Published in final edited form as:
Alcohol. 2009 November ; 43(7): 509. doi:10.1016/j.alcohol.2009.01.002.

Neurobiological Mechanisms Contributing to Alcohol-Stress-Anxiety Interactions

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Abstract

This article summarizes the proceedings of a symposium that was presented at a conference entitled “Alcoholism and Stress: A Framework for Future Treatment Strategies”. The conference was held in Volterra, Italy on May 6–9, 2008 and this symposium was chaired by Jeff L. Weiner. The overall goal of this session was to review recent findings that may shed new light on the neurobiological mechanisms that underlie the complex relationships between stress, anxiety, and alcoholism. Dr. Danny Winder described a novel interaction between D1 receptor activation and the CRF system

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that leads to an increase in glutamatergic synaptic transmission in the bed nucleus of the stria terminalis. Dr. Marisa Roberto presented recent data describing how PKCε, ethanol, and CRF interact to alter GABAergic inhibition in the central nucleus of the amygdala. Dr. Jeff Weiner presented recent advances in our understanding of inhibitory circuitry within the basolateral amygdala and how acute ethanol exposure enhances GABAergic inhibition in these pathways. Finally, Dr. Brian McCool discussed recent findings on complementary glutamatergic and GABAergic adaptations to chronic ethanol exposure and withdrawal in the basolateral amygdala. Collectively, these investigators have identified novel mechanisms through which neurotransmitter and neuropeptide systems interact to modulate synaptic activity in stress and anxiety circuits. Their studies have also begun to describe how acute and chronic ethanol exposure influence excitatory and inhibitory synaptic communication in these pathways. These findings point toward a number of novel neurobiological targets that may prove useful for the development of more effective treatment strategies for alcohol use disorders.

INTRODUCTION

There is a large and growing body of clinical and preclinical evidence suggesting an important, albeit complex, relationship between stress, anxiety and alcohol use disorders (AUDs) (Piazza and Le Moal, 1998; Kushner et al., 2000a; Roberts et al., 2000; Weiss et al., 2001). For example, clinical studies have documented a significant degree of comorbidity between anxiety disorders and AUDs (Regier et al., 1990; Kessler et al., 1997; Kushner et al., 1999). Furthermore, ethanol dependence is often viewed as a chronic relapsing disease (Heilig and Egli, 2006) and there is evidence that stress and anxiety may promote relapse and negatively influence treatment prognosis (Miller and Harris, 2000; Willinger et al., 2002; Kushner et al., 2005; Fox et al., 2007; Sinha and Li, 2007).

Although these and many other studies consistently report a strong association between anxiety and AUDs (see Kushner et al., 2000a; Bradizza et al., 2006; Cosci et al., 2007), the etiological nature of this relationship is not well understood. However, recent preclinical findings are beginning to shed light on this clinically important topic. Human and animal studies have shown that acute exposure to low to moderate doses of ethanol are anxiolytic (see Kushner et al., 2000a; Koob, 2004) for reviews) and repeated exposure and withdrawal are associated with neuroadaptive changes that may lead to persistent increases in a range of anxiety measures (Roberts et al., 2000; Valdez et al., 2002; Kliethermes, 2005; Santucci et al., 2008). Several studies have also shown that, during withdrawal, ethanol-exposed animals display significant increases in voluntary ethanol consumption (Roberts et al., 1996; Becker and Lopez, 2004; Lopez and Becker, 2005). Moreover, increased intake in ethanol-dependent animals can be effectively reduced by treatments that can attenuate withdrawal-associated anxiety (e.g. CRF1-R antagonists)(Roberts et al., 1995; Valdez et al., 2002; Chu et al., 2007). These and other recent findings have led to the recognition that ethanol use and abuse likely involve both the positive and negative reinforcing effects of this drug (Koob and Le Moal, 2005). Early on, the positive or euphoric effects of ethanol (associated with the classical activation of the mesolimbic reward circuit) may dominate. However, following prolonged ethanol exposure and/or in some individuals with pre-existing anxiety disorders (Kushner et al., 2000b; Cosci et al., 2007), the negative reinforcing effects of ethanol, including anxiolysis, may become increasingly important and play a major role in both the development of abusive drinking behavior and in relapse (Lopez and Becker, 2005; Le Moal and Koob, 2007; Koob and Le Moal, 2008).

Interestingly, although much is known about the basic neurophysiological mechanisms underlying ethanol's positive reinforcing effects, the neural substrates responsible for the negative reinforcing effects of this drug, (including relief from anxiety) are much less understood. To that end, this symposium sought to highlight recent advances in our

understanding of how synaptic communication in brain regions that regulate stress and anxiety-related behaviors (e.g. amygdala, bed nucleus of the stria terminalis) can be modulated by endogenous factors like dopamine and CRF as well as acute and chronic ethanol.

Ethanol and CRF: Which is driving GABA release in the amygdala?

Maureen Cruz, Michal Bajo, George R. Siggins, Robert O. Messing and Marisa Roberto

Corticotrophin-releasing factor (CRF) is an anxiogenic neuropeptide and an important component of the stress circuits that modulate anxiety associated with drug dependence. The anxiogenic effects of CRF are mediated by type 1 CRF receptors (CRF-R1s), which are abundantly expressed in the cortex, cerebellum, hippocampus, amygdala, olfactory bulb, and pituitary (Potter et al., 1994; Chalmers et al., 1996; Palchaudhuri et al., 1998). CRF-R1 activation also plays an important role in regulating voluntary ethanol intake. The central nucleus of the amygdala (CeA) is a pivotal site of action for both the acute positive reinforcement of ethanol addiction and for the negative reinforcement associated with ethanol abstinence (Koob and Le Moal, 2001). CRF release in the CeA is increased in alcohol-dependent animals (Merlo Pich et al., 1995; Olive et al., 2002) and appears to contribute to alcohol withdrawal-related anxiety, which can be reduced by CRF-R1 receptor antagonists injected into the CeA (Rassnick et al., 1993). CRF also contributes to increased alcohol consumption in dependent animals because their increased ethanol self-administration is reduced by CRF-R1 antagonists (Spies et al., 1995; Overstreet et al., 2004) or the deletion of the CRF-R1 (Chu et al., 2007).

GABAergic transmission in the CeA has been implicated in regulating ethanol intake (Hyytia and Koob, 1995; Roberto et al., 2004a). Most of the neurons in the rodent CeA are GABAergic inhibitory neurons with inhibitory recurrent or feed-forward connections, as well as inhibitory projections to brainstem nuclei (Sun and Cassell, 1993; Davis et al., 1994). CRF is abundant in the CeA, where it is co-expressed with GABA (Watson et al., 2001). We have previously shown that CRF and ethanol enhance GABA release from mouse CeA neurons in a CRF-R1-dependent manner (Nie et al., 2004). However, little is known about the cellular mechanisms through which GABA transmission in the CeA modulates the behavioral and motivational effects of CRF and ethanol.

Recent *in vitro* evidence indicates that PKC signaling is stimulated by CRF-R1 activation (Suzuki et al., 1984; Kim et al., 2007). PKC is a family of serine-threonine kinases that respond to lipid second messengers and have been implicated in neurobehavioral disorders, including anxiety and drug abuse (Olive and Messing, 2004). Among the PKC isozymes, we hypothesized that protein kinase C epsilon (PKCε) mediates downstream effects of CRF-R1 activation in the CeA because PKCε is expressed throughout the amygdala (Choi et al., 2002) and PKCε$^{-/-}$ mice show reduced anxiety-like behavior (Hodge et al., 2002) and reduced alcohol consumption (Hodge et al., 1999; Olive et al., 2000). To test this hypothesis, we studied the role of PKCε signaling in basal CeA GABAergic transmission and in ethanol- and CRF-induced GABA release in an *in vitro* slice preparation using both genetic and pharmacological approaches (Bajo et al., 2008). Here we examined signaling pathways downstream of the CRF-R1 in the CeA that mediate GABAergic signaling and anxiety. We characterized the effects of acute ethanol and CRF on CeA GABAergic synapses in mice with a null mutation for PKCε (PKCε$^{-/-}$) and wild type (PKCε$^{+/+}$) littermates.

Using local stimulation within the CeA, we evoked pharmacologically isolated $GABA_A$ receptor-mediated IPSPs in PKCε$^{-/-}$ mutant mice and PKCε$^{+/+}$ wild-type littermates. We found that basal GABAergic transmission is enhanced (25%) in CeA neurons from PKCε$^{-/-}$ mice when compared with neurons from PKCε$^{+/+}$ mice. To determine if this effect was presynaptic, we measured the paired-pulse facilitation (PPF) ratio of the IPSPs. Generally,

changes in PPF are inversely related to transmitter release. We found that the basal PPF ratio of IPSPs was decreased in PKCε$^{-/-}$ mice. To further characterize the enhanced GABAergic transmission in PKCε$^{-/-}$ mice, we recorded pharmacologically isolated spontaneous miniature $GABA_A$ IPSCs (mIPSCs) using whole-cell patch clamp in the presence of 1μM TTX. Compared to neurons in PKCε$^{+/+}$ mice, neurons from PKCε$^{-/-}$ mice demonstrated an increased (nearly doubled) mean baseline frequency of mIPSCs with no significant difference in the mean amplitude of mIPSCs.

To examine the role of PKCε in CRF enhancement of GABAergic transmission in the CeA, we superfused CRF (200nM) on CeA slices from both PKCε$^{-/-}$ and PKCε$^{+/+}$ mice. In neurons from PKCε$^{+/+}$ mice, CRF increased GABAergic transmission (43%) but this effect was absent in neurons from PKCε$^{-/-}$ mice. CRF decreased the PPF ratio in PKCε$^{+/+}$ mice, but had no effect on the PPF ratio in PKCε$^{-/-}$ mice. Furthermore, CRF increased (52%) the mean frequency of mIPSCs in PKCε$^{+/+}$ mice, but decreased (25%) the mean mIPSC frequency in PKCε$^{-/-}$ mice. CRF did not significantly alter the mean amplitude of mIPSCs in either PKCε$^{-/-}$ or PKCε$^{+/+}$ mice. To confirm the role of PKCε in CRF-induced changes in GABAergic transmission, we superfused Tat-ε V1-2 (500nM), a PKCε inhibitor peptide, onto CeA slices from PKCε$^{+/+}$ mice. The inhibitor increased the mean evoked IPSP amplitude and decreased the PPF ratio of IPSPs, and blocked the CRF effects.

We investigated whether ethanol-stimulated GABA release also involved PKCε. Ethanol (44mM) increased (47%) the mean amplitude of evoked IPSPs in PKCε$^{+/+}$ neurons but not in PKCε$^{-/-}$ neurons. Ethanol decreased the PPF ratio of IPSPs in PKCε$^{+/+}$ neurons, but this effect was absent in PKCε$^{-/-}$ neurons. Like CRF, ethanol increased (more than doubled) the mean frequency of mIPSCs in PKCε$^{+/+}$ neurons but decreased (20%) the mean mIPSC frequency in PKCε$^{-/-}$ neurons. Ethanol had no significant effect on mIPSC amplitudes in both the PKCε$^{-/-}$ and PKCε$^{+/+}$ neurons. Pretreatment of PKCε$^{+/+}$ neurons with the PKCε inhibitor Tat-ε V1-2 completely abolished the ethanol effects, confirming findings in the PKCε$^{-/-}$ CeA.

Both CRF and ethanol increased the mean amplitude of evoked GABA IPSPs and decreased the PPF ratio of IPSPs in PKCε$^{+/+}$ mice. Furthermore, CRF increased the mean frequency of mIPSCs in PKCε$^{+/+}$ neurons and decreased the mIPSC frequency in PKCε$^{-/-}$ neurons. Pretreatment with a PKCε inhibitor of PKCε$^{+/+}$ neurons blocked the CRF- and ethanol-induced effects on IPSP amplitudes and PPF. These data indicate that the PKCε isozyme has a double function. Under drug-stimulated conditions, PKCε facilitates vesicular GABA release. However, without drug treatment, a basal level of PKCε activity serves to limit spontaneous GABA release.

In conclusion, our data identify a PKCε signaling pathway in the CeA that is activated by CRF-R1 stimulation, regulates neurotransmitter release at GABAergic terminals, and may contribute to increased anxiety-like behavior (Bajo et al., 2008). Moreover, consistent with our previous observation that ethanol-induced GABA release in the amygdala is CRF-R1-dependent (Nie et al., 2004), here we also find that ethanol-stimulated vesicular GABA release depends on PKCε. Taken together, these findings indicate a signaling pathway whereby CRF, acting via presynaptic CRF-R1s in the amygdala, activates PKCε to stimulate GABA release (Bajo et al., 2008). Because CRF is anxiogenic and plays an important role in promoting alcohol drinking (Heilig and Koob, 2007), disturbance of this CRF-R1-PKCε signaling pathway in the CeA likely contributes to decreased anxiety-like behavior and decreased alcohol consumption in PKCε$^{-/-}$ mice. These studies provide insight into some of the neurobiological mechanisms that contribute to alcohol-stress-anxiety interactions. Being able to identify which enzymes are implicated in alcohol intake and dependence may be helpful in developing new and innovative preventive strategies and pharmacotherapeutic remedies for stress- and alcohol-related biomedical phenomena.

Dopamine Regulation of Synaptic Transmission in the Bed Nucleus of the Stria Terminalis

Thomas L. Kash and Danny G. Winder

Drugs of abuse, including alcohol, are thought to exert effects on behavior through modulation of neuronal activity and plasticity in specific brain regions. A great deal of effort has been focused on understanding the impact of drugs of abuse on the mesolimbic dopamine system, in particular the dopamine neurons of the ventral tegmental area (VTA) (Borgland et al., 2006) and the medium spiny neurons of the nucleus accumbens (Thomas et al., 2001), as this network is thought to serve as a common pathway for drug-seeking behavior. However, there is growing evidence that drugs of abuse can alter function in regions outside of the classical reward circuitry, and this modulation is critical for specific aspects of addiction (Koob and Le Moal, 2008).

The bed nucleus of the stria terminalis (BNST), a component of the central extended amygdala, is a region of the brain that has been implicated primarily in the regulation of stress and anxiety (Walker and Davis, 2008). A large literature suggests that corticotrophin releasing factor (CRF) signaling within this region plays an important role in these behaviors (Davis et al., 1997). Further, while not part of the classical reward circuitry, the BNST receives dopaminergic projections, from both the VTA and the periaqueductal gray (Fadda et al., 1985), and has been suggested to be an important regulator of VTA dopamine neuron firing (Georges and Aston-Jones, 2002).

In keeping with the important interconnections between the BNST and reward circuitry, studies have suggested that the BNST may also be involved with behavioral adaptations following prolonged exposure to drugs of abuse (Dumont et al., 2005; Grueter et al., 2008). Given the role that dysregulation of emotional behaviors, including fear and anxiety, is proposed to play in chronic drug abuse, the involvement of the BNST in these processes, while exciting, is not surprising. Several studies demonstrate that the BNST is also involved in the acute reinforcing actions of drugs of abuse. In particular, acute administration of ethanol, and a range of other abused drugs, leads to a significant increase in dopamine levels in the BNST (Carboni et al., 2000). Further, it has been shown that dopamine receptor antagonism in the BNST can alter operant responding for alcohol and cocaine (Watkins et al., 1999; Eiler et al., 2003). Taken together, these studies suggest that dopamine signaling in the BNST is involved in regulation of the acute actions of multiple drugs of abuse.

Based on the above findings, we hypothesized that dopamine modulates synaptic transmission in the BNST. To test this hypothesis, we examined the ability of dopamine to modulate synaptic transmission in the BNST using an *ex vivo* slice preparation. We found that a brief application of dopamine led to a transient increase in the frequency of spontaneous excitatory post-synaptic currents (sEPSCs) in BNST. This effect was blocked by the D1 dopamine receptor (D1R) antagonist SCH23390 and was absent in the D1R knockout mouse. These results strongly support the possibility that dopamine is exerting this effect through D1R-mediated signaling. In order to understand the mechanisms underlying this action of dopamine, we next examined the ability of dopamine to modulate spontaneous excitatory synaptic transmission in the presence of the sodium channel blocker tetrodotoxin (mEPSCs). Curiously, we found that dopamine had no effect on either mEPSC frequency or amplitude. Taken together, these results suggest that dopamine is enhancing glutamatergic transmission in the BNST in a D1R and activity-dependent fashion.

This lack of an effect on mEPSCs suggested that dopamine could be acting to modulate synaptic transmission by altering the excitable properties of neurons in the BNST. In order to evaluate

this possibility, we examined the ability of dopamine to modulate the membrane potential of BNST neurons. We found that in most neurons dopamine had no effect on the membrane potential. However, in a small subpopulation of neurons we found that dopamine caused a robust depolarization associated with an increase in spontaneous action potential firing.

Several studies in rat have demonstrated that dopamine fibers are associated with CRF-positive neurons in the BNST (Fadda et al., 1985; Phelix et al., 1999). Using double-label immunohistochemistry we observed a similar pattern of expression in the mouse BNST. Based on this, we reasoned that the actions of dopamine could be mediated in part through activation of the CRF system. In order to test this, we applied dopamine in the presence of the CRF-R1 antagonist, NBI27914, and found that the effect was blocked. This finding raised the possibility that dopamine is enhancing glutamatergic transmission in the BNST by causing release of CRF. We tested this possibility by examining the actions of both CRF and another CRF receptor agonist, urocortin, on sEPSCs in the BNST. Both of these compounds increased the frequency, but not amplitude, of sEPSCs in the BNST. Using selective antagonists for CRF-R1 and CRF-R2, we found that the effects of CRF and urocortin were mediated through activation of CRF-R1. Finally, in order to identify the mechanism of action, we examined the ability of CRF to modulate mEPSCs. We found that CRF significantly increased mEPSC frequency but had no effect on mEPSC amplitude, consistent with an increase in glutamate release. Taken together these results suggest that CRF enhances glutamate release via activation of the CRF-R1 in the BNST.

Our results demonstrate the dopamine, acting at the D1R, enhances fast excitatory synaptic transmission in the BNST through a CRF-R1 dependent mechanism. When taken together with previous results, our findings suggest that glutamatergic transmission in the BNST plays an important role in self-administration of drugs of abuse, including alcohol and cocaine. Moreover, these findings, particularly the functional link between dopamine and CRF signaling, support the idea that the activation of regions involved in regulation of emotion, such as the BNST, may reflect arousal independent of the valence of the event.

Acute Effects of Ethanol on Local and Lateral Paracapsular GABAergic Synapses in the Rat Basolateral Amygdala

Yuval Silberman and Jeff L. Weiner

Along with the BNST and CeA discussed earlier, the basolateral amygdala (BLA) is also an integral element of both stress/anxiety ((LeDoux, 1993; Davis et al., 1994) and reward neurocircuitry (Balleine and Killcross, 2006; Tye et al., 2008). The groups of cells within the lateral, basal and accessory basal nuclei of the amygdala are typically referred to as the BLA. This brain region consists primarily of glutamatergic pyramidal neurons (~90% of all cells in the BLA), which provide the main excitatory input to the CeA as well as many other limbic and cortical structures (Sah et al., 2003). As such, the BLA is in the unique position to serve as the major input for sensory information into the amygdala complex and is critically involved in establishing the emotional salience of environmental stimuli. While GABAergic interneurons represent only a small portion of the neurons within the BLA, they are thought to play an integral role in the regulation of excitatory transmission in this brain region (Washburn and Moises, 1992), and thus are likely to be critically involved in the regulation of anxiety-like behaviors.

Although it is clearly an oversimplification, in general, increasing excitatory output of the BLA is usually associated with increases in anxiety-like behavior whereas dampening this activity usually results in anxiolysis (Davis et al., 1994; Menard and Treit, 1999). Thus, GABAergic inhibitory tone in the BLA likely plays an integral role in regulating anxiety-like behaviors.

Since acute ethanol exposure has been shown to enhance GABAergic synaptic transmission in many brain regions (Siggins et al., 2005; Weiner and Valenzuela, 2006), our recent studies have focused on characterizing the acute effects of ethanol on GABAergic synaptic inhibition in the BLA.

A wide range of distinct classes of local interneurons have been described within the BLA, based on differences in their morphological and electrophysiological characteristics (Washburn and Moises, 1992; Woodruff and Sah, 2007) as well as the types of classical interneuronal markers that they express (McDonald and Mascagni, 2002; Mascagni and McDonald, 2003; Muller et al., 2007; Woodruff and Sah, 2007). These cells are sparsely distributed throughout the BLA and are thought to provide the majority of feedback inhibition onto BLA pyramidal neurons. Importantly, Marowsky and colleagues, using GAD-GFP transgenic mice, recently identified a novel cluster of GABAergic cells located along the external capsule-BLA border. They demonstrated that these cells are devoid of many of the classical markers of GABAergic interneurons (e.g. Parvalbumin, CCK), are excited by cortical input through the external capsule, and provide a major feed-forward inhibitory input onto BLA pyramidal neurons (Marowsky et al., 2005). Thus, we sought to confirm that lateral paracapsular (lpcs) cells are also present in the rat BLA and to characterize ethanol modulation of local and lpcs inhibition in this brain region.

Using young Sprague-Dawley rats (4–6 weeks old) and immunohistochemical techniques to look for GAD expression, we first confirmed that both local and lpcs interneurons were present in the rat BLA. As observed in the mouse, local interneurons were visualized as punctate staining throughout the BLA while lpcs cells appeared densely clustered along the BLA-external capsule border. In addition, while the majority of local interneurons stained positive for parvalbumin, lpcs interneurons were devoid of this protein (Silberman et al., 2008).

We next used whole-cell patch clamp methods to record from BLA pyramidal neurons. Using a standard paired-pulse protocol, we first demonstrated that we could discretely activate GABAergic synapses arising from local and lpcs interneurons and then examined their sensitivity to ethanol. Notably, although ethanol potentiated both local and lpcs evoked IPSCs (eIPSCs) to a similar extent, across a range of pharmacologically relevant concentrations (10–80 mM), the mechanism of ethanol action differed markedly at these two pathways. Ethanol potentiation of local eIPSCs was associated with a decrease in paired pulse ratio and could be significantly enhanced by pretreatment with a $GABA_B$ receptor antagonist, SCH-50911. In addition, pretreatment with a low concentration of the $GABA_B$ receptor agonist baclofen significantly reduced ethanol potentiation of local eIPSCs. These effects are very similar to those observed in the hippocampus where several studies have demonstrated that ethanol enhances GABAergic inhibition primarily via a presynaptic facilitation of GABA release (Ariwodola and Weiner, 2004; Li et al., 2006).

In contrast, ethanol had no effect on PPR at lpcs synapses and ethanol potentiation of lpcs-mediated inhibition was not influenced by pretreatment with either a $GABA_B$ receptor agonist or antagonist. Interestingly, bath application of a $GABA_B$ receptor antagonist alone significantly potentiated lpcs, but not local, IPSCs, possibly suggesting higher ambient GABA levels at lpcs synapses.

Taken together, these initial studies demonstrated that ethanol significantly potentiated local and lpcs-mediated GABAergic inhibition in the BLA, consistent with the well-known anxiolytic effects of this drug. Moreover, while ethanol potentiation of local GABAergic synapses appears to be mediated via a presynaptic mechanism, common to several other brain regions (Siggins et al., 2005; Weiner and Valenzuela, 2006), ethanol enhancement of lpcs

IPSCs does not involve a facilitation of terminal GABA release and may be mediated postsynaptically.

While there are many potential mechanisms through which ethanol may enhance lpcs synapses, several lines of evidence point to a possible role of the β noradrenergic receptor system (β-AR). Previous work in the cerebellum demonstrated that NE can enhance $GABA_A$ receptor function (Cheun and Yeh, 1996) and that β-AR function is required for postsynaptic facilitatory effects of ethanol on GABA-mediated inhibition of Purkinje cell firing (Lin et al., 1991). Interestingly, the BLA receives dense NE input from the locus coeruleus and other noradrenergic brain regions via inputs near the external capsule (Fallon et al., 1978; Roder and Ciriello, 1993) where lpcs interneurons are localized and β-AR activation has been shown to suppress LTP in the BLA. We therefore tested the hypothesis that ethanol potentiation of lpcs synapses may be dependent on β-AR activation. Our initial studies demonstrated that 20 μM NE significantly potentiated lpcs, but not local, IPSCs and this effect was completely blocked by pretreatment with a cocktail of α1, α2, and β-AR antagonists. In addition, although pretreatment with this antagonist cocktail had no effect on its own, this cocktail significantly and selectively reduced ethanol potentiation of lpcs synapses. Additional preliminary studies suggest that pretreatment with a β-AR antagonist alone can significantly antagonize ethanol potentiation of lpcs IPSCs.

In summary, our findings suggest that ethanol significantly enhances GABAergic synaptic inhibition arising from both local and lpcs interneurons in the BLA. Therefore, acute ethanol exposure increases both cortical feed-forward inhibition as well as local feedback inhibition onto the primary excitatory output cells of the BLA. Since increases in BLA GABAergic inhibition are associated with decreases in anxiety-like behavior, ethanol enhancement of these two inhibitory pathways likely contributes to the acute anxiolytic effects of this drug. Given the important role that anxiety is thought to play in the etiology of alcohol abuse, it will be important in future studies to further resolve the specific mechanisms through which ethanol enhances GABAergic inhibition at local and lpcs synapses and to examine how these pathways may be influenced by chronic ethanol exposure and withdrawal.

Glutamate, GABA, and Amygdala-Dependent Anxiety: Tipping the Balance with Chronic Ethanol and Withdrawal

Anna K. Lack, Marvin R. Diaz, Daniel T. Christian, Ann M. Chappell, and Brian A. McCool

The lateral/basolateral amygdala (BLA) is a central component of the brain's fear/anxiety circuit and acts as the primary input nuclei of the amygdala. For example, the BLA receives extensive input from sensory/limbic/insular cortex (Ong et al., 2000) and thalamic nuclei (Ong et al., 2000). The region in turn provides major excitatory input to the neighboring central nucleus (Nose et al., 1991), to the nucleus accumbens (North et al., 1987), and has extensive reciprocal connections with medial prefrontal and orbitofrontal cortex (Krettek and Price, 1978; Porrino et al., 1981). Communication within the context of these important anatomical relationships appears to be governed by the balance between excitatory and inhibitory neurotransmission within the BLA (Sanders and Shekhar, 1995; Sajdyk and Shekhar, 1997a).

The privileged position held by the BLA within the fear/anxiety circuit may sub serve its central role in drug abuse-related behaviors. For example, the BLA appears to be critical for cue-induced re-instatement of cocaine (Fuchs et al., 2006) and heroin (Rizos et al., 2005) seeking in rodents following chronic exposure. Consistent with these observations, long-term cocaine and morphine self-administration increases the expression of BLA glutamate-gated ion channel subunits (Panchenko et al., 1999; Brunton et al., 2005). Likewise, chronic ingestion of an ethanol-containing liquid diet increases NMDA-type glutamate receptor function measured in

acutely isolated rat BLA neurons (Samson et al., 1997). These findings suggest that altered glutamatergic signaling in the BLA following chronic drug exposure may be a common characteristic shared by drugs of abuse. Importantly, the relationships between increased glutamate receptor expression or function, BLA neurophysiology, and withdrawal-related anxiety-like behavior have been largely unexplored.

We have recently employed a chronic intermittent ethanol inhalation paradigm (Becker and Hale, 1993) to investigate this relationship. Male Sprague-Dawley rats received twelve hours of ethanol vapor for ten consecutive days. Experimental groups consisted of individuals housed in identical conditions but receiving only air during the ten day period (CON), ethanol-exposed individuals where measures were made immediately following the tenth ethanol exposure, while animals were still intoxicated (CIE), and ethanol-exposed individuals withdrawn from the ethanol treatment for twenty-four hours (WD). Alterations in BLA neurophysiology were assessed using both whole-cell patch clamp electrophysiology and field potential recordings. Behavioral manifestations within these treatment groups were assessed using a light/dark test for anxiety-like behavior. In some experiments, glutamate receptor agonists or antagonists were microinjected into the BLA using standard procedures.

Consistent with previous work showing that chronic ethanol liquid diet exposure increases NMDA receptor function in isolated BLA neurons, CIE and WD increased the function of synaptic NMDA receptors recorded from principal neurons within BLA coronal brain slices (Lack et al., 2007). This increase was evident using two independent measures. First, the ratio of NMDA- to AMPA-mediated synaptic responses, measured by first examining the amplitude of a compound synaptic response and then inhibiting the AMPA-component with the antagonist DNQX, was significantly larger in both CIE and WD neurons. Second, an NMDA-specific stimulus-response relationship was significantly greater in CIE and WD neurons across a range of stimulus intensities. These data suggest that, like many brain regions, chronic ethanol/withdrawal increase synaptic function of NMDA receptors in the BLA.

Kainate receptor (KAR)-mediated synaptic responses can be measured in BLA neurons (Li and Rogawski, 1998). And, these receptors can initiate long-term increases in synaptic strength that are distinct from other forms of synaptic plasticity in the BLA (Li et al., 1998). Importantly, KAR-mediated synaptic responses in BLA neurons are acutely sensitive to ethanol and are more potently inhibited than NMDA receptor synaptic responses expressed in these same neurons (Lack et al., 2008). KAR-mediated synaptic plasticity was likewise inhibited by acute ethanol. Chronic ethanol exposure increased KAR-mediated synaptic responses relative to both CON and WD neurons (Lack et al., 2009). Thus, CIE-dependent increases in KAR synaptic function are transient. However, KAR-mediated synaptic plasticity was diminished in both CIE and WD BLA neurons. This suggests that CIE/WD either 1) inhibits the mechanisms required to establish KAR-dependent synaptic plasticity or 2) engages the mechanisms responsible for the expression of synaptic plasticity and thus occlude the subsequent *in vitro* initiation in CIE and WD BLA slices.

Expression of synaptic plasticity depends upon AMPA receptor-dependent transmission in many brain regions including the BLA. Along these lines, CIE and WD both significantly increased spontaneous AMPA-mediated synaptic transmission in BLA neurons. This increase appeared to involve both increased postsynaptic AMPA receptor function as well as increased presynaptic release of glutamate (Lack et al., 2007). These data suggest that the CIE/WD-dependent decrease in KAR-mediated synaptic plasticity was more likely related to an activation of AMPA-related mechanisms required for the expression of synaptic plasticity. Consistent with this interpretation, BLA field EPSPs stimulus-response relationships were increased in both CIE and WD treatment groups (Lack et al., 2009).

The behavioral manifestations related to increased BLA-dependent glutamate signaling, particularly subsequent to chronic ethanol exposure or withdrawal, have not been examined. WD, but not CIE, significantly increased anxiety-like behavior measured in the light/dark apparatus (Lack et al., 2007). Importantly, this increase in anxiety-like behavior was alleviated by microinjection of the AMPA receptor antagonist, DNQX, into the BLA of WD animals. These data suggest that increased BLA glutamatergic synaptic transmission during WD may contribute to increased anxiety-like behavior in this group. In contrast, the absence of a significant anxiety-related phenotype in CIE animals contrasts with the increased glutamatergic function in the BLA of these animals. Since the balance between excitatory and inhibitory BLA neurotransmitters is known to regulate anxiety-like behavior (Sanders and Shekhar, 1995; Sajdyk and Shekhar, 1997b), our data suggests that ethanol-sensitive inhibitory systems (e.g. GABA) may make substantial contributions to the regulation of anxiety-like behavior in intoxicated animals.

In conclusion, we've recently shown that chronic intermittent ethanol inhalation and subsequent withdrawal engage and up-regulate BLA glutamate receptor systems. These alcohol-dependent alterations largely parallel those responsible for cue-dependent synaptic plasticity during classical fear learning (Walker and Davis, 2002). Together, these findings suggest that treatments or physiological/psychological paradigms that reverse or ameliorate cue-related fear learning may have some efficacy for reversing or diminishing synaptic alterations resulting from chronic ethanol exposure and withdrawal.

SUMMARY

The results of these studies provide new insight into some of the modulatory mechanisms that regulate fast synaptic communication within brain regions involved in both reward and stress/anxiety systems. In particular, CRF signaling has emerged as an important presynaptic regulator of excitatory and inhibitory synaptic transmission in some of these areas. In the BNST, activation of CRF-R1s mediates dopamine enhancement of glutamate release (Kash et al., 2008), while CRF-R1s in the CeA can influence basal GABAergic tone and enhance GABA release through a PKCε-dependent mechanism (Bajo et al., 2008). These data suggest that CRF plays an important role in setting the delicate balance between excitation and inhibition in brain circuits that likely influence ethanol self-administration, stress, and anxiety-like behaviors. In fact, at least within the CeA, CRF-R1 activation is required for ethanol enhancement of GABAergic transmission (Nie et al., 2004; Bajo et al., 2008). Additional studies will be needed to elucidate the behavioral significance of these findings and importantly, to determine how the CRF system in these, and other, brain regions adapts following chronic ethanol exposure. However, the observations that CRF-R1 antagonists can have anxiolytic properties (Takahashi, 2001; Holsboer and Ising, 2008) and are particularly effective at reducing alcohol drinking in stressed (Marinelli et al., 2007; Lowery et al., 2008) or ethanol-dependent (Funk et al., 2007; Gilpin et al., 2008) animals suggest that dysregulation of CRF-R1 signaling may play an integral role in the development of alcoholism.

In the BLA, new evidence was presented describing two distinct inhibitory pathways that regulate excitability in this brain region. Ethanol significantly enhanced both of these inhibitory pathways, consistent with this drug's well-known anxiolytic properties. However the mechanisms underlying these effects were quite different. Although ethanol enhanced local GABAergic inhibition via a presynaptic mechanism that was tightly regulated by $GABA_B$ receptor activity, ethanol potentiation of lpcs-mediated inhibition was not modulated by $GABA_B$ receptor activity nor was it associated with an increase in terminal GABA release probability (Silberman et al., 2008). In contrast, ethanol enhancement of lpcs IPSCs did require β-NE receptor activation, as previously shown for ethanol potentiation of GABA inhibition of Purkinje cell firing (Lin et al., 1991). Additional studies will be needed to further characterize

the mechanisms underlying ethanol facilitation of local and lpcs-mediated GABAergic inhibition in the BLA and, importantly, to determine how these pathways adapt following repeated ethanol exposure and withdrawal. Interestingly, baclofen (a $GABA_BR$ agonist) has been shown in animal and human studies to reduce measures of alcohol intake, craving and relapse (Addolorato et al., 2002b; Addolorato et al., 2002a; Colombo et al., 2004; Flannery et al., 2004). The observation that baclofen pretreatment significantly reduced the acute potentiating effect of ethanol of local BLA IPSCs may provide a possible neurobiological mechanism that contributes to the efficacy of this drug as a treatment for alcoholism. It will be of interest in future studies to examine the effect of intra-BLA manipulations of the $GABA_B$ and β-NE receptor systems on ethanol drinking and measures of ethanol-mediated anxiolysis.

Finally, it was shown that chronic ethanol exposure and withdrawal have profound neuroadaptive effects on excitatory synaptic transmission in the BLA. A ten day intermittent inhalation procedure resulted in significant increases in NMDA and KA receptor function as well as increased pre- and postsynaptic measures of AMPA receptor-gated synaptic excitation (Lack et al., 2007). Importantly, although enhanced glutamatergic transmission was evident immediately after the chronic ethanol treatment, increases in behavioral measures of anxiety-related behavior only emerged during withdrawal. As noted by these authors, since the expression of anxiety-related behaviors is largely governed by the balance between excitatory and inhibitory transmission in brain regions like the BLA, it seems likely that the acute facilitatory effects of ethanol on GABAergic inhibition in this, and other brain regions, may counter the hyperglutamatergic activity that develops during chronic ethanol treatment. Interestingly, several studies have demonstrated that tolerance does not develop to the acute potentiating effects of ethanol on GABAergic synapses in several brain regions within the stress/anxiety circuitry (Kang et al., 1998; Roberto et al., 2004b). The persistence of these acute effects of ethanol on GABAergic inhibition, particularly in the presence of increased glutamatergic excitation, provides a plausible neurobiological mechanism that may help explain the increased saliency of ethanol's negative reinforcing effects that is thought to emerge during the progression of alcohol dependence (Koob, 2004; Koob and Le Moal, 2008).

In conclusion, these studies demonstrate that acute and chronic ethanol exposure have profound effects on the balance between excitatory and inhibitory synaptic transmission in several key brain regions within the stress/anxiety circuitry. These studies also highlight new synaptic elements that either potently modulate synaptic communication in these regions (e.g. CRF-R1s) and/or significantly alter ethanol effects on synaptic activity in these circuits (e.g. GABAB-Rs, β-ARs). These findings, along with those of many other studies, suggest that drugs that can selectively target some of these synaptic elements may prove to be effective pharmacotherapies for the treatment of alcohol addiction.

Acknowledgments

This work was supported by NIH grants AA017039 (Y.S.), AA017576 (M.R.D.), AA017668 and AA016025 (T.L.K.), AA10994 and AA013498 (G.R.S.), AA013588 (R.O.M.), DA 019112 and AA 013641 (D.G.W.), AA016985, AA015566 and AA06420 (M.R.), AA014445, AA016671, and AA017053 (B.A.W.), AA013960 and AA017053 (J.L.W.)

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