



Review

Neuropeptide modulation of central amygdala neuroplasticity is a key mediator of alcohol dependence

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ABSTRACT

Alcohol use disorders are characterized by compulsive drug-seeking and drug-taking, loss of control in limiting intake, and withdrawal syndrome in the absence of drug. The central amygdala (CeA) and neighboring regions (extended amygdala) mediate alcohol-related behaviors and chronic alcohol-induced plasticity. Acute alcohol suppresses excitatory (glutamatergic) transmission whereas chronic alcohol enhances glutamatergic transmission in CeA. Acute alcohol facilitates inhibitory (GABAergic) transmission in CeA, and chronic alcohol increases GABAergic transmission. Electrophysiology techniques are used to explore the effects of neuropeptides/neuromodulators (CRF, NPY, nociceptin, dynorphin, endocannabinoids, galanin) on inhibitory transmission in CeA. In general, pro-anxiety peptides increase, and anti-anxiety peptides decrease CeA GABAergic transmission. These neuropeptides facilitate or block the action of acute alcohol in CeA, and chronic alcohol produces plasticity in neuropeptide systems, possibly reflecting recruitment of negative reinforcement mechanisms during the transition to alcohol dependence. A disinhibition model of CeA output is discussed in the context of alcohol dependence- and anxiety-related behaviors.

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1. Introduction

Alcoholism (i.e., substance dependence on alcohol) is a complex and dynamic disease process. The ICD-10 and DSM-IV describe alcohol dependence as a chronically relapsing disorder characterized by compulsive drug-seeking and drug-taking, loss of control in limiting intake (in terms of amount of drug per bout and number of drug-taking bouts), and the emergence of a withdrawal syndrome in the absence of the drug that includes, but is not limited to dysphoria, sleep disturbances, disruption of autonomic processes, as well as increases in anxiety and irritability (American Psychiatric Association, 1994; World Health Organization, 2007). These stages of alcohol abuse and dependence have been conceptualized in terms of cycling between alcohol binge intake and intoxication, physical and motivational withdrawal from alcohol in its absence, and preoccupation with and anticipation of alcohol consumption (Koob, 2003). These cycles are hypothesized to become progressively more exaggerated with time, whereby tolerance produces higher levels of alcohol intake, which produce gradually worsening withdrawal syndromes in the absence of the drug, thereby increasing the probability of subsequent alcohol-seeking and alcohol-drinking behavior (Koob, 2003). Many years of research have shaped the current view that excessive alcohol consumption is largely mediated by an organism's past experience (e.g., pattern and frequency) with alcohol, and is driven by the emotional disturbances, rather than the physical disturbances, associated with alcohol withdrawal/abstinence (Heilig et al., 2010).

Animal models of alcoholism aim to mimic particular components, rather than the entirety of the addiction phenotype in humans. For example, genetic animal models have been used to model the heritable aspects of spontaneous and persistent high and low alcohol preference in the offspring of animals that consume either high or low quantities of alcohol (Grahame et al., 1999; Murphy et al., 2002). Alternatively, models of chronic high-dose alcohol exposure have been utilized to produce an array of neural, emotional and motivational disturbances that reflect symptoms of alcohol dependence. Each method (e.g., alcohol vapor, alcohol liquid-diet) of inducing alcohol dependence in rats has its own set of advantages and disadvantages (for reviews, see Lieber and DeCarli (1973) and Rogers et al. (1979)). Most of the studies described below (behavior and slice electrophysiology) utilize chronic intermittent alcohol vapor inhalation for producing alcohol dependence in rats. The advantages of this procedure over others are that the dose, duration and pattern of alcohol exposure are easily controlled across rats (providing a singular independent variable) and the procedure reliably produces physical and motivational aspects of alcohol dependence. The major disadvantages of the alcohol vapor procedure are that alcohol exposure is not voluntary and that the route of administration is not the same as in humans, which can

lead to a different set of deleterious effects on peripheral physiological systems.

As described above, alcohol addiction is defined by three recurring and cyclical phases. The binge/intoxication phase has been modeled in rodents using alcohol self-administration, conditioned place preference and brain stimulation reward (i.e., intracranial self-stimulation) techniques. The withdrawal/negative affect phase has been modeled using withdrawal-induced alcohol self-administration and conditioned place aversion techniques, as well as an array of assays intended to measure anxiety-like behavior in rodents. Finally, the preoccupation/anticipation phase has been modeled using reinstatement procedures wherein animals exhibit increases in drug-seeking behavior triggered by a small priming alcohol dose, exposure to stressor, or presentation of a drug-related cue (Koob, 2009). The current review will focus on neurotransmission in the central nucleus of the amygdala (CeA) as it is relevant to the withdrawal/negative affect phase of the alcohol addiction cycle. A major conclusion of this review is that neuropeptide systems in the CeA interact with each other and with alcohol in a complex way to modulate synaptic transmission, largely by modulating pre-synaptic release, and that the transition to alcohol dependence is marked by neuroadaptations that functionally up-regulate many of these neuropeptide systems in the CeA.

2. Negative emotional state circuitry

Chronic consumption of large quantities of drugs, including alcohol, promotes a transition from casual drug use to drug dependence that is defined neurally by downregulation of dopamine signaling in the mesocorticolimbic reward system, hyperactivity of glutamate signaling, and dysregulation of brain stress systems (Koob and Volkow, 2010). Chronic alcohol effects on brain stress systems can refer to either alcohol-induced changes in neuroendocrine function (i.e., hypothalamic–pituitary–adrenal [HPA] axis; Clarke et al., 2008; Kiefer and Wiedemann, 2004) or the recruitment of extra-hypothalamic brain stress systems. The current review will focus on the circuitry of extra-hypothalamic stress systems involved in excessive alcohol-drinking phenotypes, with the ultimate goal of guiding future research on the neurobiological mechanisms of alcohol dependence. This review will detail the effects of acute and chronic alcohol on synaptic transmission and plasticity in the CeA and neighboring regions that exhibit high connectivity with the CeA, and the role of these regions in mediating alcohol-related behaviors. Throughout this review, *acute* alcohol exposure refers to *in vitro* application of ethanol to the slice preparation, whereas *chronic* alcohol exposure refers to long-duration (at least several weeks) *in vivo* alcohol exposure. Furthermore, the chronic alcohol exposure protocols utilized here reliably produce somatic and motivational signs of alcohol dependence (Gilpin et al.,

2009; Richardson et al., 2008a), therefore, the terms *chronic alcohol exposure and dependence* will be used interchangeably in this review. Finally, we will review the existing literature on peptidergic modulation of synaptic transmission in the central (and extended) amygdala, particularly because these peptides share a common cellular target, and they interact with each other and alcohol.

Many of the long-term emotional disturbances associated with alcohol abuse and dependence have been attributed to neurotransmission within a conceptual macrostructure in the basal forebrain called the “extended amygdala” (Koob, 2008). In the context of drug addiction, the major constituents of the extended amygdala are the CeA, bed nucleus of the stria terminalis (BNST), and the nucleus accumbens (NAc) shell (Heimer and Alheid, 1991). These regions exhibit similar morphology, a high degree of interconnectivity, and overlapping afferents from limbic cortices, hippocampus, and basolateral amygdala (BLA). Data from the BNST and BLA will be used throughout the review to support findings in the CeA, but full review of alcohol effects on synaptic transmission in those regions is beyond the scope of this review and can be found elsewhere (e.g., McCool et al., 2010). The outputs of the extended amygdala project largely to effector regions, including lateral hypothalamus and various brainstem regions that produce changes in behavior, most prominently those related to fear and anxiety responses (Davis, 1998).

The role of the extended amygdala in fear and anxiety behaviors has been well described (Davis et al., 2010). Many years of work have contributed to the notion that the CeA and BNST play prominent roles in phasic fear and sustained fear (i.e., anxiety). The BLA receives significant sensory input from thalamus and cortex, sends prominent glutamatergic projections to CeA and BNST, and is integral in both conditioning (Phelps and LeDoux, 2005) and extinction (Quirk and Mueller, 2008) processes. The CeA is composed mostly of gamma-aminobutyric acid (GABA)ergic projection neurons and interneurons (Sun and Cassell, 1993; Veinante and Freund-Mercier, 1998), and a major target of CeA projection neurons is the BNST (Krettek and Price, 1978; Sun et al., 1991; Sun and Cassell, 1993; Veinante and Freund-Mercier, 1998; Weller and Smith, 1982). Of major relevance for the data discussed below, connections between CeA and BNST often contain neuropeptide co-transmitters, for example, the CeA is a major source of corticotropin-releasing factor (CRF) in the BNST (Sakanaka et al., 1986).

Neuropeptides in the extended amygdala have been attributed a prominent role in the negative affective aspects of addiction to drugs, including alcohol (Koob, 2008). More specifically, these peptides have been conceptually divided into pro-stress peptides and anti-stress peptides that respectively promote and rescue negative affective disturbances during drug abstinence following heavy drug use. Pro-stress peptides include CRF, dynorphin, hypocretin/orexin, and vasopressin, whereas anti-stress peptides include neuropeptide Y (NPY) and nociceptin. It is becoming increasingly evident that these peptides interact in a complex way in the extended amygdala to modulate excitatory and inhibitory transmission, and that dysregulation of these peptide systems by alcohol alters the way in which they modulate synaptic neurotransmission (see Fig. 1).

3. Inhibitory (GABAergic) transmission in amygdala and alcohol

For the slice electrophysiology data presented in this section, it is important to note that in the preparation used in our lab, inhibitory transmission is pharmacologically isolated and post-synaptic potentials are evoked by local electrical stimulation in the CeA (Fig. 1). Because projection neurons out of the CeA are also inhibitory, evoked GABAergic transmission from CeA afferents and

interneurons inhibits the activity of GABAergic neurons projecting out of CeA. Conversely, decreases in GABAergic transmission from CeA afferents and interneurons would increase the activity of GABAergic neurons projecting out of CeA, thereby facilitating the release of GABA onto downstream targets. As such, recorded increases in GABAergic transmission reflect a disinhibition of downstream target regions (e.g., hypothalamus, periaqueductal gray, locus coeruleus, nucleus of the solitary tract, pedunculopontine tegmental nucleus), whereas recorded decreases in GABAergic transmission reflect a net inhibition of downstream target regions. The overall hypothesis, then, is that increases and decreases in local GABAergic transmission in CeA produce decreases and increases in inhibitory output from the CeA to downstream effector regions, and increases and decreases in anxiety-like behavior, respectively.

3.1. GABAergic transmission and alcohol-related behaviors

GABA, the major inhibitory transmitter in the brain, acts on two classes of GABA receptors: GABA_A (which includes GABA_A-rho subclass – formerly GABA_C) and GABA_B. GABA_A receptors are ligand-gated ion channels, whereas GABA_B receptors are G protein-coupled receptors. There is considerable evidence that GABAergic transmission mediates some aspects of alcohol-drinking behavior, but there is some ambiguity in the literature with respect to the directions of these effects. Early studies showed that systemic administration of GABA_AR agonists increased voluntary alcohol drinking whereas GABA_AR antagonists and benzodiazepine inverse agonists decreased alcohol drinking (Boyle et al., 1993; Rassnick et al., 1993a). A subsequent experiment showed that infusion of both GABA_AR agonists and antagonists into the NAc suppress alcohol drinking by non-dependent rats (Hodge et al., 1995). A more recent study showed that systemic administration of a GABA_BR agonist suppresses alcohol drinking by all rats, but that alcohol-dependent rats are more sensitive to this effect, suggesting an upregulation of GABA_BR function in those animals (Walker and Koob, 2008). These studies confirm the long-standing notion that chronic alcohol produces neuroadaptations in inhibitory neurotransmission, as supported by findings that [1] GABA mediates somatic disturbances associated with acute alcohol withdrawal (Cooper et al., 1979; Frye et al., 1983) and [2] animals exhibit changes in sensitivity to GABAergic compounds following chronic alcohol exposure (Martz et al., 1983; Rassnick et al., 1993a).

Behavioral studies have highlighted a role for brain GABA circuitry in alcohol-drinking behavior, particularly in regions implicated in the negative reinforcing properties of the drug (i.e., extended amygdala). One such study examined the effects of GABA_AR antagonism in the major constituent regions of the extended amygdala: CeA, BNST, and NAc shell (Hyytia and Koob, 1995). In each of these regions, antagonism of GABA_ARs suppressed alcohol drinking by non-dependent rats, but this suppressive effect on alcohol consumption was most potent and most selective for alcohol when infused into the CeA. Another study showed that antagonism of GABA receptors in the BNST reverses decreases in alcohol drinking produced by D₂ receptor antagonism in the ventral tegmental area (VTA) of alcohol-preferring (P) rats (Eiler and June, 2007). Interestingly, infusion of a GABA_A agonist directly into the amygdala suppresses alcohol drinking by alcohol-dependent rats without affecting intake by non-dependent controls (Roberts et al., 1996). There are considerable methodological differences between these studies, but what is clear is that GABAergic neurotransmission modulates alcohol drinking, and in the case of excessive alcohol consumption by alcohol-dependent rats, the amygdala is a strong candidate region for localization of these effects.

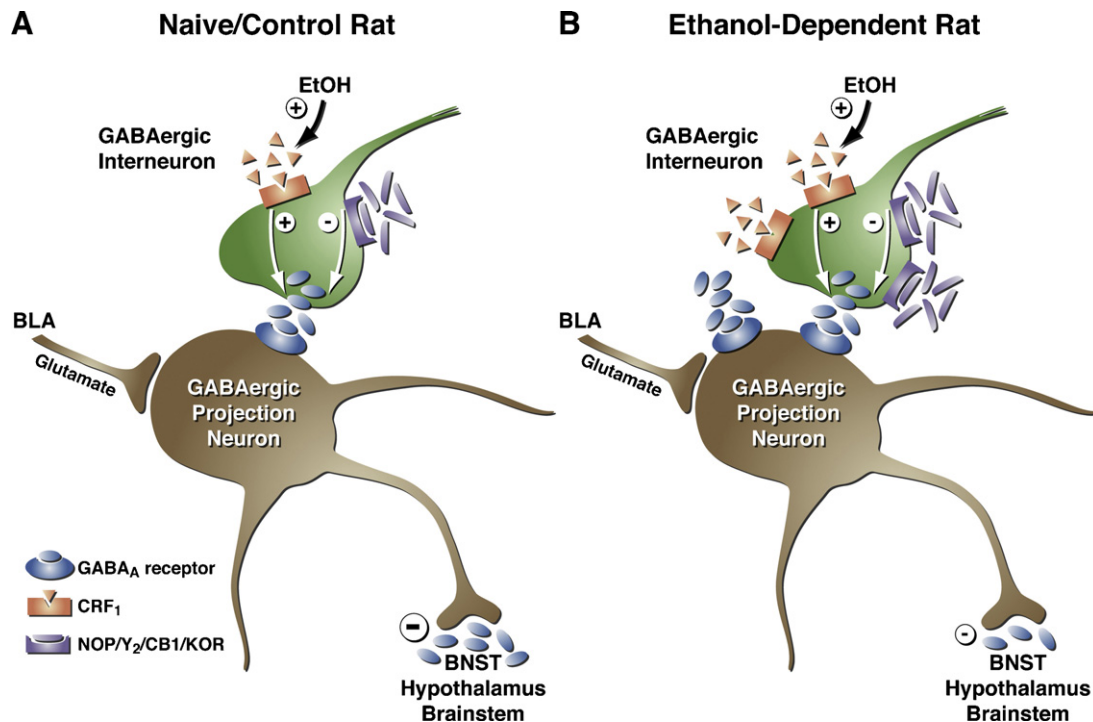


Fig. 1. Acute and chronic effects of alcohol on GABAergic synaptic transmission in the medial aspect of the CeA. Schematic diagram of a medial CeA GABAergic synapse, including pre-synaptic GPCRs that modulate neurotransmitter release and post-synaptic ionotropic receptors located at synapses. GABAergic afferents in the medial CeA arise largely from the lateral aspect of the CeA and the intercalated GABA cells. Many of these neurons contain CRF and/or other neuropeptides as co-transmitters. Glutamatergic inputs to medial CeA arise largely from BLA. (A) In the medial CeA synapse of an alcohol-naïve rat, the predominant pre-synaptic effect of acute alcohol is potentiation of GABA release, most likely via effects on neuromodulators such as CRF. It is thought that activation of pre-synaptic CRF₁Rs and downstream signaling pathways (e.g., AC and PKC ϵ) increases the probability of vesicle fusion and GABA release. Acute alcohol enhances GABA release from CeA interneurons or afferent projections to the CeA (green neuron) either via release of CRF onto the pre-synaptic terminal or via direct activation of CRF₁Rs. Thus, CRF and acute alcohol both augment the inhibition of CeA projection neurons (brown neuron), leading to excitation (i.e., disinhibition) of downstream (e.g., BNST, hypothalamus, PAG, LC) neurons. Activation of pre-synaptic nociceptin (NOP) receptors, NPY-Y₂ receptors, CB₁ receptors, and KORs reduces GABA release onto CeA inhibitory projection neurons, increasing inhibition of downstream targets. (B) Following chronic alcohol exposure (i.e., the development of alcohol dependence), the GABA release is increased at medial CeA synapses, leading to more inhibition of GABA projection neurons out of CeA and net disinhibition of downstream target regions (smaller negative symbol in right panel). This increased GABA release may be due to increased CRF levels in the CeA, since CRF₁R antagonists reverse basal increases in GABA release of alcohol-dependent rats. There is also evidence for increased function/quantity of pre-synaptic NOP and Y₂Rs in the medial CeA of alcohol-dependent rats.

3.2. Acute alcohol effects on inhibitory transmission in CeA

In vitro brain slice preparations provide a number of highly sensitive experimental strategies that allow for investigation of the synaptic effects of ethanol (for reviews of these approaches, see Siggins et al. (2005)). Unlike many cell culture models, the interneuronal connections between neurons are largely preserved. While not all the afferent and efferent projections are maintained in brain slices, the synapses that are preserved have developed in their native environment and are representative of the anatomical relationships that are observed in vivo. The stability and experimental access afforded by the brain slice preparation facilitates single-cell and whole-cell recordings that are extremely difficult to carry out in intact animals.

Acute alcohol potentiation of GABA_A receptor function has been extensively studied (for review, see Lovinger and Roberto, *in press*). Alcohol (1–100 mM) selectively potentiates the function of GABA_A receptors that contain particular subunit compositions, but findings have been inconsistent across laboratories examining alcohol effects in heterologous systems (reviewed in (Lovinger and Homanics, 2007; Aguayo et al., 2002)). In general, alcohol enhances synaptic inhibition by increasing GABA release from pre-synaptic terminals (reviewed in Siggins et al. (2005)) and also by activating post-synaptic GABA_A receptors. Alcohol increases both evoked and spontaneous GABAergic synaptic transmission in the CeA (Roberto et al., 2003; see Fig. 2) and BLA (Zhu and Lovinger, 2006). More specifically, ethanol increases the amplitudes of evoked

GABA_A-mediated inhibitory post-synaptic potentials and currents (IPSP/Cs) (Fig. 2) and miniature IPSC (mIPSC) frequencies (Fig. 3). These effects are rapid and reversible, and have a significant pre-synaptic component. The post-synaptic contribution to alcohol effects in the CeA is evidenced by acute alcohol-induced increases in mIPSC amplitudes as well as increases in the response to exogenous GABA in the presence of tetrodotoxin (TTX; in approximately two-thirds of cells tested; Roberto et al., 2003).

Although the precise mechanism(s) by which alcohol potentiates GABA release have yet to be identified, past studies have examined the role of intracellular signaling pathways in the facilitatory effect of acute alcohol on GABAergic transmission. It is well established that activation of adenylyl cyclase (AC) or protein kinase C (PKC) potentiates transmission at synapses throughout the nervous system (Cruz et al., 2011; Leenders and Sheng, 2005; Nguyen et al., 2000; Kelm et al., 2010, 2011). It is logical to speculate that these signaling molecules might play a role in acute alcohol-induced facilitation of GABAergic release from pre-synaptic terminals since, for example, the enhancing effect of alcohol on GABA release is impaired in CeA of mice that lack PKC epsilon (PKC ϵ ; Bajo et al., 2008), suggesting that PKC ϵ facilitates vesicular GABA release in the presence of alcohol. It is important to note that basal GABA release is increased in PKC ϵ knockout mice prior to alcohol application, suggesting that under normal conditions in wild-type neurons, PKC ϵ limits spontaneous GABA release. Because developmental changes or compensatory effects of other gene products may confound studies in gene-targeted mice, we

pharmacologically confirmed the role of PKC ϵ in regulating GABA release from CeA neurons by using a PKC ϵ inhibitor peptide, Tat- ϵ V1–2 (Qi et al., 2007). Superfusion of the inhibitor alone onto slices from PKC ϵ wild type mice increased the mean evoked IPSP amplitude and decreased the PPF of IPSPs in CeA neurons. These results suggest a constitutive role for PKC ϵ in tonically inhibiting GABA release at CeA synapses that may account, in part, for increased basal GABAergic transmission seen in PKC ϵ knockout mice. Moreover, pretreatment of CeA neurons with the Tat- ϵ V1–2 peptide blocked the ethanol and CRF-induced GABA release. These results resemble those obtained in CeA neurons from PKC ϵ knockout mice and support the conclusion that PKC ϵ mediates ethanol-induced GABA release in CeA. Therefore, PKC ϵ serves at least two roles in the CeA: (1) limit baseline GABA release, and (2) mediate alcohol-stimulated release of GABA.

3.3. Chronic alcohol effects on pre-synaptic GABA release in CeA

Chronic alcohol exposure affects pre-synaptic release of GABA, and most of these data come from in vitro brain slice preparations, an approach that allows for multiple methods of detecting pre-synaptic changes in transmitter release (for review, see Siggins et al., 2005; Weiner and Valenzuela, 2006). Although the current review focuses on alcohol effects in the amygdala, the effects of alcohol on GABA transmission in other brain regions has been reviewed elsewhere (Criswell and Breese, 2005; Siggins et al., 2005; Weiner and Valenzuela, 2006). Our lab has shown that chronic in vivo alcohol exposure facilitates GABA release in the CeA, mainly via actions at pre-synaptic GABAergic terminals (Roberto et al., 2004a, 2010a,b). Relative to alcohol-naïve controls, alcohol-dependent rats exhibit larger baseline amplitudes of evoked IPSP/Cs, smaller baseline paired-pulse facilitation (PPF) of GABA_A IPSCs, and higher baseline frequency of mini IPSCs in CeA neurons, suggesting augmented GABA release in CeA following chronic alcohol exposure. Interestingly, acute alcohol increases IPSCs, decreases PPF ratio of IPSCs, and increases mIPSC frequency similarly in alcohol-dependent and alcohol-naïve rats, suggesting a lack of tolerance for the acute effects of alcohol (Roberto et al., 2004a). This unaltered sensitivity of GABA_ARs to acute alcohol in vitro following chronic in vivo alcohol exposure is confirmed by recordings from BLA neurons of monkeys with a long history of alcohol self-administration (Anderson et al., 2007). Although animals develop tolerance to many of the behavioral effects of alcohol in vivo, the observed lack of tolerance to the acute effects of alcohol on GABA release in CeA of alcohol-dependent rats is not necessarily surprising in light of the highly region-specific effects of acute and chronic alcohol on synaptic transmission.

Consistent with in vitro electrophysiological results, in vivo microdialysis studies have measured a four-fold increase of baseline dialysate GABA concentrations in the CeA of alcohol-dependent rats relative to alcohol-naïve controls, as well as lack of tolerance for alcohol-induced increases in dialysate GABA levels in alcohol-dependent rats (Roberto et al., 2004a). These results strongly suggest that both acute and chronic alcohol alter pre-synaptic components of GABAergic synapses in the CeA, although it is not clear at this point whether the microdialysis data are attributable to changes in release or uptake or both. Future studies should determine the molecular mechanisms responsible for chronic alcohol-induced adaptations in CeA neurons, and the behavioral implications of these neuroadaptations in the alcohol-dependent and/or alcohol-withdrawn organism. For example, acute and chronic alcohol each produce changes in histone acetylation in CeA (in opposite directions) that have been functionally linked to some of the peptide systems discussed below (Pandey et al., 2008). Future studies will help to elucidate the mechanism(s) responsible for reductions in alcohol withdrawal-related

hyperexcitability produced by GABA-mimetic drugs (Breese et al., 2006; McCown et al., 1985; Roberto et al., 2008; Ticku and Burch, 1980), and may have implications for treatment of pathological excessive alcohol-drinking behaviors.

3.4. Chronic alcohol effects on post-synaptic GABA_AR transmission in CeA

Chronic alcohol exposure produces tolerance to many behavioral effects of the drug, including the anxiolytic, sedative, ataxic, and positive reinforcing effects of alcohol (Kumar et al., 2004, 2009). Chronic alcohol also produces physical and motivational dependence, and alcohol withdrawal is associated with increased neuronal excitability (Kliethermes, 2005; Weiner and Valenzuela, 2006). These chronic alcohol effects are thought to reflect, at least in part, compensatory adaptations to the facilitatory effects of alcohol on GABAergic synapses (Siggins et al., 2005; Weiner and Valenzuela, 2006). Using in vitro brain slice preparations, we have shown that evoked IPSCs in CeA slices from alcohol-dependent rats are significantly increased relative to alcohol-naïve controls (Roberto et al., 2004a). This result differs, for example, from the GABA_AR tolerance seen in BLA of monkeys following long-term alcohol consumption (Floyd et al., 2004). Slices from alcohol-dependent rats also exhibit increased mIPSC amplitude relative to naïve rats, indicative of a post-synaptic effect of chronic alcohol. Substantial evidence suggests that alcohol-induced behavioral and neural adaptations are attributable to marked changes in GABA_AR subunit assembly rather than decreases in the number of GABA_ARs (Devaud et al., 1995; Eckardt et al., 1998; Grobin et al., 1998; Kumar et al., 2004, 2009; Morrow et al., 1992). That said, there is considerable ambiguity in the literature (both within and across species) regarding which GABA_AR subunits are most critical for mediating alcohol actions (e.g., Borghese and Harris, 2007; Hemby et al., 2006; Korpi et al., 2007), and the critical subunits are likely to vary across brain regions (Grobin et al., 2000).

3.5. Acute and chronic alcohol effects on GABA_BR transmission in CeA

It has been suggested that the ability of alcohol to facilitate GABAergic neurotransmission is limited by GABA feedback onto pre-synaptic GABA_BRs (Ariwodola and Weiner, 2004). For example, acute alcohol facilitates GABAergic transmission in hippocampus (Ariwodola and Weiner, 2004; Wan et al., 1996; Wu and Saggau, 1994) and nucleus accumbens (Nie et al., 2000) only if GABA_B receptors are blocked. However, in the CeA, GABA_B receptor blockade is not required for the enhancement of IPSPs by acute alcohol nor does it potentiate this effect (Roberto et al., 2003). Thus, the involvement of GABA_B receptors in alcohol-induced GABA release may be brain region-specific, and may depend on the presence or absence of pre-synaptic GABA_B receptors in particular brain regions (Ariwodola and Weiner, 2004).

Our lab has reported neuroadaptations in CeA GABA_BRs following chronic alcohol exposure (Roberto et al., 2008). More specifically, the sensitivity of GABA IPSCs to GABA_BR agonists and antagonists is decreased following chronic alcohol, suggesting downregulation of this system. In alcohol-naïve rats, a GABA_BR antagonist significantly increases the amplitude of evoked IPSCs and decreases the PPF ratio of IPSCs in CeA, suggesting a tonic activation of pre-synaptic GABA_BRs. Conversely, a GABA_BR agonist markedly depresses evoked IPSC amplitudes and increases the PPF ratio of IPSCs in the CeA of alcohol-naïve rats, indicative of decreased pre-synaptic GABA release. These effects of GABA_BR agonists and antagonists are either absent or greatly attenuated in the CeA of alcohol-dependent rats, suggesting a chronic alcohol-induced downregulation of the GABA_BR system that may help

to explain the increased GABAergic tone observed in the CeA of alcohol-dependent rats (Roberto et al., 2008). These alcohol dependence-induced neuroadaptations of the GABA_BR system may account for chronic alcohol-induced changes in gabapentin effects on inhibitory transmission in CeA. Gabapentin, a structural analogue of GABA (Sills, 2006), increases the amplitude of evoked IPSCs in CeA neurons from non-dependent rats (an effect that is blocked by a GABA_BR antagonist), but decreases IPSC amplitude in CeA of alcohol-dependent rats. Likewise, gabapentin infused directly into the CeA reverses alcohol dependence-induced increases in operant alcohol responding, but tends to increase alcohol drinking by non-dependent rats (Roberto et al., 2008).

4. Excitatory (glutamatergic) transmission in amygdala and alcohol

4.1. Glutamatergic transmission and alcohol-related behaviors

Glutamate, the major excitatory neurotransmitter in the brain, has long been implicated in the acute reinforcing actions of alcohol. Glutamate receptors include three major classes of ionotropic receptors (iGluRs) that are cation-permeable ion channels, with varying ratios of selectivity for Na⁺, K⁺ and Ca²⁺. The ionotropic glutamate receptors include α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA), N-Methyl-D-aspartate receptors (NMDARs), and kainate receptors (KARs). In contrast, the various sub-classes of metabotropic glutamate receptors (mGluRs) are G protein-coupled receptors (GPCRs).

In contrast to its effects on GABA, alcohol inhibits glutamatergic neurotransmission in the brain (for review, see Lovinger and Roberto, *in press*) and in fact, animals generalize the interoceptive cues (i.e., feelings) associated with alcohol to NMDA receptor antagonists (Colombo and Grant, 1992). Dysregulation of brain glutamate systems is thought to contribute to hyperexcitability and craving associated with alcohol withdrawal (Pulvirenti and Diana, 2001). Acamprosate, a pharmacotherapeutic approved for treatment of alcoholic patients, modulates glutamatergic transmission via actions at NMDA receptors and/or metabotropic glutamate receptors (Blednov and Harris, 2008; Mann et al., 2008). Acamprosate dampens increased glutamate levels in abstinent alcoholics as measured by magnetic resonance spectroscopy (Umhau et al., 2010) and reduces excessive alcohol drinking in those individuals, presumably by reducing craving and negative affect (for review, see Littleton, 2007). AMPARs are another category of ionotropic glutamate receptors that appear to be important in regulating relapse-like behaviors without having a central role in alcohol consumption *per se* (Sanchis-Segura et al., 2006).

Excitatory transmission in the CeA appears to mediate some of the aversive aspects of withdrawal from drugs of abuse (e.g., morphine; Watanabe et al., 2002). In the CeA of P rats, long-term consumption of high quantities of alcohol produces increased levels of metabotropic glutamate receptors, NMDA receptor subunits, and a scaffolding protein that regulates expression of these receptors on the cell membrane (Obara et al., 2009). It has also been suggested that group II metabotropic glutamate receptors block stress-induced and cue-induced reinstatement of alcohol-seeking behavior via neuronal activation in CeA or BNST (Zhao et al., 2006).

4.2. Acute alcohol effects on excitatory transmission in CeA

Acute alcohol (2–100 mM) exerts inhibitory effects on ionotropic glutamate receptor function, the best characterized of which may be inhibition of NMDARs (Dildy and Leslie, 1989; Hoffman et al., 1989; Lima-Landman and Albuquerque, 1989; Lovinger et al., 1989) and reduction of NMDAR-mediated synaptic

responses (Lovinger et al., 1990; Morrisett and Swartzwelder, 1993; Wang et al., 2007; Roberto et al., 2004b). Low concentrations (5–50 mM) of alcohol also inhibit AMPAR function (Akinshola et al., 2001; Dildy-Mayfield and Harris, 1992; Moykkynen et al., 2003; Wirkner et al., 2000). Our lab has shown that acute alcohol (5–66 mM) decreases excitatory post-synaptic potentials (EPSPs) and currents (EPSCs) in the CeA, and that these effects are mediated by both NMDAR and non-NMDAR mechanisms (Roberto et al., 2004b). In the BLA, high concentrations of acute alcohol suppress NMDAR-mediated EPSCs, but lower concentrations suppress basal and evoked EPSCs mediated by kainate receptors (Läck et al., 2008). In contrast to alcohol facilitation of GABA release, the vast majority of studies indicate that acute alcohol either has no effect on or inhibits glutamate release (for review, see Siggins et al., 2005).

4.3. Chronic alcohol effects on excitatory transmission in CeA

Chronic alcohol exposure produces neuroadaptive changes in glutamatergic synaptic transmission. Specifically, chronic alcohol produces upregulation of NMDAR function and NMDAR-mediated glutamatergic synaptic transmission (Cebers et al., 1999; Grover et al., 1998; Gulya et al., 1991; Läck et al., 2007; Smothers et al., 1997). In the context of increased basal NMDAR-mediated glutamatergic transmission following chronic alcohol exposure, acute alcohol produces similar or greater suppression of NMDAR function in multiple brain regions including CeA, relative to alcohol-naïve rats (Floyd et al., 2003; Roberto et al., 2006, 2004b, but see Grover et al., 1998; Miyakawa et al., 1997). Specifically, acute alcohol produces greater suppression of NMDAR-mediated EPSPs and EPSCs in the CeA of alcohol-dependent rats relative to alcohol-naïve controls. With local applications of NMDA, acute alcohol elicits a greater inhibition of NMDA currents in slices taken from alcohol-dependent rats, suggesting that alcohol dependence sensitizes NMDA receptors to ethanol (Roberto et al., 2004b, 2006).

Severe alcoholism in humans affects synthesis and expression of multiple NMDAR subunits (e.g., in cortex; Ridge et al., 2008). In rodent studies, the NR2B subunit appears to be very sensitive to chronic alcohol exposure, particularly in limbic brain regions (Carpenter-Hyland et al., 2004; Floyd et al., 2003; Kash et al., 2009; Roberto et al., 2004b). In the CeA and BNST, chronic alcohol exposure produces increases in NR2B mRNA and/or protein levels (Kash et al., 2009; Roberto et al., 2006), although alcohol does not produce such increases in all brain regions (Cebers et al., 1999; Floyd et al., 2003; Läck et al., 2005). The molecular mechanisms that underlie NR2B subunit changes are not yet well understood, and it also not yet clear what portion of alcohol-induced increases in NMDAR function is attributable to changes in NR2B subunit expression.

Microdialysis experiments indicate that chronic alcohol exposure produces increases in extracellular glutamate levels in brain, especially after single or repeated withdrawals (Dahchour and De Witte, 1999, 2003; Roberto et al., 2004b; Rossetti and Carboni, 1995). These increases may be due to chronic alcohol-induced decreases in glutamate uptake (Melendez et al., 2005), or perhaps changes in cystine/glutamate exchange (Kalivas, 2009). In the amygdala, several studies have reported increases in glutamate release following chronic alcohol exposure (at 2–8 h withdrawal in CeA, [Roberto et al., 2004b]; at 24 h withdrawal in BLA, [Läck et al., 2007]). In particular, our lab showed that prior chronic alcohol exposure unmasks the ability of acute alcohol to increase pre-synaptic glutamate release (Roberto et al., 2004b), and that this effect persists two weeks into abstinence (Roberto et al., 2006). Collectively, these results suggest that multiple factors contribute to increased extracellular glutamate levels and increased glutamatergic transmission following chronic alcohol exposure and withdrawal.

5. Central amygdala neuromodulators and alcohol dependence

The extended amygdala is densely populated by pro-stress and anti-stress neuropeptides. Each of the neuropeptides described below has robust effects on anxiety-like and alcohol-drinking behaviors. These peptides play particularly prominent roles in regulating such behaviors in individuals that are either alcohol-dependent, genetically vulnerable to develop excessive alcohol drinking (e.g., selective breeding), repeatedly cycled through periods of alcohol withdrawal and/or innately anxious. It is interesting that pro-stress peptides (e.g., CRF, dynorphin) and anti-stress peptides (e.g., nociception, NPY) exhibit a high degree of neuroanatomical overlap, and opposite behavioral profiles (Koob, 2008). The effects of these peptides on anxiety-like and alcohol-related behaviors have been localized to the amygdala and neighboring regions, and are likely attributable to their modulation of excitatory and inhibitory transmission in those regions (see Table 1).

A wealth of evidence points to the CeA as a major point of convergence for peptide neuromodulators of excitatory and inhibitory transmission, and amygdalar stress systems are heavily recruited during the development of alcohol dependence. The CeA is the major output center for the amygdala but is not a homogeneous neuroanatomical structure. It can be subdivided into lateral and medial aspects that differ in terms of their neuropeptide content, origin of incoming afferents, and target sites of efferent projections (for review of amygdala anatomical organization, see Pitkänen (2000)). The lateral portion of the CeA contains a much higher density of neuropeptides (e.g., CRF; Cassell et al., 1999, 1986; Shimada et al., 1989; Veening et al., 1984) than the medial CeA, receives input from cortical areas and thalamus, and projects to the substantia innominata and quite heavily to the lateral portion of the BNST. The medial portion of the CeA, on the other hand, receives prominent inputs from other amygdaloid nuclei, and sends dense projections to effector regions such as hypothalamus and brainstem nuclei (Pitkänen, 2000), although medial CeA also projects to BNST (Sun et al., 1991). Medial CeA projection neurons receive excitatory

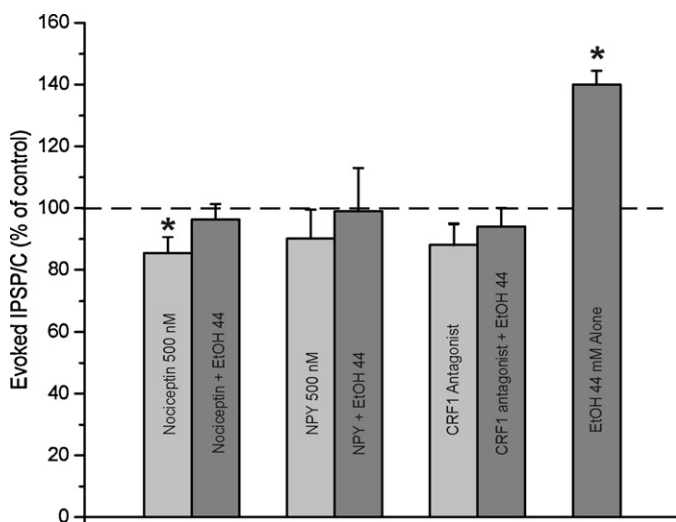


Fig. 2. Histogram summarizing the effects of ethanol (44 mM), nociceptin (500 nM), NPY (500 nM), and various CRF₁R antagonists on evoked GABA IPSP/Cs in medial CeA. Also depicted are the effects of ethanol in combination with nociceptin, NPY, and CRF₁R antagonists on evoked GABA IPSP/Cs in CeA. Ethanol produces increases in evoked CeA IPSP/Cs that are reliably prevented and reversed by nociceptin, NPY and CRF₁R antagonists.

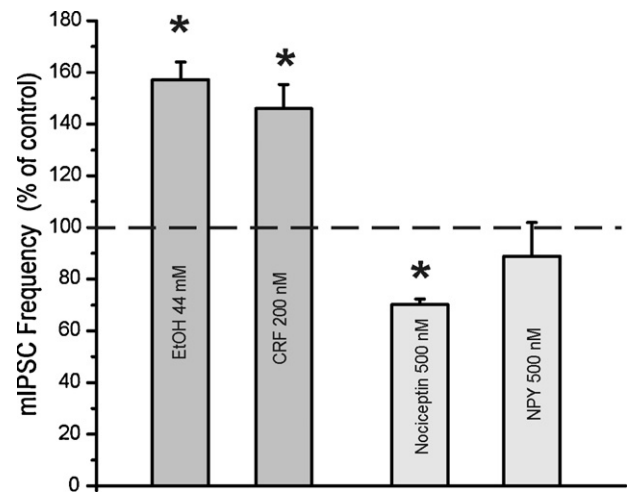


Fig. 3. Histogram summarizing the effects of ethanol (44 mM), CRF (200 nM), nociceptin (500 nM) and NPY (500 nM) on miniature IPSC frequencies in medial CeA. Ethanol and CRF facilitate mIPSC frequency, indicative of increased pre-synaptic GABA release. Nociceptin decreases mIPSC frequency, indicative of decreased GABA release. NPY alone produces a non-significant tendency toward decreased mIPSC frequency, although a Y₂R antagonist produces decreases in paired-pulse ratio of GABA IPSPs (not shown), indicative of increased GABA release. Together, these data suggest that ethanol and these neuromodulators have a pre-synaptic site of action in medial CeA.

inputs from BLA as well as inhibitory inputs from lateral CeA and intercalated GABA cells, although it is not well understood at this point which synaptic connections govern emotion- and alcohol-related behavior (Ehrlich et al., 2009; Pape and Pare, 2010). In general, the amygdala receives strong inputs about the external environment and projects lateromedially (both between nuclei and within the CeA) to convert sensory information into appropriate behavioral and physiological responses. In our electrophysiological studies, we use local stimulation in the medial subdivision of CeA and we record from neurons in the same subdivision.

5.1. Pro-stress neuromodulators

5.1.1. CRF effects on alcohol-related behavior

CRF is a peptide that plays a central role in arousal as well as the hormonal, sympathetic, and behavioral responses to stress. The CeA, BNST, and BLA are abundant in CRF neurons and receptors (De Souza et al., 1984; Sakanaka et al., 1986). Hyperfunction of CRF systems in the CeA, BLA, and BNST produce increases in anxiety-like behavior (Lee et al., 2008; Rannin et al., 2004; Sajdyk et al., 1999a). Extracellular CRF levels in CeA are elevated following exposure to stress and also following development of alcohol dependence (Merlo-Pich et al., 1995; Zorrilla et al., 2001). Withdrawal/abstinence from chronic alcohol in dependent rats produces increases in CRF transcript in CeA (Roberto et al., 2010a; Sommer et al., 2008), decreased CRF immunoreactivity in CeA (perhaps reflecting increased exocytosis; Funk et al., 2006), and increased extracellular CRF in BNST (Olive et al., 2002), the latter of which is normalized by alcohol consumption. CRF receptor antagonists suppress alcohol dependence-induced increases in alcohol drinking during acute withdrawal and protracted abstinence (Valdez et al., 2002), and also block the increased sensitivity to stress-induced anxiety during protracted abstinence from chronic alcohol (Valdez et al., 2003). CRF repeatedly administered into the CeA, BLA, and dorsal (but not ventral) BNST “kindles” or exaggerates the increases in anxiety-like behavior produced by alcohol withdrawal, and this effect is due to CRF action at CRF₁ receptors (CRF₁Rs; Huang et al., 2010). Conversely, antagonism of CRF receptors in the CeA attenuates the increases in anxiety-like

Table 1
The effects of neuropeptides/neuromodulators on GABAergic transmission in the central nucleus of the amygdala (CeA) as assessed in our lab using slice electrophysiology techniques. Up arrows indicate a facilitatory effect on inhibitory transmission in CeA and down arrows indicate a suppression of inhibitory transmission in CeA. The table shows the effects of neuropeptides in CeA of alcohol-naïve and chronic alcohol-exposed rats, and also alone and in combination with acute alcohol application. The last two columns describe the site (pre- vs. post-synaptic) and mechanism (receptor subtype) of action for each of these neuropeptides.

	Alcohol-naïve		Chronic alcohol		Pre vs. post	Mechanism
	Basal	Acute alcohol	Basal	Acute alcohol		
CRF	–	↑	↑	↑	PRE	CRF ₁ Rs
NPY	–	↑↑	↑↑	↑↑	PRE	Y ₂ Rs
Nociceptin	↓	Block	↓	Block	PRE	NOP
Dynorphin	↓	Block	↓	Block	PRE	KOR
Galanin	↓	Partial Block	Not known	Not known		
Population 1	↑	↑↑	Not known	Not known	POST?	Gal ₃ R
Population 2	↓	Block			POST?	Gal ₂ R
eCBs	↓	Block	Not known	Not known	PRE	CB ₁ R

behavior observed in rats during withdrawal from chronic high-dose alcohol exposure (Rassnick et al., 1993b).

Recent research has highlighted the role of CRF₁Rs in mediating the effects of limbic CRF on anxiety-like behavior and alcohol drinking. CRF₁R antagonists block the anxiogenic effects of many stressors (including alcohol withdrawal) in tests of open field exploration, elevated plus-maze, light-dark box, and defensive withdrawal (Arborelius et al., 2000; Zorrilla et al., 2007). CRF₁R antagonists also block increases in alcohol self-administration produced by stressors and alcohol withdrawal (Funk et al., 2007; Gehlert et al., 2007; Hansson et al., 2006; Lowery et al., 2008; Marinelli et al., 2007; Richardson et al., 2008b), and chronic antagonism of CRF₁Rs abolishes dependence-induced escalation of alcohol drinking in rats chronically exposed to high doses of alcohol (Roberto et al., 2010a). Likewise, stressors and alcohol withdrawal produce increases in CRF₁R synthesis and expression in limbic brain regions (Aguilar-Valles et al., 2005; Sommer et al., 2008). Similarly, rats selectively bred for high alcohol preference exhibit increased anxiety-like behavior and CRF₁R levels (Ciccocioppo et al., 2006), and CRF₁R knockout (KO) mice exhibit decreased anxiety-like behavior (Muller et al., 2003), as well as decreased alcohol drinking following withdrawal from chronic high-dose alcohol exposure (Chu et al., 2007).

5.1.2. CRF effects on synaptic transmission in CeA

Our lab has shown that CRF produces robust increases in GABAergic transmission in CeA of rats and mice (Nie et al., 2004; Roberto et al., 2010a; see also Figs. 2 and 3). Pre-synaptic GABA release is increased by CRF and decreased by antagonism of CRF₁Rs, the latter of which reflects a tonic facilitation of GABA release by CRF in the CeA. CRF₁R antagonists also block the ability of acute alcohol to augment GABAergic transmission in CeA (Fig. 2). In some CeA neurons from alcohol-naïve rats, CRF and acute alcohol produce additive increases in evoked IPSC amplitudes (Roberto et al., 2005). The ability of CRF and acute alcohol to augment GABAergic transmission in CeA is contingent on the integrity of PKC ϵ intracellular signaling pathways (Bajo et al., 2008). Alcohol-dependent rats exhibit heightened sensitivity to the effects of CRF and CRF₁R antagonists on GABA release in CeA, suggesting an upregulation of the CRF–CRF₁R system. These electrophysiological findings are further corroborated by increased CRF and CRF₁R mRNA levels in the CeA of alcohol-dependent rats, as well as reversal of alcohol dependence-induced elevations in amygdalar GABA dialysate by a CRF₁R antagonist (Roberto et al., 2010b).

CRF also increases GABAergic transmission in the BNST, likely via actions at post-synaptic CRF₁Rs (Kash and Winder, 2006). The juxtacapsular nucleus of the anterior division of the BNST (jcBNST) receives robust glutamatergic and dopaminergic projections from BLA and midbrain (Hasue and Shammah-Lagnado, 2002;

Larriva-Sahd, 2004), and sends GABAergic projections to the medial division of the CeA (Dong et al., 2000). Protracted withdrawal from chronic alcohol and repeated CRF treatment each produce impairments in long-term potentiation of the intrinsic excitability (LTP-IE) of jcBNST neurons in response to high-frequency stimulation (HFS), and these effects are dependent on the functionality of CRF₁Rs but not CRF₂Rs (Francesconi et al., 2009). Induction of LTP-IE of jcBNST neurons is dependent on the presence of intact D₁R function (Francesconi et al., 2009), a result that is especially interesting in light of the finding that integrity of CRF₁Rs is essential for enhancement of glutamatergic transmission in BNST produced by either exogenous dopamine application or history of cocaine exposure (Kash et al., 2008). Therefore, disruption of excitatory transmission in the BNST during protracted abstinence from alcohol may be dependent on specific and concurrent activation CRF and DA signaling in that region. A possible downstream effect of impaired intrinsic neuronal plasticity in the jcBNST might be reduced inhibition of the CeA and the emergence of a negative affective state during abstinence following the development of alcohol dependence (Francesconi et al., 2009).

5.1.3. Dynorphin/kappa opioid receptor (KOR) effects on alcohol-related behavior

Dynorphin is an opioid peptide that acts primarily at KORs. Dynorphin/KOR systems generally promote anxiety-like behavior and may have implications for addictive behaviors (Bruchas et al., 2010; Wee and Koob, 2010). Dynorphin and KORs are abundantly expressed throughout the extended amygdala (Mansour et al., 1996; Marchant et al., 2007), and acute alcohol injection produces increases in extracellular concentrations of dynorphin in both CeA (Lam et al., 2008) and NAc (Marinelli et al., 2006). Variation in the gene that encodes for the KOR is associated with alcohol dependence in humans (Beadles-Bohling et al., 2000; Edenberg et al., 2008; Xuei et al., 2006). KOR knockout mice consume less ethanol than wild-type mice (Kovacs et al., 2005). The KOR antagonist, nor-BNI, selectively suppresses alcohol drinking by alcohol-dependent rats, but does not affect alcohol drinking by non-dependent rats (Walker et al., 2010). Finally, brain dynorphin and CRF systems appear to work together to produce the aversive effects (e.g., anxiety, dysphoria) associated with stress exposure and drug withdrawal, but it is not yet clear precisely how these systems interact since there is evidence that CRF systems drive dynorphin systems and vice versa (e.g., Land et al., 2008; Valdez et al., 2007).

5.1.4. Dynorphin/KOR effects on and synaptic transmission in CeA

Our lab has recently presented findings that the dynorphin/KOR system modulates GABAergic transmission in the CeA of alcohol-naïve rats (Gilpin et al., unpublished data). Dynorphin decreases GABA_A-mediated synaptic transmission and attenuates acute

alcohol-induced increases in GABAergic transmission in CeA by reducing pre-synaptic GABA release. Furthermore, antagonism of KORs augments GABAergic transmission in CeA, suggesting a tonic inhibitory effect of endogenous dynorphin on inhibitory transmission in CeA (Gilpin et al., unpublished data). These results correlate well with recent findings that dynorphin decreases GABAergic transmission in BNST by reducing pre-synaptic GABA release, an effect that is mimicked by a KOR agonist and blocked by a KOR antagonist (Kash et al., 2009). It is not yet clear what implications these findings have for in vivo effects of dynorphin/KOR manipulations on anxiety- and alcohol-related behaviors.

5.2. Anti-stress neuromodulators

5.2.1. Nociceptin/orphanin FQ effects on alcohol-related behavior

Nociceptin is a peptide that structurally resembles endogenous opioids (Meunier et al., 1995; Reinscheid et al., 1995) and acts at opioid-like (NOP) receptors, although nociceptin does not appear to bind at endogenous opioid receptors and endogenous opioids do not bind to the NOP receptor. Nociceptin is abundantly expressed in the CeA and BNST (Neal et al., 1999) and has been described as a functional CRF antagonist (Ciccocioppo et al., 2004a). Nociceptin and NOP receptor agonists have a general anxiolytic-like profile in animal studies (Jenck et al., 1997, 2000). Nociceptin knockout mice exhibit increased anxiety-like behavior (Köster et al., 1999) and are more sensitive to social stress (Ouagazzal et al., 2003). Nociceptin KO mice also exhibit higher basal and post-stress corticosterone levels, indicating that the nociceptin system tonically inhibits activity of the HPA stress axis (Köster et al., 1999). Nociceptin also tonically inhibits norepinephrine (NE) release from afferents arriving in the amygdala, which may constitute an important site of entry for stress/arousal inputs into the extended amygdala circuitry (Kawahara et al., 2004).

Nociceptin suppresses alcohol drinking and prevents relapse-like behavior in rats (Ciccocioppo et al., 2004b; Kuzmin et al., 2007). High doses of buprenorphine also suppress alcohol drinking via activation of nociceptin receptors (Ciccocioppo et al., 2007). Nociceptin reverses stress-induced anorexia (Ciccocioppo et al., 2002), and blocks reinstatement of alcohol-seeking behavior produced by footshock stress (Martin-Fardon et al., 2000; a behavior mediated by brain CRF systems) and cues predictive of alcohol availability (Ciccocioppo et al., 2004b). Nociceptin also prevents the development and expression of conditioned place preference for alcohol in rats and mice (Ciccocioppo et al., 1999; Kuzmin et al., 2003). In general, rats selectively bred for high alcohol intake exhibit heightened sensitivity to the suppressive effects of nociceptin on alcohol drinking and related behaviors (Economidou et al., 2008). Relative to genetically heterogeneous rats, alcohol-preferring rats have higher levels of nociceptin in CeA and BNST, and nociceptin transmission in CeA appears to be most important in regulating alcohol drinking in these rats (Economidou et al., 2008). Human alcoholics exhibit decreased levels of mRNA for the nociceptin OPRL1 receptor in the CeA (Kuzmin et al., 2009), suggesting that the CeA is a critical brain site for nociceptin effects on alcohol-related behaviors.

5.2.2. Nociceptin effects on synaptic transmission in CeA

Our lab has shown that nociceptin dose-dependently and reversibly reduces GABA_A-mediated IPSCs in CeA (Roberto and Siggins, 2006). Nociceptin increases the PPF ratio of IPSCs and decreases the frequency of mIPSCs in CeA (Fig. 3), suggesting decreased pre-synaptic GABA release in that brain region. Interestingly, nociceptin both prevents (when applied before acute alcohol; Fig. 2) and reverses (when applied after acute alcohol) the ability of acute alcohol to increase evoked IPSC amplitudes and mIPSC frequencies, and decrease PPF ratio by opposing alcohol-induced increase in GABA release in CeA. Furthermore, the ability

of nociceptin to decrease GABAergic transmission in CeA is augmented following a history of alcohol dependence, suggesting that nociceptin systems in the CeA undergo neuroadaptations during chronic alcohol exposure (Roberto and Siggins, 2006). It has yet to be determined whether these effects also occur in other regions of the extended amygdala.

5.2.3. NPY effects on alcohol-related behavior

The amygdala contains a wealth of NPY fibers and receptors (Allen et al., 1984; De Quidt and Emson, 1986; Dumont et al., 1990; Gustafson et al., 1997; Migita et al., 2001). NPY exerts robust anxiolytic effects that are mediated by amygdala, and it appears that both the CeA (Heilig et al., 1993) and BLA (Sajdyk et al., 1999b) contribute to this effect. Rats selectively bred for high alcohol preference exhibit low levels of NPY mRNA and NPY in CeA that are restored by voluntary alcohol consumption (Pandey et al., 2005). Alcohol-withdrawn rats exhibit increases in anxiety-like behavior and decreased amygdalar NPY, perhaps via decreases in histone acetylation (i.e., gene transcription; Pandey et al., 2008; Roy and Pandey, 2002). It has therefore been hypothesized that rescue of impaired histone acetylation in amygdala might block withdrawal-related increases in alcohol consumption and anxiety-like behavior via restoration of NPY levels (Pandey et al., 2008; Zhao et al., 2007).

Of particular relevance to this review, it has been suggested that the ability of ICV NPY administration to block stress-induced reinstatement of alcohol-seeking behavior is mediated by activation of inhibitory neuronal populations in CeA (Cippitelli et al., 2010), a hypothesis that is supported by data showing that NPY activation in the CeA suppresses alcohol consumption in alcohol-dependent rats and abstinent alcohol-preferring rats (Gilpin et al., 2008a,b), as well as rats that exhibit innately high levels of anxiety-like behavior (Primeaux et al., 2006). Both post-synaptic Y₁ and pre-synaptic Y₂ receptors have been implicated in the effects of NPY on anxiety-like behavior and alcohol consumption. Early studies described roles for both Y₁ (Heilig et al., 1993) and Y₂ receptors (Sajdyk et al., 2002) in amygdala in anxiety-like behavior. Studies in mice have indicated that Y₁ receptors are responsible for mediating the suppressive effects of NPY on alcohol drinking (Eva et al., 2006; Sparta et al., 2004; Thiele et al., 2002). Likewise, acute stress and alcohol withdrawal produce increases in amygdalar Y₁ receptor expression in rodents (Eva et al., 2006).

5.2.4. NPY effects on synaptic transmission in CeA

Our lab has shown that NPY prevents and reverses acute alcohol-induced increases in evoked GABAergic transmission in CeA (Gilpin et al., 2011; Fig. 2). NPY blocks alcohol-induced decreases in PPF ratio and increases in mIPSC frequency, suggesting that the ability of NPY to block alcohol effects is due to suppression of pre-synaptic GABA release. Pharmacological probes with Y₁R and Y₂R antagonists confirmed the pre-synaptic site of action and suggested that NPY blocks alcohol effects via actions at pre-synaptic Y₂Rs. NPY alone does not suppress GABAergic transmission in CeA unless post-synaptic Y₁Rs are blocked, suggesting that functional Y₁Rs in CeA buffer the effects of NPY at pre-synaptic Y₂Rs. NPY also normalizes alcohol dependence-induced increases in GABA release in CeA, suggesting that chronic alcohol exposure produces neuroadaptations in NPY systems that affect inhibitory transmission in that brain region. These results are consistent with findings that NPY modulates GABA release in BNST via activation of pre-synaptic Y₂Rs (Kash and Winder, 2006), supporting the notion that Y₂Rs function not only as autoreceptors regulating NPY release (Chen et al., 1997), but also as heteroreceptors regulating the release of other neurotransmitters (Greber et al., 1994). These findings concerning NPY-GABA circuitry in the CeA are supported by recent data from our lab that infusion of a Y₂R antagonist into CeA selectively increases alcohol drinking in alcohol-dependent rats (Gilpin et al.,

unpublished data), as well as findings that infusion of a Y₁R antagonist into CeA reduces alcohol self-administration in rats (Schroeder et al., 2003).

5.3. Other neuromodulators

5.3.1. Endocannabinoids

Endocannabinoids have recently been implicated in regulation of alcohol-related behavior, particularly via their actions at cannabinoid type 1 receptors (CB₁Rs; Colombo et al., 2002, 2005; Hungund et al., 2003; Wang et al., 2003). Agonists and antagonists of CB₁Rs produce neuronal activation in CeA (Patel et al., 2005) and activation of CB₁Rs produces decreases in anxiety-like behavior that are dependent on the integrity of brain opioid systems (Zarrindast et al., 2008). Recently, our group showed that activation of CB₁Rs in the CeA produces dose-dependent decreases in GABA_AR-mediated transmission, and also blocks acute alcohol-induced increases in GABA_AR-mediated transmission by modulating GABA release (Roberto et al., 2010b). Conversely, CB₁R antagonists augment GABA_AR-mediated IPSPs and this effect is additive with acute alcohol-induced increases, suggesting a tonic inhibitory effect of endocannabinoids and non-CB₁R site of action for alcohol.

5.3.2. Galanin

Galanin, like the opioid peptides, is widespread in diverse brain regions, including amygdala where it is co-localized and co-released with vasopressin (Han and De Vries, 1999; Miller et al., 1993) and norepinephrine (Hokfelt et al., 1998; Skofitsch and Jacobowitz, 1985). High levels of Gal1, Gal2 and Gal3 receptors are expressed in amygdala (Branchek et al., 2000; Waters and Krause, 2000). Galanin administered into the ventricles is anxiolytic (Bing et al., 1993) and galanin infused directly into CeA reduces stress-induced anxiety (Khoshbouei et al., 2002; Barrera et al., 2006; but see Moller et al., 1999). Stressful stimuli release galanin (Weiss et al., 2005) and increase galanin mRNA in CeA (Palkovits, 2000). Recent electrophysiological data from our group (Bajo et al., in press) suggest that galanin has differential effects in mouse CeA neurons (see also Sharkey et al., 2008) according to the presence of galanin receptor subtypes. Galanin produces an early increase in IPSP amplitudes in one subpopulation of CeA neurons, but a later and more pronounced IPSP decrease in other CeA neurons, all in the absence of robust effects on GABA release. Augmented GABA transmission is likely mediated by post-synaptic GalR3 receptors, whereas depression of IPSPs is likely mediated by GalR2 receptors (Bajo et al., in press). Of particular relevance to the current review, galanin and alcohol produces additive facilitatory effects on IPSP amplitudes. More specifically, alcohol attenuates the ability of galanin to decrease IPSP amplitudes, but potentiates the ability of galanin to augment IPSP amplitudes, suggesting that alcohol and galanin act via different mechanisms. Future studies will clarify the behavioral relevance of the complex interaction between galanin and alcohol in CeA.

5.4. Neuropeptide interactions

5.4.1. CRF–nociceptin

Nociceptin in the brain opposes the actions of CRF and antagonizes the pro-anxiety effects of CRF. Nociceptin opposes the anorexia induced by stress and CRF (Ciccocioppo et al., 2001, 2004a) and this effect has been localized to the BNST (Ciccocioppo et al., 2003). Nociceptin infused directly into the BNST blocks the anxiogenic effects of CRF as measured on the elevated plus maze (Rodi et al., 2008).

Because excessive alcohol drinking is selectively suppressed by intra-CeA infusion of both CRF receptor antagonists and nociceptin

(Funk et al., 2006; Economidou et al., 2008), there has been recent interest in potential nociceptin–CRF interactions in the CeA. Recent data from our lab show that nociceptin prevents and reverses CRF-induced increases in pre-synaptic GABA release via effects on pre-synaptic adenylate cyclase (Cruz et al., in press). That study also confirms previous findings that nociceptin and CRF effects are each augmented in the CeA of alcohol-dependent rats, raising the possibility that the interaction between these peptides in CeA is enhanced following chronic alcohol exposure.

5.4.2. CRF–NPY

CRF and NPY have long been considered to exist in an opposing relationship, particularly because of their high degree of neuroanatomical overlap and their opposite behavioral profiles. CRF produces decreases in feeding behavior (Levine et al., 1983) via effects in the PVN (Krahn et al., 1988), increases in arousal (Koob et al., 1984), perhaps via actions in amygdala (Lee and Tsai, 1989; Liang et al., 1992) and/or interactions with noradrenergic neurons in the locus coeruleus (Butler et al., 1990), and increases in anxiety-like behavior (Koob and Thatcher-Britton, 1985) via effects in amygdala (Rassnick et al., 1993b). Conversely, NPY produces robust increases in feeding behavior (Levine and Morley, 1984) via effects in the PVN (Gilpin et al., 2004a; Stanley and Leibowitz, 1985), increases in sedation (Gilpin et al., 2004b; Heilig and Murison, 1987) via effects in the posterior hypothalamus (Naveilhan et al., 2001), and decreases in anxiety-like behavior (Heilig et al., 1989, 1992) via effects in the amygdala (Heilig et al., 1993; Sajdyk et al., 2002). It has long been hypothesized that CRF and NPY interact in a complex way to regulate emotion (Heilig et al., 1994; Sajdyk et al., 2004) and modulate alcohol dependence-related behaviors (Cowen et al., 2004; Valdez and Koob, 2004), an interaction that may occur in the amygdala.

CRF facilitates GABAergic transmission in CeA (Roberto et al., 2010b) and BNST (Kash and Winder, 2006) via actions at CRF₁Rs, whereas NPY opposes GABAergic transmission in CeA (Gilpin et al., 2011) and BNST (Kash and Winder, 2006) via actions at pre-synaptic Y₂Rs. In the CeA, CRF₁R antagonists and NPY block the effects of acute alcohol on inhibitory transmission, and neuroadaptations occur in both CRF and NPY systems during the development of alcohol dependence (Gilpin et al., 2011; Roberto et al., 2010a), reflecting recruitment or upregulation of those systems. The fact that CRF and NPY effects on inhibitory transmission are similar in multiple sub-regions of the extended amygdala suggests that these neuropeptide systems may converge on GABA neurons in CeA and BNST to affect anxiety- and alcohol-related behaviors.

6. Disinhibition model of CeA output

Most neurons in the CeA are GABAergic inhibitory projection neurons or interneurons that co-transmit GABA and one of several neuromodulators (see Fig. 1). It may seem initially counterintuitive that pro-anxiety pro-alcohol-drinking peptides (e.g., CRF) increase GABAergic transmission in CeA, whereas anti-anxiety anti-alcohol-drinking peptides (e.g., nociceptin, NPY) decrease GABAergic transmission in the same region. However, in our slice preparation, electrical stimulation and recording occur locally in the CeA and recordings of GABAergic transmission reflect the activity of inhibitory CeA interneurons and/or afferent projection neurons from other brain regions to the CeA. Therefore, observed increases in GABAergic transmission from CeA interneurons/afferents (e.g., following application of acute alcohol or CRF) inhibit the activity of GABAergic neurons projecting out of CeA. Conversely, observed decreases in GABAergic transmission from CeA interneurons/afferents (e.g., following application of nociceptin or NPY) reduce inhibition of GABAergic neurons projecting out of CeA,

thereby facilitating the release of GABA onto downstream targets. As such, recorded increases in GABAergic transmission reflect a disinhibition of downstream target regions (e.g., BNST, hypothalamus, periaqueductal gray), whereas recorded decreases in GABAergic transmission reflect a net inhibition of downstream target regions. Therefore, increases and decreases in inhibitory output from the CeA to downstream effector regions may produce decreases and increases in anxiety-like behavior, respectively (see also Davis et al., 2010; Paré et al., 2004; Tye et al., 2011).

There are two apparent exceptions to the otherwise consistent relationship between the effects of a compound on anxiety-related behavior and inhibitory transmission in CeA. First, acute alcohol, a presumed anxiolytic, produces increases in GABAergic transmission in CeA. However, even acute doses of alcohol can produce anxiogenic-like dysphoric-like effects during and after elimination of alcohol from the blood following the peak of blood-alcohol levels (e.g., see Roberto et al., 2008). Furthermore, alcohol has profound effects on excitatory transmission in CeA, particularly via effects at NMDA receptors (Roberto et al., 2004b, 2006). NMDA receptors may act as cellular 'toxicity stressors' and/or contribute to the behavioral effects of acute alcohol and alcohol withdrawal, lending at least partial resolution to this conundrum. Finally, alcohol may alter the release of local opioids (e.g., Lam et al., 2008) and/or cannabinoids (Roberto et al., 2010b) in CeA that in turn leads to increased GABA-mediated inhibition of downstream target areas. A second exception to the relationship between anxiety-related behavior and inhibitory transmission in CeA is that dynorphin, a presumed endogenous anxiogenic, produces decreases in GABAergic transmission in CeA. In the brain, dynorphin appears to interact in a complex way with CRF, which may account for this counterintuitive result. It is not yet clear what the implications of this finding are for which brain region(s) mediate dynorphin/KOR effects on anxiety- and alcohol-related behaviors.

7. CeA is an important locus for alcohol-related neuroplasticity

The data summarized here support the hypothesis that the CeA is a critical locus of neuroadaptation during the transition to alcohol dependence. Alcohol has persistent effects, particularly on inhibitory transmission, in the CeA of alcohol-dependent animals. Neuropeptides are present in high quantities in the CeA and these peptides profoundly alter inhibitory transmission, and potentially excitatory transmission. The ability of these neuropeptides to affect neurotransmission in the CeA either alone or in combination with alcohol is often up-regulated in the alcohol-dependent organism. Although manipulation of many of these systems affects alcohol drinking exclusively in the alcohol-dependent organism, it is not surprising that these neuropeptides affect basal neurotransmission in alcohol-naïve animals, particularly because the activity of all these peptides in the CeA contributes to anxiety-related behavior independent of alcohol exposure history. This final point also contributes to our understanding of *why these* neuropeptide systems are recruited and/or up-regulated during the transition to alcohol dependence, a dynamic disease state defined largely by a negative emotional state in the absence of the drug.

Electrophysiological approaches have contributed substantially to our understanding of the neuroadaptations that occur during the transition to alcohol dependence. Multiple neuropeptide and neuromodulator systems appear to be recruited in the alcohol-dependent organism, and these systems may converge on GABA circuitry in the CeA to produce alcohol-related behaviors (e.g., self-administration and anxiety-related behavior). Manipulation of neural systems that are recruited and/or functionally up-regulated during the transition to alcohol dependence produces

more robust effects on *both* post-dependent CeA neurotransmission and alcohol-related behaviors. Future studies will unravel the complex interaction effects of these neuropeptides on synaptic transmission in this brain region, and more closely examine the downstream effects of modulating these peptidergic inputs to GABAergic transmission in the CeA.

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