determine if any presynaptic neuroadaptations resulted from CIE treatment. After the equilibration period, the stimulation intensity was determined as before. Then the ratios

were calculated by recording 15 sweeps consisting of two paired eEPSCs at -80 mV with a 25, 50, or 250 millisecond interstimulus interval for each interstimulus interval tested. Each sweep was separated by a 30 second period of time. For analysis of the paired pulse ratios, the peak amplitudes of both eEPSCs were calculated in Clampfit 10.3 (Axon Instruments, Foster City, CA) and exported to a spreadsheet for determination of the ratio of the second eEPSC to first eEPSC.

Except where noted, all chemicals were obtained from Sigma Aldrich (St. Louis, MO).

Statistics

All data are reported as the mean ± the standard error of the mean. All data were compiled and organized in Microsoft Excel spreadsheets and exported to R 2.15.0 (www.R-project.org) or IBM SPSS Statistics 21 (IBM, Armonk, NY) for analysis; specifically, MANOVAs were completed in SPSS and all other statistical tests were conducted in R.

Single-factor, repeated measures ANOVAs with Bonferroni post hocs were used to examine differences in mEPSC frequency, mEPSC amplitude, and AMPA/NMDA ratios in the acute CART peptide electrophysiological experiments. For the light/dark box validation experiment, a single-factor (restraint stress/non-stress) Multivariate ANOVA (MANOVA) was used for the behavioral experiments and an unpaired t-test was used to examine for differences in the corticosterone ELISA in this experiment. For the examination of basal anxiety-like behaviors between genotypes, a single factor MANOVA was used. For the experiment examining stress responsivity between genotypes, a two-factor (restraint stress/non-stress and genotype) MANOVA was used

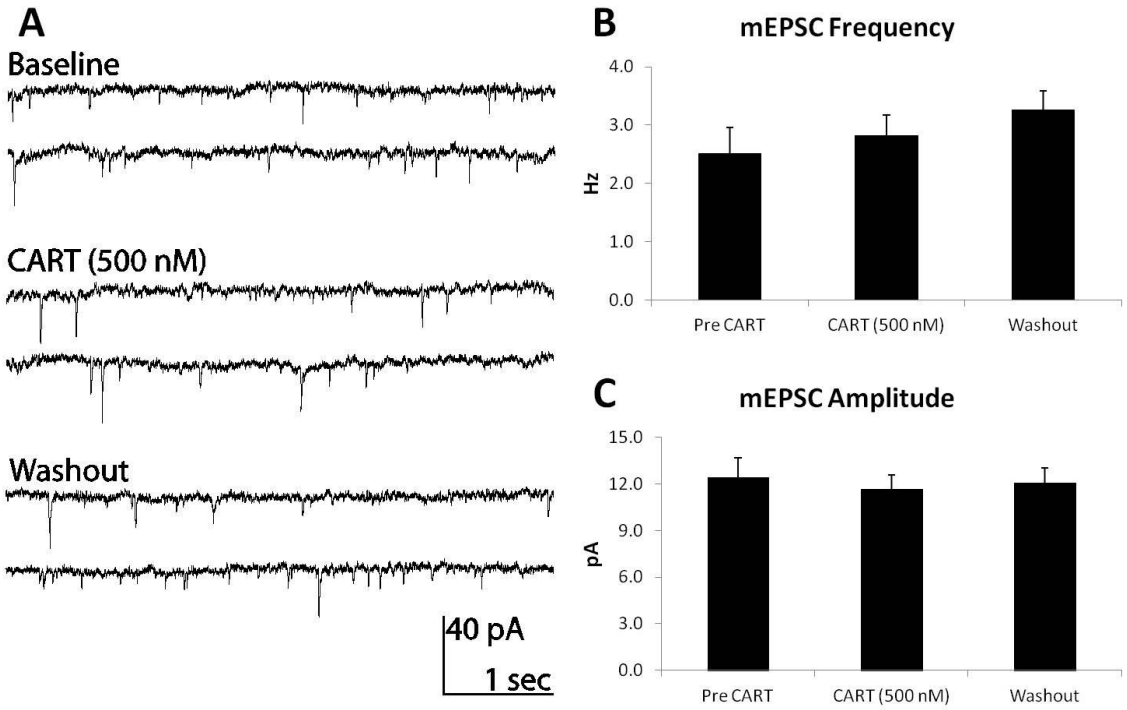
for the behavioral light/dark box data and a two-factor ANOVA was used to determine any statistical differences in the corticosterone ELISA data. For the ethanol consumption data in the CIE experiment, a two-factor (CIE treatment and genotype) repeated measures (baseline and post-CIE) ANOVA was used. For the light/dark box behavioral data and paired pulse ratio data in the CIE experiment, two-factor MANOVAs were used. Two-factor (CIE treatment and genotype) ANOVAs were used to examine the corticosterone ELISA data, sEPSC peak amplitudes, sEPSC frequencies, and AMPA/NMDA ratios.

RESULTS

Effects of acute CART peptide on mEPSCs and AMPA/NMDA ratios

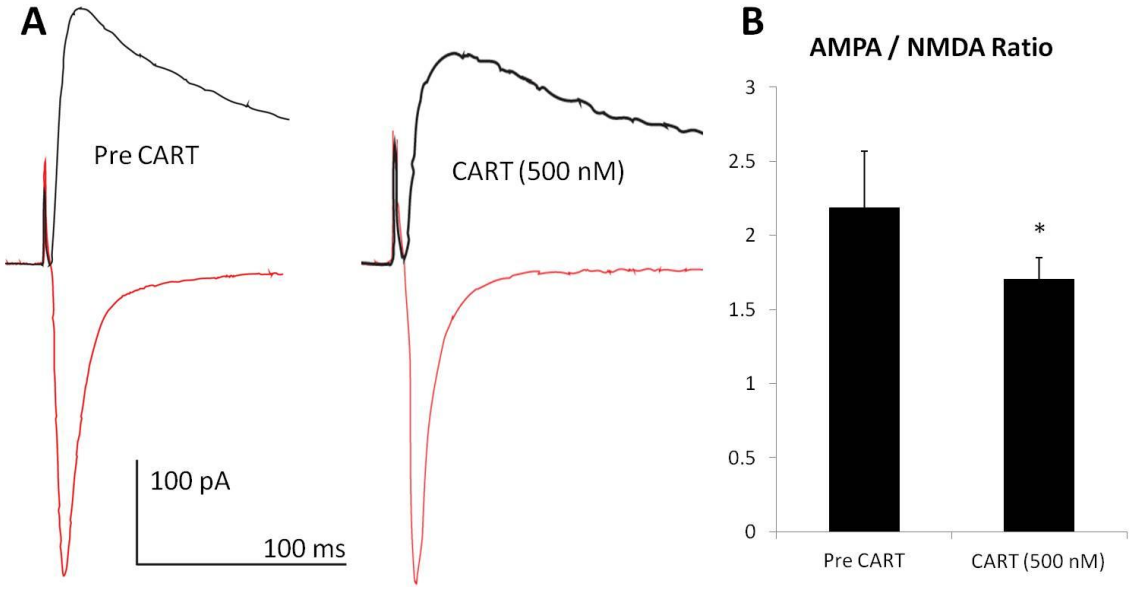
Acute application of 500 nM CART peptide did not have any significant effects on mEPSCs. Neither the average event peak amplitude nor the average event frequency was altered in the presence of CART peptide or after a ten minute washout period (Figure 6). The same 500 nM dose of CART peptide did result in a significant decrease in the AMPA/NMDA ratio ($F(1,5)=9.024$, $p<0.05$; figure 7). Given that CART had no effect on mEPSC amplitude and that the mEPSCs recorded were AMPA receptor-mediated (i.e. holding potential was -80 mV and the currents were DNQX-sensitive; data not shown) we can presume that the CART effect on AMPA/NMDA ratios is driven by a CART enhancement of NMDA receptor-mediated currents and not any effects of CART on AMPA receptor-mediated currents.

Figure 6. CART peptide (500 nM) had no effect on mEPSC peak amplitude or frequency



Neither the average event frequency nor the average event peak amplitude was altered in the presence of CART peptide. Washout of CART peptide also had no effect on mEPSCs. (A) Sample mEPSC activity recorded under baseline conditions, in the presence of CART, and after a washout period. (B) A bar graph representing the average mEPSC frequencies in all three conditions. (C) A bar graph representing the average mEPSC peak amplitude in all three conditions. n=10 cells from 8 mice.

Figure 7. CART peptide (500 nM) reduced AMPA/NMDA ratios in central amygdala neurons



Application of CART peptide reduced AMPA/NMDA ratios of central amygdala neurons. (A) Sample traces of the AMPA receptor-mediated currents collected at -80 mV (in red) and the compound AMPA & NMDA receptor-mediated currents collected at +40 mV (in black) under baseline (pre CART) and CART peptide conditions. (B) A bar graph representing the average AMPA/NMDA ratio under each condition. The average AMPA/NMDA ratio was significantly reduced in the presence of 500 nM CART peptide. n=6 cells from 6 mice. *, $p<0.05$

Validation of the light/dark box for assessment of anxiety-like behaviors

Before beginning any long term studies with an untested behavioral assay (at least in our lab), we decided to perform a positive control experiment. We briefly exposed mice to an empty, novel cage or an acute mild restraint stress and then assessed anxiety-like behaviors in the light/dark box assay.

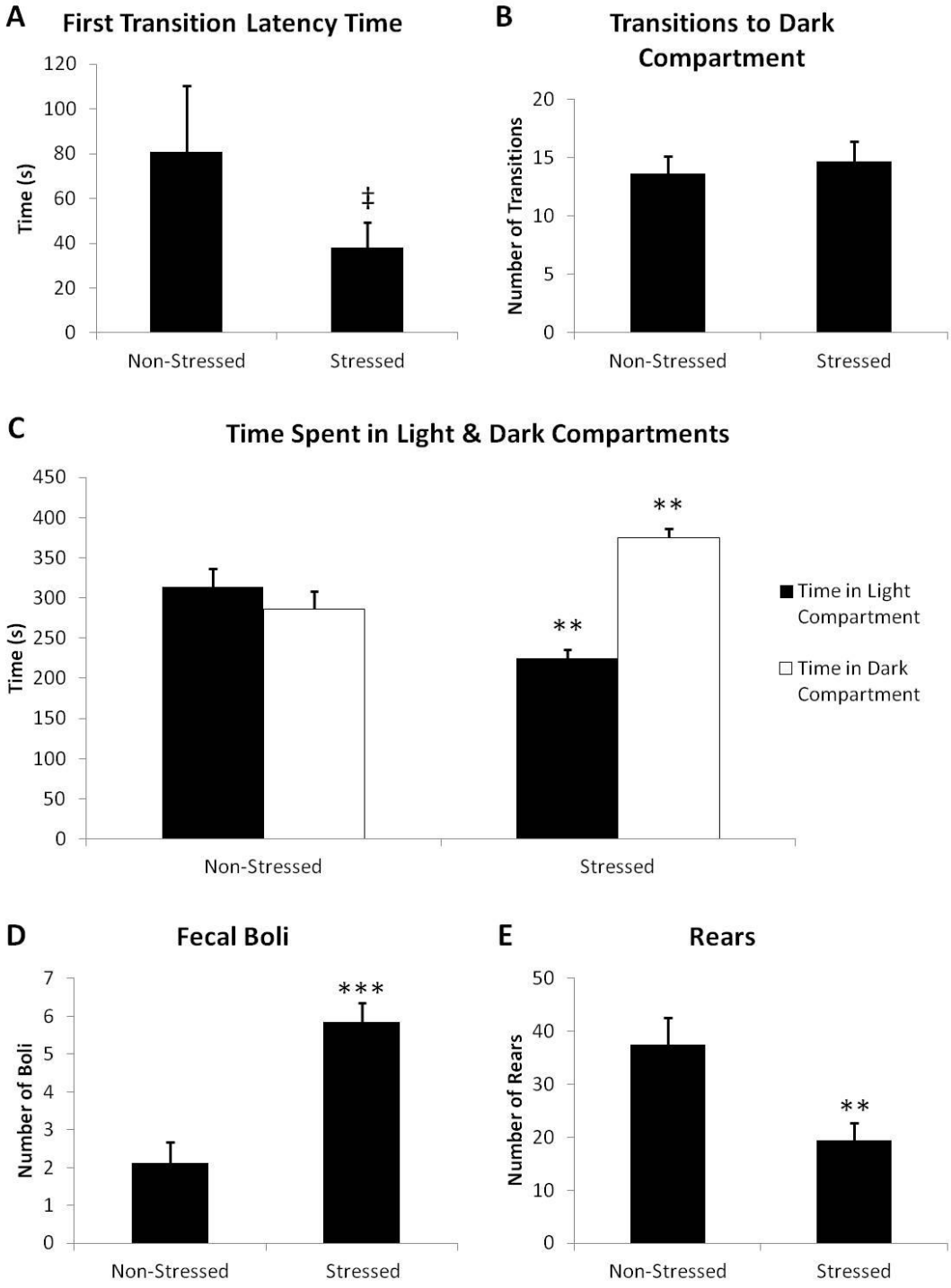
The latency time to the first transition into the dark compartment was compared between the two groups and a trend towards a decreased latency time was found in stressed mice ($F(1,13)=1.829$, $p<0.1$, Figure 8A). Given that mice begin the assay in the light compartment, it is believed that a lower latency time may reflect an increase in anxiety. The number of transitions between the light and dark compartments was compared between the two treatment groups to determine if there was any difference in general locomotor or exploratory activity. The average number of transitions did not differ between the two groups (Figure 8B). The total amount of time spent in the light and dark compartments was compared between the treatment groups. Acutely restrained mice displayed significantly less time in the light compartment than their non-stressed counterparts ($F(1,13)=13.4$, $p<0.01$; Figure 8C). This decrease in time spent in the light compartment was accompanied by a significant increase in time spent in the dark compartment of the apparatus ($F(1,13)=13.4$, $p<0.01$; Figure 8C). Given the natural aversion of rodents to brightly lit, open areas, we can infer that the decrease in time spent in the light compartment (and concomitant increase in time spent in the dark compartment) in stressed mice reflects an increased anxiety state relative to control mice. The number of fecal boli deposited in the DecapiCone™ and the light/dark box were counted. Stressed mice deposited significantly more fecal boli than their non-stressed counterparts ($F(1,13)=28.5$, $p<0.001$; Figure 8D). Exploratory rearing behaviors were

also tabulated and found to be significantly decreased in stressed mice ($F(1,13)=9.5$, $p<0.01$, Figure 8E).

Immediately following the light/dark box assay trunk blood samples were collected, the plasma isolated, and stored until assaying for corticosterone content. A corticosterone ELISA of the plasma samples determined that acutely restrained mice had a significantly higher plasma corticosterone content than their non-stressed counterparts ($t(16)=-4.7$; $p<0.001$; Figure 9).

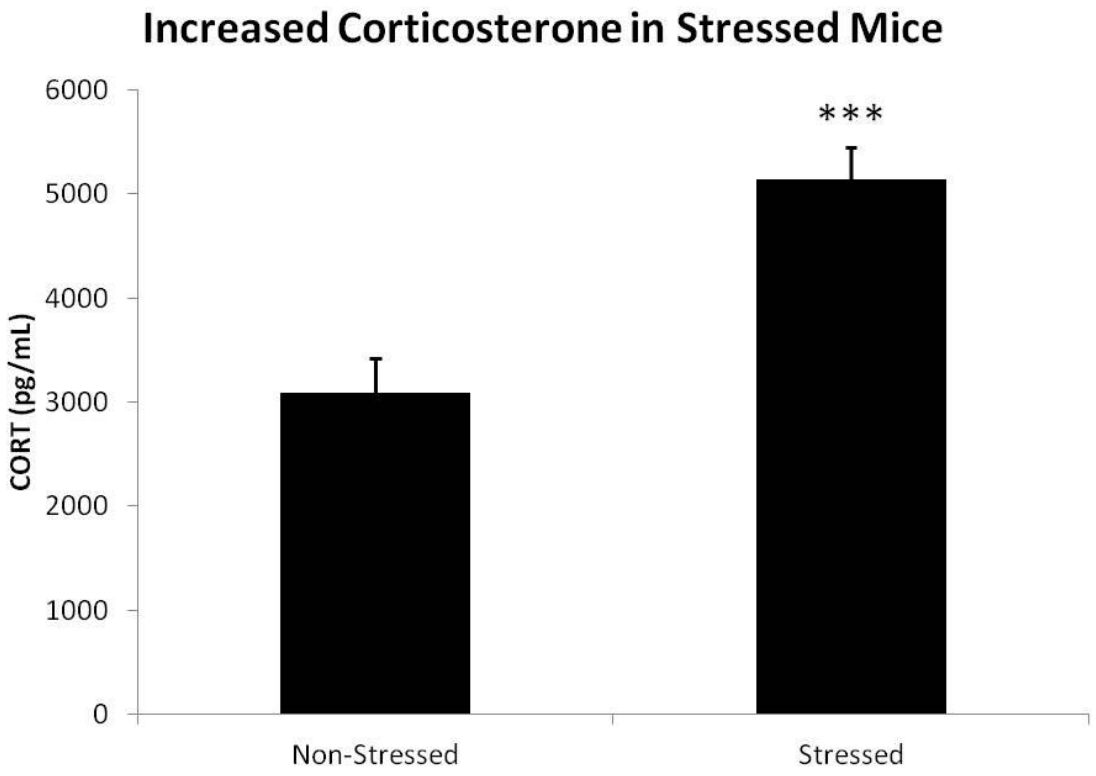
Taken together, the behavioral and physiological measures (corticosterone content) support the conclusion that mice with an increased anxiety state will display increases in anxiety-like behaviors in the light/dark box assay and further, they will have greater plasma corticosterone levels than mice with a decreased anxiety state.

Figure 8. An acute restraint stress exposure increased anxiety-like behaviors in the light/dark box assay



Acutely restrained mice displayed increased anxiety-like behaviors in the light/dark box assay. Bar graph representations of the average latency time of the first transition into the dark compartment of the box (A), the average number of transitions between the light and dark compartments (B), the average amounts of time spent in the light and dark compartments (C), the average number of fecal boli deposited (D), and the average number of exploratory rearing behaviors (E). n=8 and 7 for non-stressed and restraint stressed groups, respectively. ‡, $p<0.1$; **, $p<0.01$; ***, $p<0.001$ compared to non-stressed controls

Figure 9. Plasma corticosterone was increased in acutely restrained mice



Acutely restrained mice had increased plasma corticosterone content relative to their non-stressed counterparts. n=9 per treatment group. ***, $p<0.001$

Comparison of basal anxiety-like behaviors between CART KO and WT mice

Given that these studies would be the first to examine anxiety behaviors in CART KO mice, we first sought to compare baseline anxiety levels in CART KO and WT mice using the light/dark box assay. We found no significant genotype differences in any of the light/dark box parameters examined (Table 1). These findings suggest that there are no unforeseen compensatory effects in the (global) CART knockout mice under basal conditions.

Table 1. Basal anxiety-like behaviors do not differ between CART KO and WT mice

	Genotype	
Light/Dark box parameter	CART Wild Type	CART Knockout
Latency to first transition, s	159.4 ± 85.8	160.8 ± 98.4
Number of transitions	5.14 ± 1.32	6 ± 2.2
Time in light compartment, s	225.6 ± 72.5	262 ± 83
Time in dark compartment, s	374.4 ± 72.5	338 ± 83
Fecal Boli	1 ± 0.6	2.2 ± 1.3
Rears	12.7 ± 2.1	13 ± 3.3

Values are mean ± SEM. No significant differences were detected across any of the parameters examined. n=6 for CART knockout mice and n=7 for CART wild type mice

Anxiety/stress responsivity in CART KO and WT mice

Given the presence of CART at every level of the HPA axis and its putative role in the stress response, we performed an additional experiment to examine anxiety/stress responsivity in CART KO and WT mice. To that end, mice of both genotypes were exposed to an acute restraint stress or a clean novel cage before being tested in the light/dark box assay and having trunk bloods collected for later determination of corticosterone content.

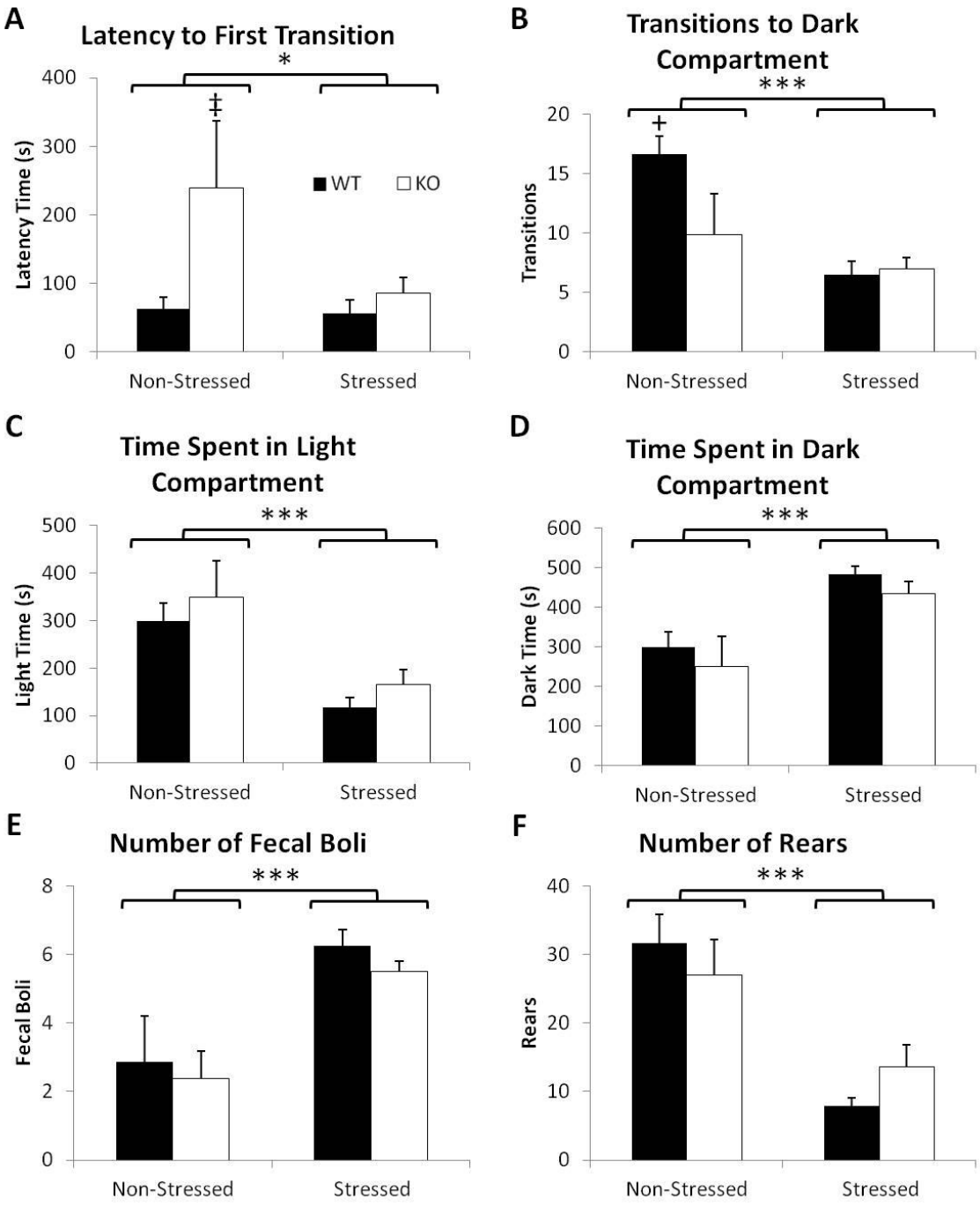
We found a significant main effect of restraint stress treatment on the latency to first transition ($F(1,28)=4.24$, $p<0.05$; Figure 10A), the number of transitions to the dark compartment ($F(1,28)=16.24$, $p<0.001$; Figure 10B), the amount of time spent in the light compartment ($F(1,28)=23.63$, $p<0.001$; Figure 10C), the amount of time spent in the dark compartment ($F(1,28)=23.8$, $p<0.001$; Figure 10D), the number of fecal boli ($F(1,28)=19.35$, $p<0.001$; Figure 10E), and the number of rears ($F(1,28)=32.56$, $p<0.001$; Figure 10F). There was a significant effect of genotype on the latency to the first transition ($F(1,28)=7.04$, $p<0.05$) and a trend towards a genotype effect on the number of transitions to the dark compartment ($F1,28)=3.75$, $p<0.1$). There was also a significant treatment by genotype interaction effect ($F=(1,28)=5.05$; $p<0.05$) on the number of transitions and a trend towards a significant treatment by genotype effect ($F1,28)=3.49$; $p<0.1$) on the latency to the first transition. Taken together, these results suggest that acutely stressed mice of both genotypes displayed an increase in anxiety-like behaviors relative to their respective non-stressed counterpart control mice.

After assaying the plasma corticosterone content of each mouse, we found a significant main effect of restraint stress treatment ($F(1,33)=57.56$, $p<0.001$) such that corticosterone was increased in restraint stress treated mice. We also found a significant main effect of genotype ($F(1,33)=5.25$, $p<0.05$) and a significant treatment by genotype

interaction effect ($F(1,33)=13.44$, $p<0.01$) such that CART KO mice seemed to have a potentiated corticosterone response compared to their WT counterparts (Figure 11).

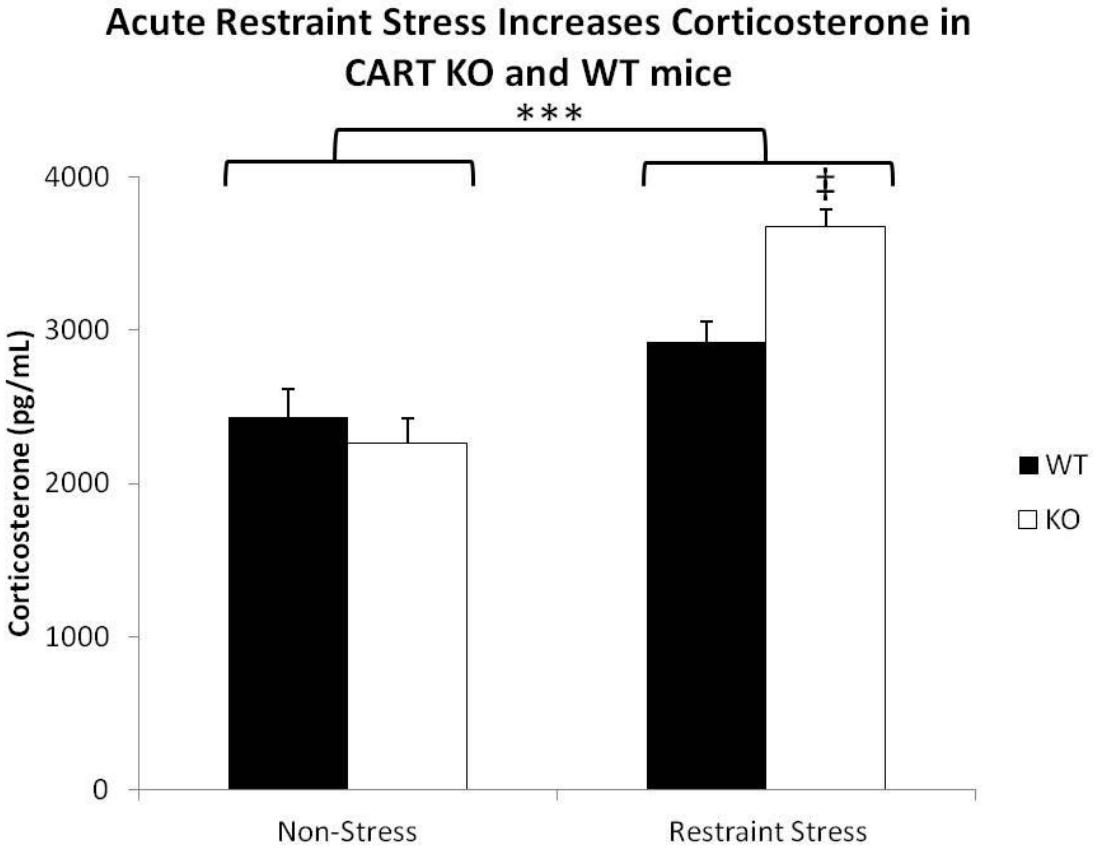
Taken together, the behavioral observations show no evidence for a significant difference between CART KO and WT mice in an acute restraint stress test. However, the corticosterone data are not in accord with the behavioral data for CART KO mice. That is to say, the behavioral data do not reflect the potentiated corticosterone response observed in CART KO mice relative to WT mice and could represent a limitation of the behavioral assay. It is also possible that HPA axis function is dysregulated in CART KO mice such that changes in anxiety states may not be accurately reflected or accompanied by changes in corticosterone content.

Figure 10. Acute restraint stress increased anxiety-like behaviors in CART KO and WT mice



Acutely restrained mice of both genotypes exhibited increases in anxiety like behaviors. Bar graph representations of the average latency time of the first transition into the dark compartment of the box (A), the average number of transitions between the light and dark compartments (B), the average amounts of time spent in the light and dark compartments (C), the average number of fecal boli deposited (D), and the average number of exploratory rearing behaviors (E). There was a main effect of treatment (non-stressed versus stressed) in all of the parameters examined (denoted by asterisks; *, $p<0.05$; ***, $p<0.001$). There was a significant main effect of genotype ($p<0.05$) on the latency to the first transition and a trend towards significance ($p<0.1$) for a treatment by genotype interaction (denoted by ‡). There was a trend towards a main effect of genotype ($p<0.1$) and a significant treatment by genotype interaction effect ($p<0.05$) on the number of transitions to the dark compartment (denoted by +). n=8-9 per group

Figure 11. CART KO and WT mice had increased plasma corticosterone in response to an acute restraint stress



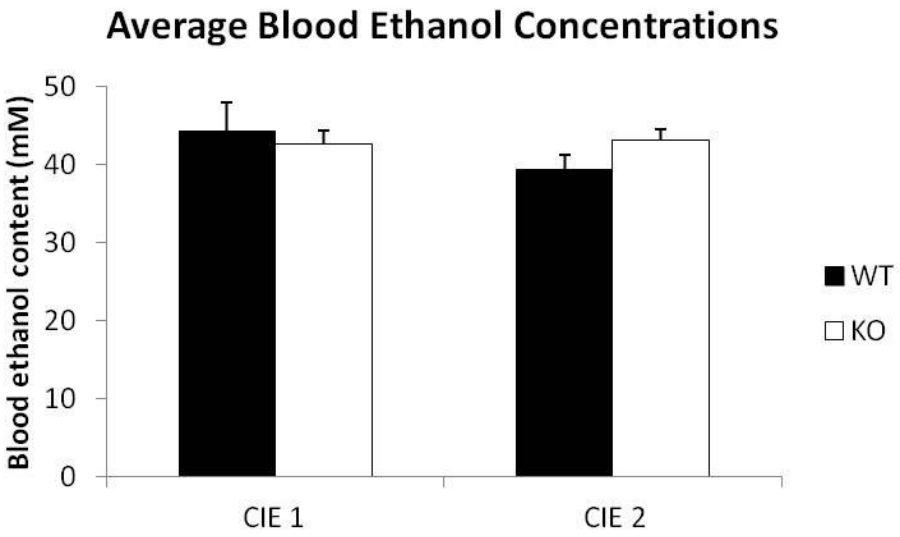
Corticosterone was increased in acutely restrained mice of both genotypes. CART KO mice displayed a potentiated corticosterone response relative the CART WT mice in response to the acute restraint stress session. n=8-10 per group. ***, $p<0.001$; ‡ denotes a significant treatment by genotype interaction ($p<0.01$) and a significant main effect of genotype ($p<0.05$).

Chronic intermittent ethanol vapor exposure increased ethanol consumption

In order for the CIE procedure to be effective, the blood ethanol content must be consistently between 175-225 mg/dL or 38-48 mM. The average BEC for all mice in the CIE groups was 42.4±2.2 mM (n=36), well within the necessary levels to achieve a CIE effect on ethanol consumption. There were no differences in BEC between genotypes or across CIE cycles (Figure 12). The average BEC for mice in the Air control group was always well below the limit of detection of our standard curve (~2.5 mM) and could not be distinguished from background noise levels on chromatographs.

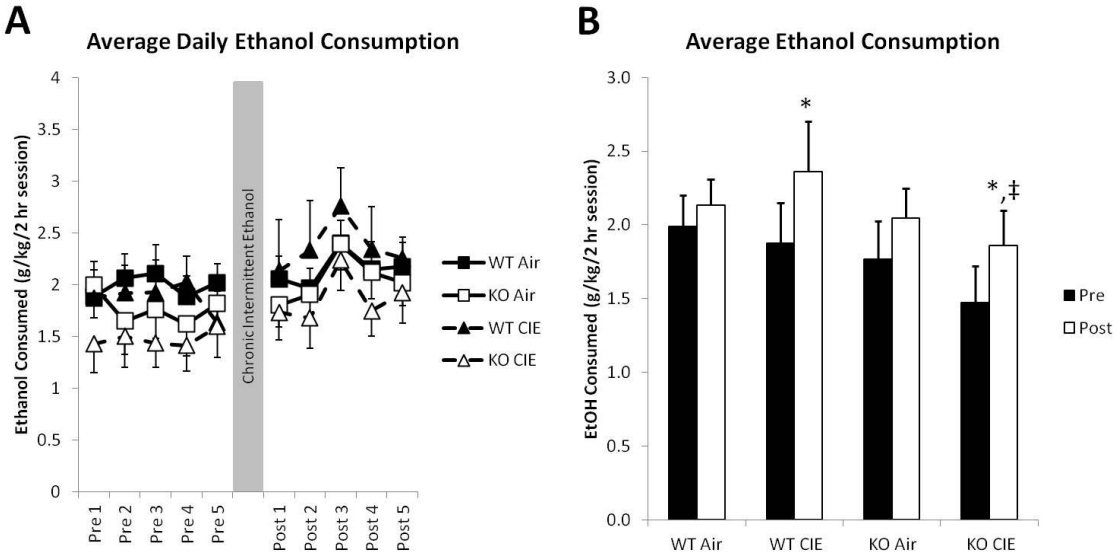
Mice in all of the treatment groups had an increase in their average ethanol consumption (Pre-CIE versus Post-CIE, $F(1,39)=8.46$, $p<0.01$); however, there was a main effect of treatment (Air versus CIE, $F(1,39)=6.96$, $p<0.05$) such that only CIE-treated mice (of both genotypes) had significant escalations in ethanol consumption. There were trends towards a genotype effect ($F(1,39)=3.95$, $p<0.1$) such that CART KO mice may consume less ethanol that WT mice. There was also a trend for a treatment by genotype interaction effect ($F(1,39)=3.58$, $p<0.1$) such that CART KO mice in the CIE treatment group, specifically, may consume less ethanol than CIE treated WT mice. The average daily ethanol consumption levels for each group are shown in figure 13A and the average ethanol consumption over the measured, five day, pre- and post-CIE drinking periods is summarized in figure 13B.

Figure 12. Blood ethanol concentrations were consistent during both CIE cycles



Blood ethanol concentrations did not differ between genotypes or CIE cycles. Air control mice had no detectable blood ethanol concentrations. n=8-14 per group

Figure 13. Chronic intermittent ethanol increased ethanol consumption



Mice that underwent chronic intermittent ethanol treatment of both sexes increased ethanol intake. There was a trend towards CART KO mice consuming less ethanol than their WT counterparts. (A) Ethanol consumption broken down by daily consumption and (B) bar graph representation of the average total ethanol consumption before and after CIE treatment. n=8-14 per group. *, $p<0.05$ relative to pre-CIE consumption; ‡, $p<0.1$ relative to WT CIE

Withdrawal from chronic intermittent ethanol reduces exploratory behaviors in the light/dark assay

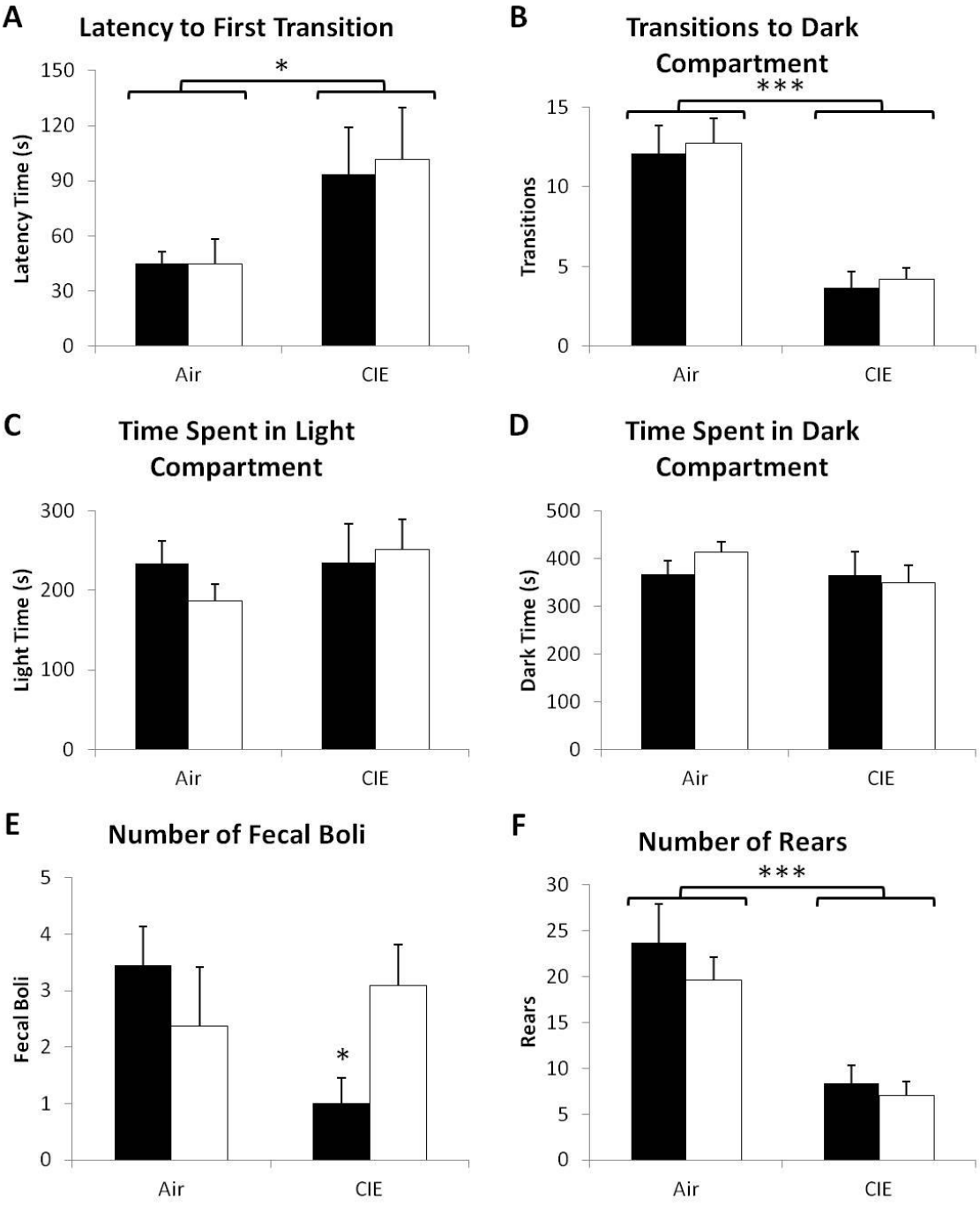
Chronic intermittent ethanol treatment resulted in significant decreases in exploratory behaviors in mice of both genotypes. The number of transitions between compartments (Figure 14B) and the number of exploratory rears (Figure 14F) were significantly reduced following CIE treatment ($F(1,33)=52.01$, $p<0.001$ & $F(1,33)=29.83$, $p<0.001$, respectively, for number of transitions and rears). There was also a significant increase in the latency to the first transition (Figure 14A) in CIE-treated mice ($F(1,33)=5.9$, $p<0.05$), however, this result may just be a reflection of the overall decrease in the number of transitions between compartments. There was a significant genotype by treatment interaction ($F(1,33)=4.64$, $p<0.05$) such that WT mice in the CIE treatment group had fewer fecal boli than the other treatment groups (Figure 14E). There were no significant genotype, treatment, or interaction effects on the amount of time mice spent in the light and dark compartments of each box (Figure 14C & D).

Withdrawal from two bouts of chronic intermittent ethanol treatment had significant inhibitory main effects on exploratory behaviors in both genotypes. The number of transitions between compartments was decreased in CIE-treated mice ($F(1,29)=20.96$, $p<0.001$; Figure 15B) relative to Air-treated mice. Similarly, the rearing exploratory behaviors were also significantly decreased ($F(1,29)=23.08$, $p<0.001$; Figure 15F) in CIE-treated mice of both genotypes when compared to the Air-treated mice. There was no significant effect of CIE on the total amount of time spent in the light or dark compartments; however, there was a significant effect of genotype such that CART KO mice spent a greater amount of time in the light compartment ($F(1,29=7.02$, $p<0.05$; Figure 15C) than WT mice in both the Air and CIE treatment groups. Accordingly, CART KO mice spent less time in the dark compartment ($F(1,29)=7.02$, $p<0.05$; Figure

15D) than WT mice in both treatment groups. There was also a trend towards a genotype effect on the latency time to the first transition such that CART KO mice in both treatment groups had a greater latency time ($F(1,29)=3.27$, $p<0.1$; Figure 15A) than WT mice. There were no significant difference in the number of fecal boli between genotypes or treatment groups (Figure 15E).

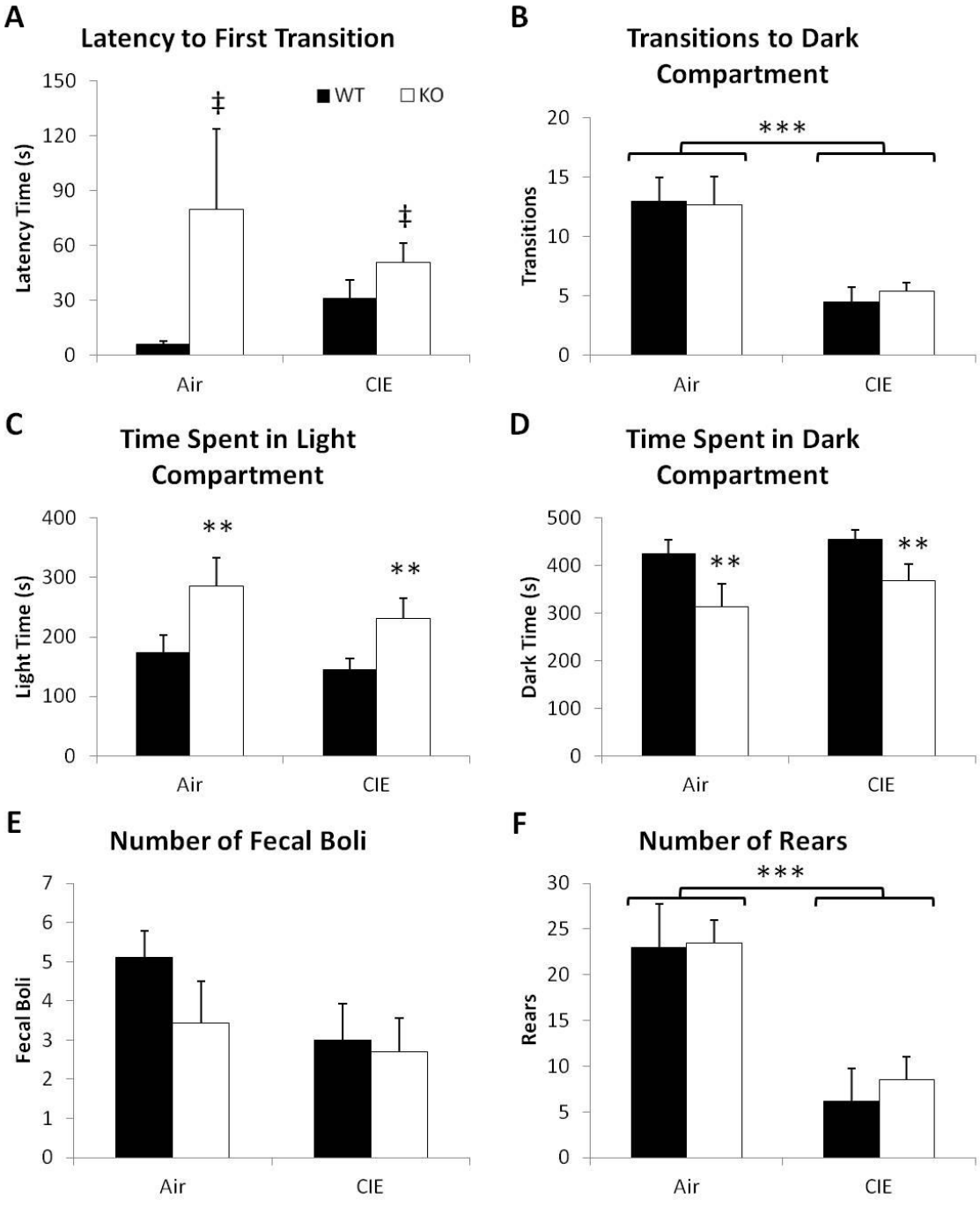
Overall, it appears that one or two bouts of CIE treatment and acute withdrawal result in a modest increases in anxiety-like behaviors. Specifically, exploratory behaviors were significantly inhibited during withdrawal while the overall amount of time spent in the light and dark compartments was unaffected by CIE treatment. Interestingly, a genotype difference in anxiety-like behaviors arose during the course of the experiment such that CART KO mice in both treatment groups spent significantly more time in the light compartment than WT mice following the second CIE bout.

Figure 14. Withdrawal from one bout of chronic intermittent ethanol reduces exploratory behaviors in the light/dark box assay



Acute withdrawal from chronic intermittent ethanol decreases exploratory behaviors in the light/dark box assay. Bar graph representations of the average latency time of the first transition into the dark compartment of the box (A), the average number of transitions between the light and dark compartments (B), the average amounts of time spent in the light and dark compartments (C), the average number of fecal boli deposited (D), and the average number of exploratory rearing behaviors (E). n=8-11 mice per group. *, $p<0.05$; ***, $p<0.001$

Figure 15. Light/Dark box behaviors during the second withdrawal from chronic intermittent ethanol

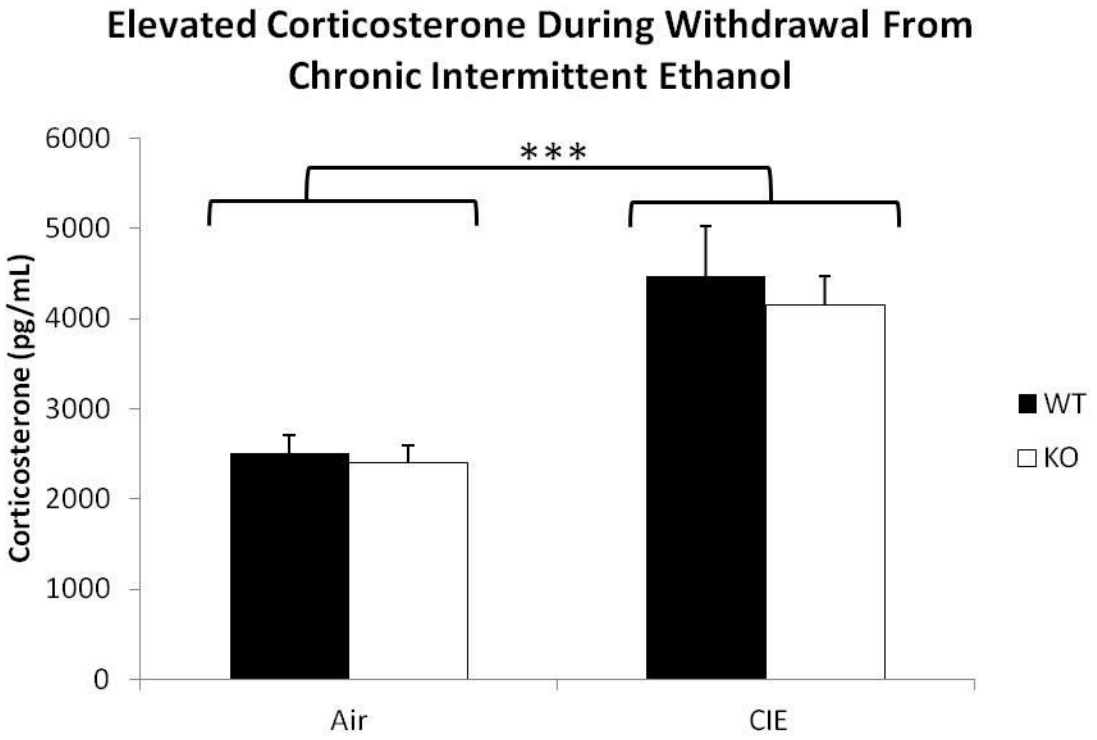


Withdrawal from two bouts of chronic intermittent ethanol significantly decreased the number of transitions between compartments and exploratory rearing behavior in mice of both genotypes. Bar graph representations of the average latency time of the first transition into the dark compartment of the box (A), the average number of transitions between the light and dark compartments (B), the average amounts of time spent in the light and dark compartments (C), the average number of fecal boli deposited (D), and the average number of exploratory rearing behaviors (E). n=6-10 mice per group. ‡, $p<0.1$ compared to WT mice; **, $p<0.01$ compared to WT mice; ***, $p<0.001$ compared to Air treatment.

Corticosterone is increased during withdrawal from chronic intermittent ethanol

Plasma corticosterone levels were significantly increased in mice of both genotypes during withdrawal from chronic intermittent ethanol ($F(1,35)=35.45$, $p<0.001$; Figure 16). Interestingly, there was no effect of genotype on plasma corticosterone levels in either treatment. There was also no significant genotype by treatment interaction effect. These results match the behavioral observations in that they demonstrate an increase in stress in CIE-treated mice; however, they do not reflect the genotype effect observed for CART KO mice.

Figure 16. Plasma corticosterone was increased during withdrawal from chronic intermittent ethanol vapor exposure



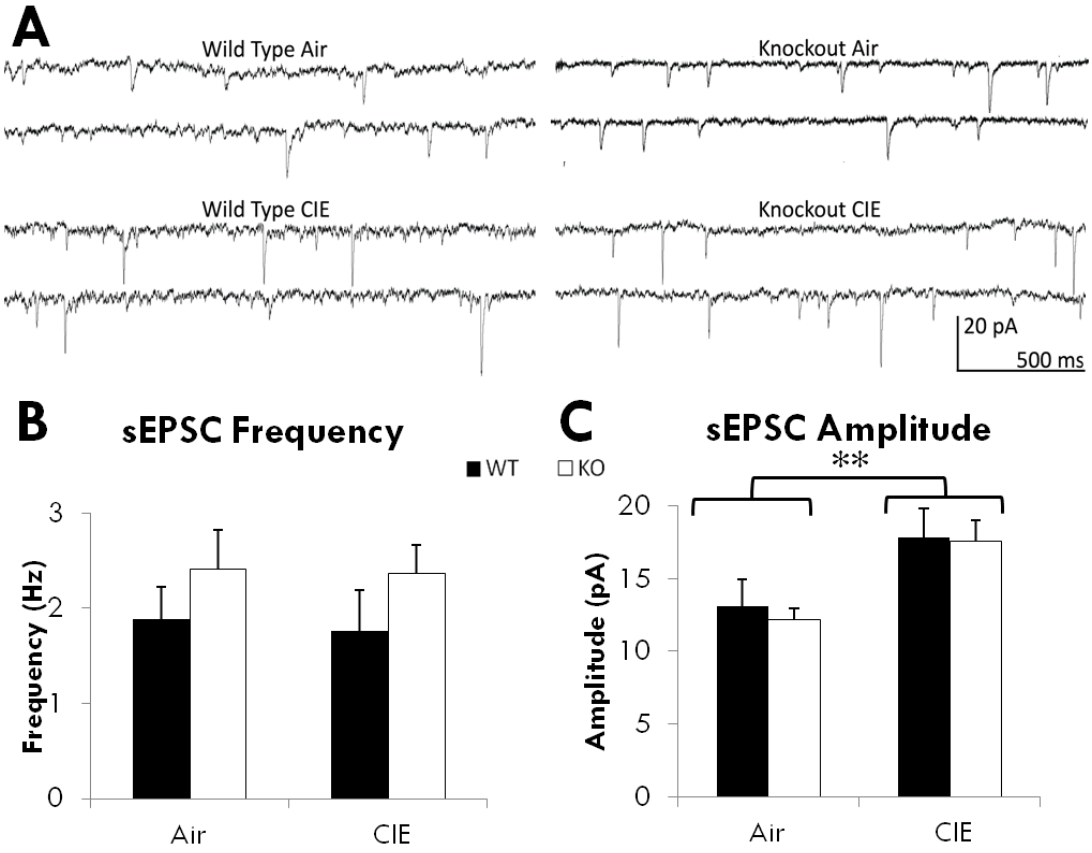
Withdrawal from chronic intermittent ethanol treatment significantly increased plasma corticosterone levels in CART KO and WT mice. n=9-12 per group. ***, $p<0.001$

Chronic intermittent ethanol increases sEPSC amplitudes in mice of both genotypes

Central amygdala sEPSC activity was measured in mice from all treatment groups (representative traces in Figure 17A). We found no significant main effects of treatment, genotype, or a treatment by genotype interaction on the frequency of sEPSCs (Figure 17B); however, there was a main effect of chronic intermittent ethanol treatment on sEPSC amplitude ($F(1,26)=13.42$, $p<0.01$; Figure 17C) such that CIE-treated mice had a greater average sEPSC amplitude than Air treatment controls in both genotypes. There was no significant effect of genotype or a treatment by genotype effect on sEPSC amplitude.

The observed increase in sEPSC amplitude in CIE-treated mice of both genotypes is indicative of a post synaptic modification in glutamatergic neurotransmission. Furthermore, since sEPSCs recorded at -80 mV are completely inhibited by DNQX, an AMPA receptor antagonist, we may presume that the post synaptic modification involves AMPA receptors. This neuroadaptation could be involved in mediating the observed increases in ethanol consumption following CIE exposure or in modulating the anxiety-like behaviors observed during withdrawal from CIE.

Figure 17. Chronic intermittent ethanol increases sEPSC amplitude

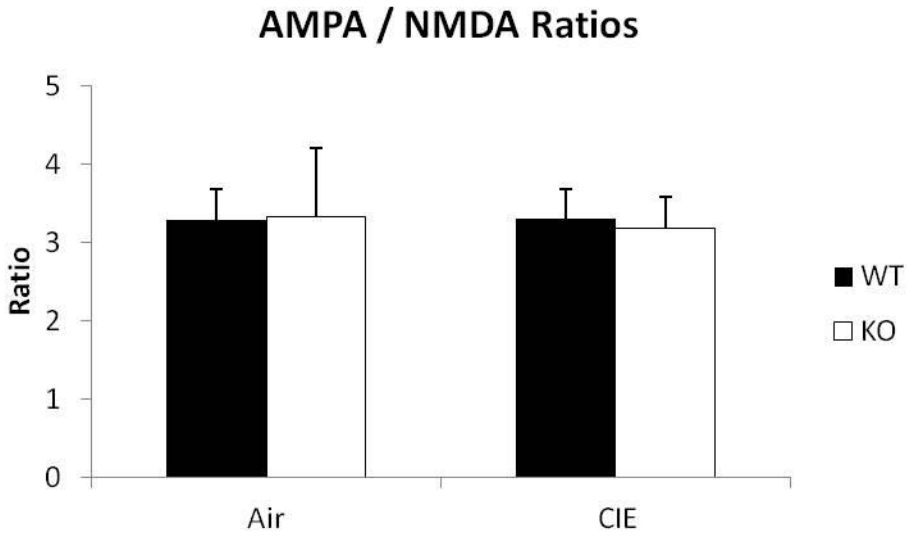


(A) Sample traces of spontaneous EPSC activity from all four treatment conditions. (B) Bar graph representation of sEPSC frequencies. There were no differences in sEPSC frequency between genotype or treatment groups. (C) Bar graph representation of sEPSC amplitudes. Chronic intermittent ethanol treatment significantly increased sEPSC amplitudes in both CART KO and WT mice. n=6-10 cells per group from 3-8 mice per group. **, $p<0.01$

Chronic intermittent ethanol did not affect AMPA/NMDA ratios

In addition to monitoring sEPSC activity, we also assessed the AMPA/NMDA ratio of central amygdala neurons in mice from each treatment group. We found that there were no main effects of genotype or CIE treatment on AMPA/NMDA ratios in any of the treatment groups. We also found that there was no significant genotype by treatment interaction effect on the AMPA/NMDA ratios (Figure 22).

Figure 18. AMPA/NMDA Ratios do not differ between treatment groups

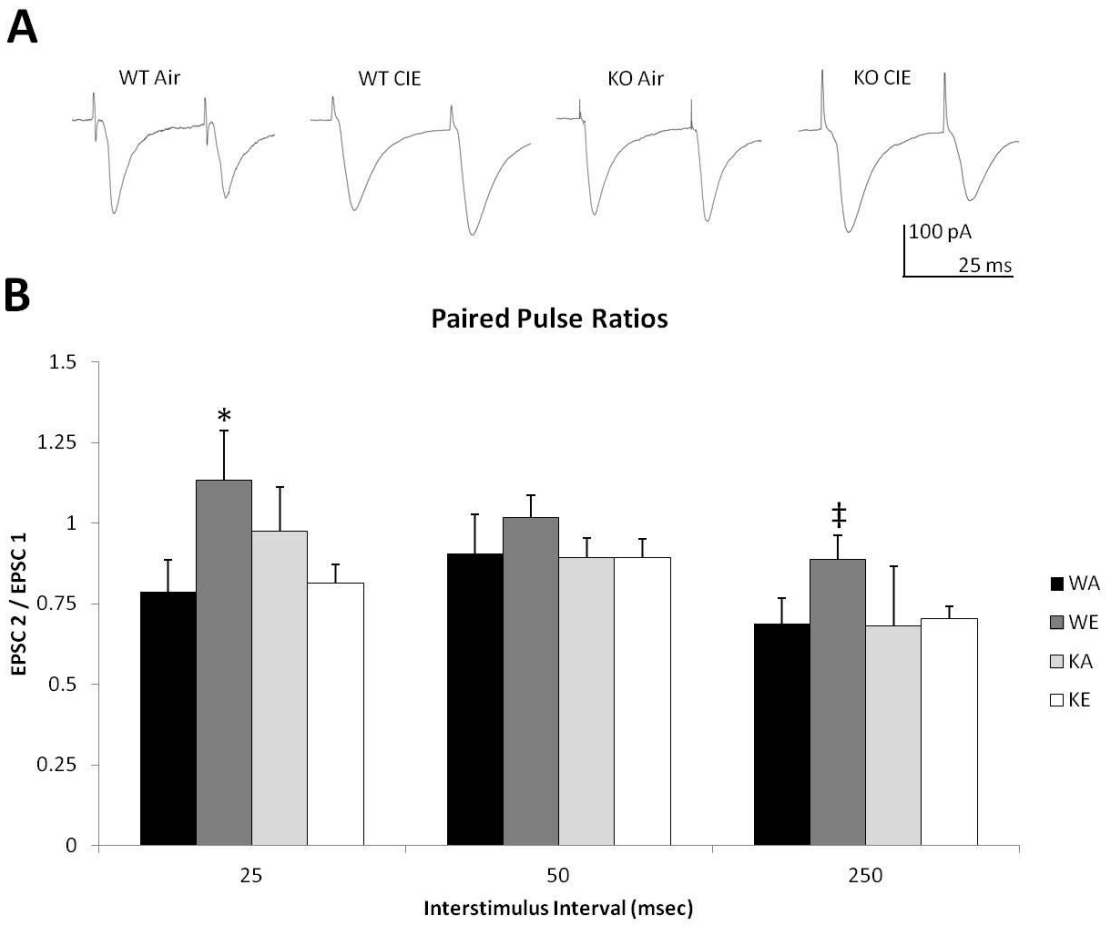


Bar graph representation of average AMPA/NMDA ratios from all treatment groups. There were no changes in the ratios in response to CIE treatment or genotype. n=7-12 cells from 4-8 mice

Chronic intermittent ethanol decreases presynaptic release probability

The final electrophysiological parameter assessed in this study was the paired pulse ratios of eEPSCs at 25, 50, and 250 millisecond interstimulus intervals (Figure 19). We found that there were no main effects of CIE treatment or genotype at any of the interstimulus intervals tested; however, chronic intermittent ethanol exposure resulted in a significant treatment by genotype interaction effect such that the paired pulse ratio was increased at the 25 millisecond interstimulus interval for WT mice ($F(1,24)=5.99$, $p<0.05$). There was also a trend for a significant treatment by genotype interaction for the 250 millisecond interstimulus interval for WT mice ($F(1,24)=2.72$, $p<0.1$). The observed increase in paired pulse ratio in this study may represent an additional neuroadaptation that contributes to the observed increase in anxiety-like behaviors during withdrawal from CIE. It is noteworthy, however, that the increase in paired pulse ratio was unique to WT mice, whereas, the increase in behavioral anxiety measures may not be.

Figure 19. Chronic intermittent ethanol increased paired pulse ratios



Chronic intermittent ethanol treatment increased paired pulse ratios in WT mice at the 25 millisecond interval, suggesting decreased presynaptic release probability. (A) Sample traces of paired pulse experiments at the 25 ms interstimulus interval for each genotype and CIE treatment. (B) Bar graph representation of paired pulse ratio data. n=4-10 cells per group from 3-7 mice. *, $p<0.05$ & ‡, $p<0.1$ relative to all other treatment groups

Table 2. Summary of results

Experiment	Result	Significance	Figure/Reference
CART peptide effects on mEPSCs and AMPA/NMDA ratio			
mEPSC frequency	No effect	None	6A
mEPSC amplitude	No effect	None	6B
AMPA/NMDA Ratio	↓	$p<0.05$	7
Acute restraint stress in CART KO and WT mice			
Latency to first transition			
Stress effect	↓	$p<0.05$	10A
Transitions between compartments			
Stress effect	↓	$p<0.001$	10B
Genotype by stress effect	↑ in NS WT	$p<0.05$	
Time spent in light			
Stress effect	↓	$p<0.001$	10C
Time spent in dark			
Stress effect	↑	$p<0.001$	10D
Fecal Boli			
Stress effect	↑	$p<0.001$	10E
Rears			
Stress effect	↓	$p<0.001$	10F
Corticosterone			
Stress effect	↑	$p<0.001$	11
Genotype effect	↑ in KO	$p<0.05$	
Genotype by stress effect	↑ in RS KO	$p<0.01$	
Ethanol consumption			
Pre-vs-post CIE	↑	$p<0.01$	13
Genotype effect	↑ in WT	$p<0.1$	
Treatment effect	↑ in CIE	$p<0.05$	
Genotype by treatment effect	↓ in CIE KO	$p<0.1$	
Anxiety-like behaviors following one bout of CIE			
Latency to first transition			
CIE effect	↑	$p<0.05$	14A

Table 2, continued

Transitions between compartments			
CIE effect	↓	p<0.001	14B
Time spent in light	No differences		14C
Time spent in dark	No differences		14D
Fecal Boli			
Stress effect	↓ in CIE WT	P<0.05	14E
Rears			
CIE effect	↓	p<0.001	14F
Anxiety-like behaviors and corticosterone following two bouts of CIE			
Latency to first transition			
Genotype effect	↑	p<0.1	15A
Transitions between compartments			
CIE effect	↓	p<0.001	15B
Time spent in light			
Genotype effect	↑	p<0.01	15C
Time spent in dark			
Genotype effect	↓	p<0.01	15D
Fecal Boli	No differences		15E
Rears			
CIE effect	↓	p<0.001	15F
Corticosterone			
CIE effect	↑	p<0.001	16
Electrophysiology measures following two bouts of CIE			
sEPSC Frequency	No differences		17B
sEPSC Amplitude	↑	p<0.01	17C
AMPA/NMDA Ratio	No differences		18
Paired Pulse Ratio 25 ms			
Genotype by treatment effect	↑ in CIE WT	p<0.05	19B
Paired Pulse Ratio 50 ms	No differences		19B
Paired Pulse Ratio 250 ms			
Genotype by treatment effects	↑ in CIE WT	p<0.1	19B

DISCUSSION

We found that chronic intermittent ethanol treatment produced a number of significant behavioral, endocrine, and electrophysiological effects. We further found that some of these effects were attenuated in CART KO mice suggesting a critical role for CART peptides in these effects.

To our knowledge, this study is the first to use CART KO mice to examine anxiety- and stress-related behaviors and the first study from our lab to examine anxiety-related behaviors. Thus we began by validating our behavioral apparatus – the light/dark box – by exposing a group of mice to an acute restraint stress and subsequently examining anxiety-like behaviors and plasma corticosterone content. We found that the light/dark box was a suitable assay and that we could reliably detect differences in anxiety-like behaviors and corticosterone levels between stressed and non-stressed mice. We next chose to compare the stress response between CART KO and WT mice since any differences in stress responsivity could have possible implications on the interpretation of subsequent studies. We found that CART KO and WT mice did not differ in their behavioral responses to an acute stressor; however, we did find that the plasma corticosterone response to an acute stressor was potentiated in CART KO mice relative to WT mice. Given the ubiquitous presence and the complex interactions of CART peptides throughout the HPA axis (for review see Koylu et al. (2006) and Rogge et al. (2008)), there are a number of possible (neuro)endocrine mechanisms contributing to this result. Determining which of these mechanisms is made difficult not only by the presence of CART peptide at every level of the HPA axis, but by the paucity of data about stress and anxiety in CART KO mice. Nonetheless, this novel finding was kept in consideration during the interpretation of all subsequent results. Interestingly, a missense mutation in the CART gene (Leu34Phe) was found to result in lower levels of bioactive

CART peptide and has been associated with increased anxiety and depression in adolescents from an Italian family (Miraglia del Giudice et al., 2006; Yanik et al., 2006). Given the potentiated corticosterone response observed in our CART KO mice following an acute stressor, it is possible that the Leu34Phe mutation present in humans, which significantly reduces bioavailable CART (Dominguez et al., 2004), may recapitulate the exaggerated glucocorticoid response to a stressor we observed in our studies. Following this line of reasoning, if every stressor encountered resulted in an aberrant stress response, it is possible that the chronic stress conditions (i.e. chronically elevated circulating glucocorticoid levels) experienced by people with this mutation contributes to or underlies the phenotypic observations of increased anxiety and depression.

Among the primary effects of CIE treatment, we observed was a significant increase in ethanol consumption after a single bout of CIE vapor exposure. Interestingly, there was a statistical trend for a genotype effect such that CART KO mice may consume less ethanol than WT mice. This result would be in accord with our previous data demonstrating a significant reduction in ethanol consumption and preference in CART KO mice (Salinas et al., 2012); however, the difference in ethanol consumption conditions used should be noted. In our previous results, we employed an unlimited access, two bottle choice assay with a number of ethanol concentrations (including the 15% concentration used in the current study). The current study (and most CIE models in general) utilized a modified drinking-in-the-dark procedure which, by design, are meant to escalate ethanol consumption to result in blood ethanol concentrations that correlate with behavioral intoxication (Rhodes et al., 2005). We therefore believe that the genotype effect of reduced ethanol consumption in CART KO mice may have been masked in this study by the use of a limited access procedure. There was also a statistical trend towards a significant genotype by treatment interaction such that CART KO mice

in the CIE group may consume less ethanol than their WT counterparts. This result may reflect a decreased efficacy of chronic intermittent ethanol treatment in CART KO mice to increase ethanol consumption and withdrawal anxiety-like behaviors.

Behaviorally, we found that early withdrawal (~6.5 hours) from a single bout of CIE treatment resulted in a significant decrease in exploratory behaviors, suggesting an increase in anxiety. There was also a significant increase in the latency to the first transition in CIE-treated mice. This latter result might imply a decrease in anxiety; however, when taken in the context of a reduction in the total number of transitions between compartments (which is also a measure of overall locomotor activity), this interpretation becomes questionable. Another possible explanation for this result is that the increase in latency time is just a byproduct of the overall reduction in the number of transitions between compartments/exploratory activity. Unexpectedly, we found a decrease in the number of fecal boli in CIE WT mice. Normally, we would interpret this as a sign of reduced anxiety, but upon examination of the boli we noticed a marked change in texture such that we believe CIE-treated mice may be dehydrated during early withdrawal. Thus, we feel that under these circumstances the number of fecal boli may not be a representative measure of an anxious or stressed state.

Acute withdrawal following two bouts of CIE treatment also resulted in a significant decrease in exploratory behaviors (number of transitions between compartments and number of exploratory rears), suggesting an increase in anxiety. Interestingly, a significant genotype effect on the total amount of time spent in the light and dark compartments was unmasked after two CIE cycles such that CART KO mice spent more time in the light compartment (with a concomitant decrease in the amount of time spent in the dark compartment) suggesting a decrease in anxiety. This effect was present in both Air and CIE treatment conditions to an equal extent. There was also a

statistical trend towards a genotype effect in the latency to the first transition such that CART KO mice in both treatment groups had increased latency times. Given that there were no differences in the number of transitions/overall locomotor activity between compartments within treatment conditions, the possible increase in latency times in CART KO mice may also be interpreted as a decrease in anxiety.

We then examined the corticosterone content of mice undergoing withdrawal from two bouts of CIE immediately following their trial in the light/dark box assay. We found that mice undergoing withdrawal from CIE had nearly twice the amount of corticosterone than their respective counterparts in the Air treatment groups, potentially supporting the observed behavioral increases in anxiety as measured by decreases in exploratory behaviors. The corticosterone results may not reflect the potential decrease in anxiety indicated by the increased amount of time CART KO mice spent in the light compartment; however, given our earlier results showing a potentiated corticosterone response in acutely restrained CART KO mice, one might presume that the increases in anxiety (as evidenced by decreases in exploratory behaviors) and concomitant potentiated corticosterone response in CART KO mice may be masking any genotype differences in anxiety (as evidenced by the increase in light time) such that the corticosterone levels in CIE treated mice are similar. That is to say, if CART KO mice are experiencing an intermediate level of anxiety compared to WT mice, the potentiated endocrine response may be raising corticosterone levels of CART KO mice to approximately match those of WT mice. Further studies, however, would be required to examine this possibility.

Electrophysiologically, we found that acute application of CART peptide onto central amygdala neurons of ethanol naïve mice had no effect on mEPSC frequency or amplitude, indicating that CART peptide does not have a postsynaptic effect on AMPA receptor-mediated transmission. The lack of a CART peptide effect on mEPSC

frequency also indicates that CART peptide has no presynaptic actions. We did, however, observe a significant effect of CART peptide application on AMPA/NMDA ratios such that the ratio was decreased. When taken together with our mEPSC data and the previous literature showing that CART does not affect AMPA receptor-mediated neurotransmission, we can infer that the observed effect of CART peptide on AMPA/NMDA ratios was mediated by a relative increase in NMDA receptor-mediated currents. This finding is in accord with previous studies showing that CART peptide enhances NMDA receptor-mediated neurotransmission (Dun et al., 2006; Hsun Lin et al., 2005). Furthermore, when considered alongside the finding that CART peptide was increased in the central amygdala during ethanol withdrawal (Dandekar et al., 2008a), this CART peptide-mediated enhancement of central amygdala glutamatergic signaling may represent the cellular mechanism through which CART peptides exert their anxiogenic effects *in vivo* and support our overall hypothesis that CART peptide-mediated increases in glutamatergic signaling modulate ethanol withdrawal-induced anxiety.

Examination of the spontaneous excitatory postsynaptic currents from mice in the chronic intermittent ethanol study revealed no genotype or CIE effects on sEPSC frequency; however, we did observe a significant increase in the average event amplitudes in the CIE treated mice of both genotypes. Given that these sEPSCs were collected at -80 mV (at which the Mg^{2+} block on NMDA receptors should be intact) and that these currents were DNQX-sensitive (an AMPA receptor antagonist), we can assume that the currents were strictly AMPA receptor-mediated and that the effects of CIE were specifically on postsynaptic AMPA receptors.

We found no differences in the AMPA/NMDA ratios in any of the treatment groups. Given our finding that CIE treatment increased AMPA receptor-mediated sEPSC

amplitudes, we might expect an increase in the AMPA/NMDA ratios of CIE-treated mice. One possibility for the lack of an observed difference in the AMPA/NMDA ratios between treatment groups is that there was an undetected concomitant increase in NMDA receptors such that any potential increase in AMPA receptor-mediated transmission was countered by an increase in NMDA receptors, thus leaving the relative ratio of AMPA to NMDA receptors unchanged. Increases in NMDA receptor function and/or subunit expression are among the most common neuroadaptations brought about by chronic ethanol treatments (Chandler et al., 1993; Hendricson et al., 2007; Kalluri et al., 1998; Kroener et al., 2012; Lack et al., 2007; Roberto et al., 2006; Trevisan et al., 1994; Wang et al., 2012). Despite this, we did not directly measure NMDA receptor-mediated currents or expression in the current study so we cannot confirm any speculation apropos our observed AMPA/NMDA ratio results.

We also examined paired pulse ratios in mice from the CIE experiments at 25, 50, and 250 millisecond interstimulus intervals. Increases in the paired pulse ratio are thought to reflect a decrease in presynaptic release probability, while decreases in PPR are thought to represent an increase in presynaptic release probability. We observed a significant increase in PPR25 and a statistical trend towards an increase in PPR250 in wild type CIE-treated mice. This decrease in presynaptic release probability was absent in all of the other treatment groups and thus may reflect a unique CART peptide-dependent neuroadaptation that contributes to either (or both) the enhanced ethanol intake or the increased anxiety-like behaviors observed in WT mice.

Our behavioral and electrophysiological findings are in accord with a number of studies demonstrating chronic ethanol and withdrawal effects on anxiety-like behaviors and AMPA receptor-mediated currents. Lack et al. (2007) found that 12 hours per day of ethanol vapor exposure for ten consecutive days resulted in increased anxiety-like

behaviors in rats immediately following their final ethanol exposure, as well as, following 24 hours of withdrawal. Similar to our findings, this group found significant decreases in exploratory rearing and compartment transition behaviors during withdrawal. Corresponding to these increased anxiety-like behaviors, they also found increased sEPSC amplitudes in basolateral amygdala neurons from Sprague-Dawley rats immediately following the final ethanol vapor exposure and mEPSC amplitudes following 24 hours of withdrawal. They finally demonstrated that the observed increases in anxiety-like behaviors were AMPA receptor-dependent by microinjecting DNQX bilaterally into the basolateral amygdala and showing a decrease in anxiety-like behaviors. A subsequent study from the same group found an increase in asynchronous EPSC (aEPSCs) amplitudes in rats after 24 hours of withdrawal, but not in rats that did not undergo withdrawal (Christian et al., 2012). This effect may have been caused by concomitant increases in the surface expression of GluA1 and GluA2/3 AMPA receptor subunits. These aEPSCs were also found to be DNQX-sensitive, demonstrating that they were mediated exclusively by AMPA receptors.

Several studies have described *in vivo* chronic ethanol (and withdrawal) treatment-induced increases in AMPA receptor function or expression. Haugbol et al. (2005) found that after 84 hours of continuous ethanol exposure and 12 hours of withdrawal, AMPA receptor binding in the cerebrum increased 94% above control levels. Wang et al. (2012) found that chronic ip ethanol injections increased synaptic protein levels of the AMPA receptor subunits, GluR1 and GluR2, relative to saline-treated control mice in the dorsal medial striatum (DMS). This same study found that inhibition of AMPA receptors in the DMS reduced operant self administration of ethanol but not sucrose. Marty and Spigelman (2012) found that a chronic intermittent ethanol treatment paradigm produced an enhancement of AMPA receptor mEPSC amplitudes in the

nucleus accumbens core, ostensibly due to a CIE-induced increase in GluA2 lacking AMPA receptors. A recent study from our lab found that a single three day bout of CIE in adolescent mice resulted in a metaplastic shift from LTD to LTP in AMPA receptor-mediated EPSCs (Jeanes et al., 2012). Further studies have found that *in vivo* modulation of AMPA receptors exert significant effects on ethanol consumption. For example, subcutaneous administration of topiramate, which is known to act as an antagonist at AMPA & kainite glutamate receptors, dose-dependently decreased ethanol consumption in a two bottle choice drinking paradigm (Nguyen et al., 2007). Another group found that enhanced AMPA receptor activity could facilitate ethanol consumption (Cannady et al., 2012). This group found that pretreatment of alcohol preferring rats with aniracetam, a positive allosteric modulator of AMPA receptors, increased operant pressing for ethanol compared to control groups. They further demonstrated the AMPA receptor specificity of the effect by abolishing the effect with DNQX treatment.

Taken together, our data support the following conclusions: (1) CART peptide enhancement of NMDA receptor-mediated currents in the central amygdala may be the mechanism of action mediating its anxiogenic effects, (2) a putative role for aberrant pre- and postsynaptic neuroadaptations of central amygdala AMPA receptor-mediated neurotransmission in ethanol withdrawal-induced anxiety and alcoholism, and (3) a role for CART peptide as a modulator of CIE-induced increases in ethanol consumption and withdrawal-induced anxiety behaviors.

Chapter 4:

Concluding remarks and future studies

These studies were undertaken in an attempt to elucidate the role of CART peptides in addiction and alcoholism in particular. The distribution of CART peptides and results from behavioral pharmacology experiments posit a role for CART peptides in the modulation of aberrant or excessive dopamine signaling (e.g. in response to administration of drugs of abuse). Specific lines of evidence supporting that idea include the findings that administration of psychostimulants or ethanol, both of which increase dopamine levels in the nucleus accumbens (Di Chiara and Imperato, 1988; Weiss et al., 1993), increase CART mRNA and peptide expression (Douglass et al., 1995; Hunter et al., 2005; Salinas et al., 2006) in the nucleus accumbens. Ultrastructural analyses have shown that CART peptide colocalizes with GABA presynaptically and synapses containing both form functional synapses onto accumbal (Smith et al., 1997) and ventral midbrain neurons (Dallvechia-Adams et al., 2002). Thus, it is possible that in response to increases in dopamine signaling, CART peptide levels are increased, and released synaptically onto accumbal and midbrain neurons. Given the nature of the putative CART receptor (i.e. Gi/o), it is possible that its activation would result in an inhibitory effect (Jones and Kuhar, 2008; Vicentic et al., 2006). Indeed, intra-accumbal injection of CART peptide was found to blunt the effect of subsequently administered psychostimulants to increase locomotor activity (Jaworski et al., 2003; Kim et al., 2003). Furthermore, intra-PVT injections with either TTX or CART peptide were shown to inhibit cocaine-primed reinstatement – a behavior thought to be mediated by dopamine signaling (James et al., 2010). Presumably this effect was due to negative modulation of PVT efferents, which have been previously shown to enhance dopamine release in the

NAc (Parsons et al., 2007). Finally, CART peptide was shown to inhibit L-type calcium channels in hippocampal neurons (Yermolaieva et al., 2001). Despite these inhibitory effects, CART peptide injections into the VTA (and to a lesser extent the substantia nigra) were found to increase locomotor activity in a haloperidol-sensitive (D2 antagonist) manner (Kimmel et al., 2000). This study also found that intra-VTA CART peptide administration induced a conditioned place preference, suggesting that CART peptide itself may be reinforcing. Seemingly, the psychostimulant-like actions of CART peptide argue against the hypothesis that CART peptides function as a homeostatic regulator of dopamine signaling in response to drugs of abuse. However, these psychostimulant-like effects may be mediated by CART peptide inhibition of GABAergic interneurons in the VTA and subsequent disinhibition of dopaminergic neurons. Indeed, approximately 70% of CART peptide containing synaptic terminals in the VTA appose GABAergic interneurons (Dallvechia-Adams et al., 2002). Further supporting these results are the findings that icv or intra-VTA injections of CART peptide increase the concentrations of dopamine or its metabolites, DOPAC & HVA, in the nucleus accumbens (Kuhar et al., 2005; Shieh, 2003; Yang et al., 2004). It should be noted that under normal conditions, the levels of CART peptide administered in these studies would never be reached unless stimulated by external events (e.g. drug administration) and that these studies were focused on specific brain region effects of CART peptide, neglecting the fact that CART peptides do not normally act at a single brain region at a time.

Further obfuscating a clear role for CART peptides in the dopamine system are studies from CART KO mice which have demonstrated complete suppression or attenuation of psychostimulant induced behaviors acutely (increased locomotor activity) and chronically (locomotor sensitization) (Couceyro et al., 2005; Moffett et al., 2006).

This group further found that cocaine self administration was also reduced in CART KO mice. Our lab's contribution to this stew came in the form of a report showing a reduction in ethanol consumption in CART KO mice (Salinas et al., 2012). The collective finding is that CART KO mice may find drugs of abuse to be less reinforcing or rewarding than WT mice. This conclusion may have been foreshadowed by the previously mentioned ability of intra-VTA CART peptide to produce increased locomotor activity and a conditioned place preference. As before, however, it should be noted that conditions of complete loss of CART peptide are unlikely and that the effects of removing the CART gene from mice may result in unforeseen compensatory events. It would be interesting to examine if psychostimulants and ethanol can still induce increases in dopamine dialysates in the NAc of CART KO mice. Given their decreased propensity to self administer or consume normally reinforcing drugs of abuse, one might expect a reduction or suppression of accumbal dopamine in response to a drug challenge. It would also be interesting to examine the ability of a non-drug reinforcer like sucrose to increase accumbal dopamine given that CART KO and WT mice do not differ in their consumption or preference for it. In all, we can safely presume a role for CART in the dopamine system; that role, however, is still unclear and likely to remain so until the putative CART receptor can be cloned and specific modulators for said receptor found and made available for research.

Fueled by my interest in anxiety and stress, I decided to spend the final portion of my studies investigating the effects of chronic intermittent ethanol (CIE) vapor exposure on withdrawal-induced anxiety and whether there would be any differences between CART KO and WT mice. Given the role of CART peptides in anxiety and stress, I hypothesized that CART KO mice would have attenuated levels of anxiety and a blunted stress response compared to WT mice during withdrawal.

These studies would be the first to examine anxiety and stress in CART KO mice, as well as, the first from our lab to examine anxiety-like behaviors. Thus it was necessary to conduct a few control experiments. I began by testing the behavioral apparatus (light/dark box) with WT mice exposed to either a clean novel cage (handling control) or a mild restraint stress and examining anxiety-like behaviors and plasma corticosterone content. The results from this experiment validated the light/dark box in our hands for detecting differences in anxiety-like behaviors and our ability to detect changes in plasma corticosterone content related to anxiety behaviors. Given the anxiogenic properties of CART, one might expect that basal anxiety levels would differ between CART KO and WT mice. They did not. We then compared stress responses between genotypes in response to a mild restraint stress. We expected that there would be an attenuation of anxiety-like behaviors and plasma corticosterone in response to a stressor in CART KO mice. Behaviorally, we observed no differences in anxiety-like behaviors between genotypes. Plasma corticosterone content, however, was significantly higher in CART KO mice than in WT mice when exposed to a stressor. This result was surprising and could not have been predicted by reports in the literature.

The lack of behavioral differences under basal and acutely stressed conditions was welcomed as it seemed to suggest a normal basal function of anxiety and stress systems in CART KO mice – of concern was the potentiated corticosterone response. This finding may reflect a dysregulation of the HPA axis in CART KO mice and suggests that CART peptides are required for normal HPA axis function. Potentially related to this point is a study of an Italian family with a known CART gene polymorphism. It was reported that adolescents from this family carrying the CART gene polymorphism exhibited increased anxiety and depression relative to their family members not carrying the mutation (Miraglia del Giudice et al., 2006). This CART gene polymorphism *in vitro*

was shown to result in dysfunctional processing and decreased release of CART peptides (Dominguez et al., 2004). It was subsequently shown that the mutation also resulted in a deficiency of bioavailable CART peptide in humans (Yanik et al., 2006). Given the similar phenotype (i.e. hypo-CART function/levels), one could expect that the people with the CART gene polymorphism might have a similar, potentiated corticosterone response to mild stressors. If this held true, then perhaps the observed depression might be the result of a dysfunctional HPA axis that overreacts to everyday mild stressors, resulting in chronic abnormally elevated circulating corticosterone levels. Indeed, in preclinical studies, chronic stress paradigms are often used to mimic some aspects of clinical depression including consistently elevated circulating cortisol (corticosterone analog in humans) levels (Tafet and Bernardini, 2003). A more comprehensive study of stress in CART KO mice would be required to determine if this hypothesis holds water. The first experiment would involve determining how long corticosterone levels in acutely restrained CART KO and WT mice remain elevated following the stressor. Then a second experiment would involve chronically stressing CART KO mice and determining if a depression-like phenotype could be more readily induced than in WT mice. This study would be completed by a final experiment aimed at determining if HPA axis responsivity is altered in chronically stressed CART KO mice; that is to say, would circulating corticosterone levels be elevated under basal conditions in chronically stressed mice and would the corticosterone response still be potentiated in response to a stress challenge in chronically stressed mice? The answers to these questions may not necessarily be relevant to alcoholism but certainly might have an impact on the treatment of clinical depression or anxiety disorders. If in fact, hypofunction of CART peptide in humans contributes to the development or expression of depression (outside of those people with the aforementioned CART gene polymorphism) in the general population,

then CART peptides should be explored as an anti-depressant treatment. Given the ability of CART peptides to readily pass the blood-brain-barrier, the treatment might consist of a slightly a modified version of the CART peptide to aid against degradation by endoproteases.

Following a single bout of CIE, we found enhanced ethanol consumption in mice of both genotypes relative to air treated controls with statistical trends towards a reduction in ethanol consumption in CART KO mice compared to WT mice and an additional statistical trend towards reduced ethanol consumption in CIE treated CART KO mice relative to CIE treated WT mice. Some of these results were expected and others were not. In the former category was the lack of a genotype effect on ethanol consumption. We have previously observed reduced ethanol consumption in CART KO mice in an unlimited access paradigm. The mice in these experiments were on a limited access paradigm and thus had a driving force to increase ethanol consumption. This effect led to approximately matched levels of ethanol consumption between CART KO and WT mice overall. Unfortunately, we did not observe any differences in anxiety-like behaviors after one bout of CIE between genotypes. There was, however, a significant decrease in exploratory behaviors in CIE treated mice of both genotypes – evidence of increased anxiety.

Following a second bout of CIE treatment, we again observed decreases in exploratory behaviors in mice of both genotypes, as well as, other behaviors indicating a reduction in anxiety in CART KO mice. Taken together, it appears that CART KO mice have reduced anxiety relative to their WT counterparts following two bouts of CIE or Air treatment. It is possible that the hypothesized reductions in anxiety in CART KO mice required chronic stress conditions (i.e. the repeated handling and injections associated with CIE treatment) to be revealed. This idea, however, is not directly supported by the

corticosterone levels observed. The corticosterone levels show approximately equal increases in CIE treated mice of both genotypes. One potential explanation is that the previously observed potentiated corticosterone response is still in effect here such that though experiencing decreased anxiety relative WT mice, the CART KO mice have enhanced corticosterone levels to approximately match those levels found in the WT mice.

The final components of this study examined glutamatergic neurotransmission in the central amygdala in response to acute CART peptide application and following two bouts of CIE and early (~6.5 hours) withdrawal. This study, to our knowledge, is the first to examine chronic intermittent ethanol vapor exposure effects in central amygdala neurons. It is also the first study, to our knowledge, to examine ethanol consumption, withdrawal-induced anxiety behaviors, and electrophysiological properties in the same adult mice.

Our first result was that CART peptide led to a decrease in AMPA/NMDA ratios. Given that CART peptide had no effect on AMPA receptor-mediated transmission otherwise, we presume that the effect was strictly NMDA receptor-mediated. This is in accord with the literature showing CART peptide-induced enhancement of NMDA receptor mediated currents in spinal cord preparations (Dun et al., 2006; Hsun Lin et al., 2005).

Following two bouts of CIE treatment, we found increased sEPSC amplitudes in CART KO and WT mice. This result suggests that CIE leads to an enhancement of postsynaptic AMPA receptor-mediated neurotransmission in the CeA. Presynaptically, we found that CIE treatment increased the paired pulse ratio of WT mice suggesting a decrease in presynaptic release probability. This finding was unique to CIE-treated WT mice and may contribute to the observed differences in anxiety between CIE-treated

CART KO and WT mice. The most similar studies in the CeA to our own have been conducted immediately following the termination of the ethanol exposure and found that pharmacologically-isolated AMPA receptor-mediated currents were reduced at higher stimulus intensities (Roberto et al., 2004b); however, it should be noted that these animals never underwent withdrawal and any concomitant changes in neurobiology associated with ethanol withdrawal. Our studies, on the other hand, were designed to coincide with peak withdrawal-induced hyperexcitability as determined by peak handling-induced convulsion scores (Becker and Hale, 1993). Studies in a neighboring region, the BLA, conducted after 24 hours of withdrawal did have similar findings to our own postsynaptically, but not presynaptically (Christian et al., 2012; Lack et al., 2007). There are several differences between our studies, but I believe the most notable are the difference in withdrawal time, the fact that their studies used rats, and the fact that their studies were conducted in juveniles. Any of these could potentially account for the differences in presynaptic release probability observed in our studies. It appears that CART KO mice had reduced withdrawal-induced anxiety compared to CART WT mice. If we were to try and match our electrophysiology results with this behavior, the most likely candidate would be our finding that presynaptic release probability was reduced in CIE-treated WT mice only. However, since mice of both genotypes exhibited decreased exploratory behaviors following CIE treatment, one could speculate that perhaps behaviors that are exploratory in nature are governed by AMPA receptor-mediated neurotransmission in the CeA. An alternative explanation for this result could be glucocorticoid-mediated enhancement of glutamatergic neurotransmission and, in particular, increased membrane trafficking of AMPA receptors (Liu et al., 2010; Yuen et al., 2009; Yuen et al., 2011).

Taken all together, these studies have demonstrated that CART peptides: (1) enhance NMDA receptor-mediated currents, (2) play a role in ethanol appetitive behaviors, (3) regulate the normal function of the HPA axis in response to an acute stressor, (4) may modulate CIE-induced increases in ethanol consumption, (5) reduce anxiety-like behaviors during early withdrawal from CIE, and (6) contribute to presynaptic alterations in glutamatergic neuroadaptations induced by CIE.

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Vita

Armando Salinas completed his undergraduate studies at the University of Texas at Austin in May of 2005 with a Bachelor of Science in Neurobiology and a Bachelor of Arts in Government. He then began working as a research technician in the laboratory of Richard A. Morrisett for a year and a half before beginning graduate school in the program of Pharmacology & Toxicology in the College of Pharmacy at the University of Texas at Austin. During his graduate school tenure, he held leadership positions in various graduate student organizations and was the recipient of several awards including the B. Bernard Matthew Scholarship, Pharmacy Graduate Student Association travel awards, Graduate School professional development award, Johnson & Johnson endowed graduate fellowship in pharmacy, as well as a three-year Ruth L. Kirschtein National Research Service Award for Individual Predoctoral Fellows from the National Institute on Alcohol Abuse and Alcoholism (NIAAA F31AA017834) of the National Institutes of Health. Armando plans to continue his training in the neurobiology of addiction with a post doctoral position and plans to pursue a career in academia.

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