A STUDY ON THE EFFECTS OF CHRONIC ALCOHOL AND
WITHDRAWAL ON THE GLUTAMATE, GABA, AND DOPAMINE SYSTEMS IN
THE BASOLATERAL AMYGDALA OF SPRAGUE DAWLEY RATS

BY

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List of Abbreviations

α1 – Alpha 1 GABAA subunit
α2 – Alpha 2 GABAA subunit
α3 – Alpha 3 GABAA subunit
α4 – Alpha 4 GABAA subunit
γ2 – Gamma 2 GABAA subunit
AMPA - Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APV - 2-amino-5-phosphonovaleric acid
β1 – Beta 1 GABAA subunit
BLA – Basolateral amygdala
Ca+ - Calcium ions
cAMP – Cyclic adenosine monophosphate
CeA – Central amygdala
CET – Chronic ethanol treated
CIE/CE – Chronic intermittent ethanol
Cl- - Chloride ions
CON - Control
CRF – Corticotropin releasing factor
DA – Dopamine
DAT – Dopamine transporter
D1R – Dopamine D1 receptor
D2R – Dopamine D2 receptor
D3R – Dopamine D3 receptor
D5R – Dopamine D5 receptor
DNQX - 6,7-Dinitroquinoxaline-2,3-dione
EC – External capsule
EPSC - Excitatory postsynaptic currents
EPSP - Excitatory postsynaptic potential
fEPSP - Field excitatory postsynaptic potential
Gi/o - Gi/o alpha subunit
Gs – Gs alpha subunit
GIRKs - G-protein-coupled inwardly rectifying potassium channels
GABA - Gamma-aminobutyric acid
GluR – Glutamate receptor
ICM – Intercalated cell masses
i.p. – Intraperitoneal
IPSC – Inhibitory postsynaptic current
IPSP – Inhibitory postsynaptic potential
K+ - Potassium ions
KA-R – Kainate receptor
LA – Lateral amygdala
LTP – Long-term potentiation
mEPSC - Miniature excitatory postsynaptic currents
Mg+ - Magnesium ions
mIPSC – Miniature inhibitory postsynaptic currents
mRNA - Messenger ribonucleic acid
Na+ - Sodium ions
NMDA - N-methyl-D-aspartic acid
NMDAR - N-methyl-D-aspartic acid receptor
NR – NMDA receptor subunit
PKA – Protein kinase A
PFC - prefrontal cortex
sEPSC - Spontaneous excitatory postsynaptic currents
sIPSC – Spontaneous inhibitory postsynaptic currents
TTX - Tetrodoxin
VTA - Ventral tegmental area
WD - Withdrawal
Abstract

Diaz, Marvin R.

THE EFFECTS OF CHRONIC ALCOHOL AND WITHDRAWAL ON THE GLUTAMATE, GABA, AND DOPAMINE SYSTEMS IN THE BASOLATERAL AMYGDALA OF SPRAGUE DAWLEY RATS

Dissertation under the directions of

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Alcoholism, a significant issue in society, has been correlated with anxiety disorders. Specifically, severe anxiety experienced during withdrawal from alcohol significantly increases the relapse rate in recovering alcoholics. Therefore, understanding the changes that occur in the brain regions involved in anxiety may help in developing new pharmacological therapies to treat alcohol withdrawal-induced anxiety. The amygdala, in particular the basolateral amygdala (BLA), has been shown to be an important brain region in initiating and processing sensory information pertaining to anxiety-related stimuli in humans and rodents. The BLA also plays a major role in alcohol exposure as different exposures to alcohol can have profound effects on BLA physiology, in particular synaptic transmission. The primary BLA neurotransmitter systems that influence BLA-output consist of the glutamate (excitatory) and GABA
(inhibitory) systems. Extensive evidence suggests that these two systems are significantly altered by chronic alcohol and withdrawal, resulting in ‘hyperexcitable’ BLA-output and anxiety-like behavior. Furthermore, the BLA inhibitory GABA system is modulated by the midbrain via the dopamine system, suggesting another potential target for the effects of chronic alcohol and withdrawal on anxiety-like behavior. Therefore, the main goal of this study was to examine the effects of chronic ethanol exposure and withdrawal on the BLA glutamate and GABA systems, and to characterize how the dopamine (DA) system modulates BLA GABAergic transmission. In chapter II we investigated the effects of chronic intermittent ethanol (CIE) and withdrawal (WD) on glutamatergic synaptic transmission in the BLA. This set of studies suggests that glutamatergic function is increased at BLA synapses in CIE and WD treated animals, albeit via distinct mechanisms. Furthermore, we found that manipulations of the glutamate system altered anxiety-like behaviors. In chapter III we examined the effects of CIE and WD on two populations of GABAergic inhibitory interneurons in the BLA. We found that CIE and WD differentially affect GABAergic transmission from local feed-back interneurons and feed-forward lateral paracapsular cells (LPCs), resulting in an overall suppression of GABAergic inhibition during withdrawal. In addition, the effects of acute ethanol on the two interneuron populations are not altered by CIE or WD. In chapter IV we characterized DA modulation of alcohol-naïve BLA GABAergic transmission at local and LPC synapses. This study revealed that inhibition of DA D3 receptors (D3Rs) in the BLA of alcohol-naïve adolescent Sprague Dawley rats can significantly alter anxiety-like behavior. Moreover, these results led us to uncover a functional role of D3Rs at local and LPC synapses as well as a concentration dependent effect of DA at local synapses.
Finally, in chapter V we focused on determining the effects of CIE and WD on the DA modulation of GABAergic transmission. Preliminary data suggest that there are age-dependent changes in DA modulation of BLA GABAergic transmission. Additionally, CIE and WD appear to differentially alter DA modulation of GABAergic transmission from local and LPC synapses in older animals. Taken together, these studies suggest that chronic ethanol exposure and withdrawal shift the balance between glutamate and GABA, potentially through alterations in the dopamine system, resulting in an increase in withdrawal-induced anxiety-like behavior.
1. Introduction

Alcoholism and Alcohol Withdrawal

Alcoholism is a major problem in society that affects many people. Every year, 17 million adults experience problems related to alcohol (Brachtesende, 2006). Furthermore, alcohol-related costs in the U.S. (i.e. health care and court costs) exceed $180 billion/year (Harwood, 2000). These statistics demonstrate the significant issues that alcoholism entails, yet does not address the psychological and emotional side-effects that also arise. A main detriment of alcoholism is that it interferes with one’s home life, job, and health (Grant, 2004). Given the negative impact of these issues, their occurrence may persuade an alcoholic to quit drinking. Unfortunately, the decision to abstain from alcohol after a chronic history can have severe long term effects.

Alcohol withdrawal is a leading cause of relapse in recovering alcoholics due to the harsh symptoms that emerge following cessation of alcohol. These withdrawal symptoms can range from altered perceptions to motor impairments. Specifically, seizures, hallucinations, and delirium tremens (DT) have been shown to occur during withdrawal from alcohol (Becker, 2000). More importantly, severe anxiety has been shown to arise following withdrawal from alcohol (Becker, 2000). Interestingly, this anxiety can occur within a few hours of an alcohol binge and can persist for weeks. Alcohol can relieve this withdrawal anxiety, as well as other typical withdrawal symptoms. Unfortunately, this usually begins a cycle where a recovering alcoholic
reverts back to alcohol to diminish the severity of the withdrawal symptoms. This vicious cycle has been suggested to be a form of self-medication to treat withdrawal-induced anxiety that eventually leads to relapse (Breese et al., 2005). In addition, even after the initial anxiety experienced from alcohol abstinence subsides, stressful life events can draw an individual back to alcohol use (Brown et al., 1995). Therefore, due to the cyclic and complex nature of the disease, it is difficult to study alcoholism exclusively in humans.

Animal models have been a helpful tool in studying different aspects of alcohol consumption and withdrawal. These models have been shown to produce predictive validity of the disease and have therefore been extremely beneficial in studying many components of alcoholism. Specifically, the three main animal models used to study alcohol exposure include: voluntary consumption, liquid ethanol diet, and forced consumption (reviewed in (Becker, 2000) (forced consumption will primarily be discussed in this document). Forced consumption of alcohol can be employed through various methods, such as an intra-gastric infusion or alcohol vapor exposure (Becker, 2000). Although both of these methods of alcohol administration can be used for chronic ethanol exposure, the vapor model is more advantageous for several reasons. First, there is a temporal advantage, whereby the investigator knows exactly how long the alcohol exposure last and when subsequent withdrawal has began. Interestingly, the control of exposure time has raised some questions as to the appropriate exposure length for producing an adequate alcohol-dependent animal that resembles the human disease. For example, alcohol can be given continuously for 24 hours/day, or it may be divided into an intermittent exposure whereby the animal undergoes acute bouts of withdrawal
throughout the chronic exposure. Between these two methods, it has been shown that chronic intermittent exposure produces greater effects on various behavioral aspects (Becker and Baros, 2006; Diaz-Granados and Graham, 2007). As previously mentioned, another aspect of the temporal advantage for vapor exposure is that an experimenter can also manipulate when withdrawal begins, as well as the exact duration of withdrawal. This is a significant advantage because experiments can be designed to examine the effects of specific withdrawal durations. A second advantage of forced vapor exposure is that the experimenter can exactly quantify the amount of exposure. This can be accomplished by accurately measuring the ethanol vapor concentration that the rodents are inhaling. Furthermore, this ethanol vapor level can be compared to the animal’s blood ethanol concentration (BEC) to confirm that it produced relevant BECs. Aside from the advantages of intermittent vapor exposure, this animal model has good predictive validity as it produces behaviors in rodents similar to the human condition. For example, similar to the human condition, rodents exposed to a chronic intermittent ethanol vapor exposure have greater seizure incidence after WD (Becker, 2000; Matsumoto et al., 2001; Mhatre et al., 2001). Importantly for this study, intermittent exposure also significantly alters anxiety-like behavior during subsequent withdrawal (Cagetti et al., 2003). As changes in anxiety-like behavior are a definitive characteristic of alcohol exposure and subsequent withdrawal in humans and rodents, it is important to understand the brain regions involved in anxiety.
The amygdala

The amygdala is a major component of the emotional circuitry involved in fear and anxiety. It functions as a ‘hub’ and receives highly processed sensory and cognitive information from sensory cortex, thalamus, and hippocampus (LeDoux, 1996). The amygdala is comprised of multiple nuclei that each receive specific information. Of the many nuclei within the amygdala, the lateral/basolateral amygdala (BLA) receives the primary input into the amygdala/anxiety circuit. Specifically, cortical and thalamic inputs into the BLA are locally processed and the newly encoded information is projected to the central nucleus (CeA) where further processing occurs. Upon local processing of the afferent information, the amygdala sends projections to various brain regions responsible for producing the physiological and psychological manifestations of fear and anxiety. The prefrontal cortex, nucleus accumbens and bed nucleus of the stria terminalis account for a few of the brain regions that receive efferent projections from the amygdala. Importantly, these brain regions have been shown to play roles in risk-assessment (Jinks and McGregor, 1997; Simpson et al., 2001), reward (Prado-Alcala and Wise, 1984), and unconditioned fear (Walker and Davis, 1997), respectively. In addition, the hypothalamus and brain stem also receive information from the amygdala (Gray et al., 1989; Davis, 1992), and these areas are responsible for the physical signs of anxiety. To facilitate BLA function, the nuclei is equipped with several specific types of neurons.

The neurons in the BLA have many different physical and physiological characteristics that allow the nucleus to process a variety of sensory and cognitive input. 95% of the neurons in the BLA are glutamatergic projection neurons, while GABAergic interneurons account for the other 5% (McDonald, 1982). Pyramidal-shaped
Glutamatergic neurons function to relay incoming information to the output stations of the BLA. These glutamatergic neurons display low basal firing rates and long spike durations (Rosenkranz and Grace, 1999) and can be further separated into groups based on their firing characteristics, i.e. strongly adapting, regular-spiking, and late spiking (Kroner et al., 2005). GABAergic interneurons, which make up only a small percent of BLA neurons (McDonald, 1982, 1984), display different electrophysiological characteristics. For example, GABAergic interneurons have high basal firing rates and display fast spike durations (Rosenkranz and Grace, 1999). These characteristics allow interneurons to tightly regulate the flow of information through the BLA.

Interestingly, multiple populations of GABAergic interneurons are found within the BLA. Two of these populations have recently received much interest because they can regulate glutamatergic activity via distinct mechanisms. Local interneurons are dispersed throughout the BLA and function to provide feed-back inhibition onto collateral glutamatergic projections and other local interneurons (Woodruff et al., 2006; Woodruff and Sah, 2007). Local interneurons provide feed-back inhibition as they receive reciprocal projections from glutamatergic principal neurons that also activate their inhibitory function once information has been transferred. Furthermore, interneuron-interneuron connections create a strong inhibitory network that can communicate via chemical and electrical synapses, thereby creating synchronous feedback inhibitory loops (Loretan et al., 2004; Woodruff et al., 2006; Woodruff and Sah, 2007). A second GABAergic population in the BLA is the lateral paracapsular interneurons (LPCs) that border the BLA along the external capsule (EC). Their main function is to provide cortically-driven feed-forward inhibition onto glutamatergic
projection neurons in the BLA (Marowsky et al., 2005). Specifically, feed-forward inhibition takes place when cortical projections activate LPCs which then hyperpolarize BLA principal neurons before they are excited. This feed-forward inhibition limits principle neuron excitation, in turn suppressing the BLA. In addition to the differences in function between these populations, the neuroanatomy also differs. Local interneurons form perisomatic synapses onto glutamatergic neurons (McDonald and Betette, 2001; Marowsky et al., 2005; McDonald et al., 2005; Muller et al., 2007), while LPCs form axo-dendritic synapses on distal dendrites of glutamatergic neurons (Marowsky et al., 2005). LPCs provide a robust inhibitory tone over the BLA, as several LPCs can innervate single glutamatergic neurons (Marowsky et al., 2005; Muller et al., 2007). These two GABAergic populations of interneurons also differ in the expression of calcium-binding proteins and peptides, which can be a useful target for localization studies. Only local interneurons have been shown to express parvalbumin (McDonald and Betette, 2001; McDonald et al., 2005; Silberman et al., 2007), while LPCs have been shown to predominately express substance P (Levita et al., 2003) as well as somatostatin (Muller et al., 2007). Given the array of neurons in the BLA and their multiple functions, information is transferred through the BLA via numerous neurotransmitter systems.

**Glutamate Neurotransmission in the Lateral/Basolateral Amygdala**

As with the remainder of the central nervous system, the main endogenous excitatory neurotransmitter in the BLA is glutamate. Glutamate’s ionotropic type receptors are alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl D-aspartate (NMDA), and kainate. In addition, glutamate can also activate
various metabotropic receptors that will not be discussed. Although the glutamate system consists of only three ionotrophic receptors, these receptors display very distinct characteristics and functions that help shape BLA output.

The main function of AMPA and kainate receptors (AMPAR and KA-R) is to mediate fast-excitatory synaptic transmission. AMPARs are composed of four subunits, GluR1-4, and form tetramers (Greger et al., 2007). The specific subunit conformation of AMPARs dictates its ion permeability. AMPAR’s are permeable to cations, such as sodium (Na⁺) and potassium (K⁺), which rapidly depolarize neurons (MacDonald and Nowak, 1990), which allows for fast synaptic transmission. Interestingly, subsets of AMPARs are also permeable to calcium (Ca²⁺), but only when the GluR2 subunit is absent (Cull-Candy et al., 2006). Due to AMPAR’s role in fast synaptic transmission, they are found on the soma or dendrites of the postsynaptic neuron (Bolton et al., 2000). In addition, AMPAR expression can be dependent on brain activity, as observed following long-term potentiation (LTP) (Bolton et al., 2000; Cull-Candy et al., 2006), an example of in vitro synaptic plasticity (discussed below). The various characteristics of AMPARs allow these receptors to quickly depolarize neurons and continue the propagation of action potentials. Until recently, AMPA and KA-R have been grouped together due to the difficulty in pharmacologically isolating them. Biomedical advances have developed pharmacological compounds that can specifically differentiate between the two receptors in a dose-dependent manner (Lack et al., 2008). Using these compounds, various groups have now demonstrated the involvement of KA-Rs in long-term synaptic alterations involved with changes in anxiety (Li and Rogawski, 1998; Li
and Wang, 2001; Lack et al., 2008). These characteristics of AMPA and KA-Rs allow them to quickly depolarize neurons and continue the propagation of action potentials.

Glutamate is also the ligand for the NMDA receptor (NMDAR). NMDARs can be composed of NR1, NR2, and NR3 subunits that form heterodimers (Dingledine et al., 1999). These receptors are activated upon depolarization of the neuron, via AMPARs, due to the voltage-dependent release of Mg$^{2+}$ that blocks the NMDA pore (Mori et al., 1992). Upon activation, NMDARs are permeable to influxes of sodium (Na$^+$) and calcium (Ca$^{2+}$), and effluxes of potassium (K$^+$) (Scatton, 1993). In addition to its role in excitatory synaptic transmission, NMDARs play a large role in initiating certain forms of LTP. LTP can be initiated by a persistent depolarization during periods of high brain activity, that allow NMDARs to transfer sufficient Ca$^{2+}$ into the postsynaptic soma, resulting in an upregulation of AMPAR trafficking and surface receptor expression (Bolton et al., 2000; Cull-Candy et al., 2006). These NMDAR-dependent processes have been shown to occur in the BLA during fear conditioning (McKernan and Shinnick-Gallagher, 1997). Interestingly, while the expression of fear learning (Sajdyk and Shekhar, 1997; Walker and Davis, 1997) can be blocked by AMPA antagonists, the acquisition of learned fear/anxiety responses can be blocked with APV, an NMDA antagonist (Miserendino et al., 1990; Kim et al., 1992; Kim and McGaugh, 1992; Hatfield and Gallagher, 1995). Although the glutamatergic system’s function in the BLA is required for fear/anxiety responses to occur, glutamate within the BLA does not originate in the BLA.

As previously described, the BLA receives projections from various brain regions that deliver information via the glutamatergic system. For example, thalamic and cortical
projections send sensory and cognitive information to the BLA. Interestingly, it has also been shown that cortical glutamatergic inputs into the BLA have increased excitability following chronic ethanol exposure and withdrawal (Lack et al., 2009). These data suggest alternative processes that regulate glutamatergic signaling at inputs into the BLA. In addition to thalamic and cortical inputs, partially processed information from the lateral amygdala is also delivered to the BLA via glutamatergic projections (Samson et al., 2003). These inputs may underlie local processing of fear and anxiety information within the amygdala (Samson and Pare, 2006). In order to process all of the glutamatergic inputs into the BLA, other systems within the BLA function to filter and process relevant information.

GABAergic Neurotransmission in the Lateral/Basolateral Amygdala

In the BLA, the flow of information is regulated by the inhibitory γ-aminobutyric acid (GABA) system. In contrast to the glutamate system that has several ionotropic and metabotropic receptors, the GABA system is only comprised of two ionotropic receptors: GABA_A,R, GABA_C,R; and one metabotropic receptor: GABA_B,R. Similar to the glutamate receptors, the three GABARs display very distinct characteristics that allow for synchronized BLA function.

GABA_A,Rs are the most abundant GABA receptors in the brain. These receptors are comprised of five subunits and form a heteromeric ligand-gated ion channel that is permeable to anions, such as chloride (Cl\(^{-}\)) and bicarbonate ions. GABA_A,Rs mediate fast and phasic inhibitory transmission (Thompson, 1994) that is achieved by rapid hyperpolarization of principle neurons via increasing levels of Cl\(^{-}\) in the postsynaptic
compartment of these principle neurons. Similar to glutamatergic receptors, the efficacy and pharmacological properties of these receptors is dependent on subunit composition (Sieghart, 1995). Importantly, GABA$\alpha$Rs are known to be a major target for various therapeutic drugs, such as benzodiazepines that are typically used for treating generalized anxiety disorders (Roy-Byrne et al., 1990) and alcohol withdrawal-induced anxiety (Enoch, 2008) in humans. Similarly, modulation of the BLA GABA system in rodents can significantly alter anxiety-like responses. For example, microinjections of muscimol, a GABA$\alpha$R agonist, into the rat BLA can dramatically decrease anxiety-like behaviors measured in a light/dark apparatus (Lack et al., 2007). In contrast, microinjections of urocortin 1, a corticotropin releasing factor (CRF)-2 receptor agonist, into the BLA decreases social interactions – a model of social anxiety – presumably via local interneurons (Truitt et al., 2007). Given the major role of GABA$\alpha$Rs in maintaining inhibitory tone in the BLA, it follows that GABA$\gamma$Rs might also play a role in amygdala function.

Unlike GABA$\alpha$Rs, GABA$\gamma$Rs have not been as well characterized. GABA$\gamma$Rs are found in the CeA and have been reported to be localized to distal dendrites of principal neurons in the rat (Collins and Pare, 1999; Royer et al., 1999). Although their specific role is unclear, it is thought that these GABA$\gamma$Rs receive GABAergic input from medial paracapsular interneurons, or intercalated cell masses (ICM). Although there are no reports of GABA$\gamma$Rs in the BLA specifically, the BLA and CeA share similar neuroanatomy, in particular the paracapsular interneurons (Marowsky et al., 2005). It is reasonable to suggest that GABA$\gamma$Rs may also be found in the BLA and may function to modulate LPC synaptic transmission.
GABA\(_B\)Rs are the only metabotropic receptor in the GABAergic family. GABA\(_B\)Rs have multiple functions, depending on whether they are localized to pre- or post-synaptic regions (Margeta-Mitrovic et al., 1999; Sloviter et al., 1999). GABA\(_B\)Rs can function as presynaptic autoreceptors in the BLA (Silberman et al., 2007) and the central amygdala (Roberto et al., 2006) on GABAergic interneuron terminals to regulate GABA release during periods of elevated GABAergic function (Kajikawa et al., 2001). GABA\(_B\)Rs can also be found postsynaptically on somatic compartments of interneurons and function to decrease excitability by opening G-protein-coupled inwardly rectifying potassium channels (GIRKs) (Misgeld et al., 1995; Couve et al., 2000). Regardless of their localization, GABA\(_B\)Rs can have profound influences on GABAergic transmission in the BLA. Interestingly, while the GABA system can regulate amygdala output, it too is subject to regulation by other neurotransmitter systems present in the amygdala.

**Dopamine Neurotransmission in the Lateral/Basolateral Amygdala**

Although the balance between the glutamatergic and GABAergic systems controls the expression of BLA-mediated behaviors (i.e. fear and anxiety), these two systems are further modulated by the dopamine (DA) system. The DA system is comprised of five G-protein coupled receptors that are categorized into two families: DA D1-like and D2-like. Receptors in the DA D1-like family include D1 and D5 receptors and are typically considered to increase cAMP activity through G\(_s\) (Sokoloff and Schwartz, 1995). In contrast, D2-like receptors include D2, D3, and D4 and are typically considered to decrease cAMP activity through G\(_i/o\) (Sokoloff and Schwartz, 1995). The
diversity of dopamine receptor functions allows this system to influence multiple brain regions, including the BLA.

DA receptors have been shown to play a role in shaping BLA-mediated behaviors. DA cell bodies arising in the ventral tegmental area (VTA) project to the BLA (Inglis and Moghaddam, 1999; Marowsky et al., 2005). In the BLA, dopaminergic cells synapse locally within the nucleus (Rosenkranz and Grace, 1999; Kroner et al., 2005) and have been more recently reported to synapse in the EC on LPCs (Fuxe et al., 2003; Marowsky et al., 2005). The location of these projections and their synaptic connections have led several groups to characterize the role of these DA projections in the BLA. For example, blockade of BLA DA D1 receptors (D1Rs) decreases anxiety-like behaviors (de la Mora et al., 2005) and blocks fear expression in a startle paradigm (Lamont and Kokkinidis, 1998). DA has also been shown to be involved in cue-induced reinstatement of cocaine-seeking (See et al., 2001; See et al., 2003; Berglind et al., 2006; Feltenstein and See, 2008). Further studies have elucidated that dopamine’s main function in the BLA is modulation of the GABAergic system. Specifically, DA has been shown to facilitate GABAergic transmission through D1R activation of local feed-back interneurons (Rosenkranz and Grace, 1999; Kroner et al., 2005). Conversely, DA activation of LPC D1Rs can suppress LPC GABAergic transmission (Marowsky et al., 2005). DA D2 receptors (D2Rs) also play a role in the lateral amygdala (LA), where DA can dis-inhibit feed-forward inhibition, leading to decreased inhibition which results in the induction of LTP (Bissiere et al., 2003). The amygdala, in particular the BLA, is a complex brain region that is regulated by numerous neuro-transmitter systems localized to specific neuron types. Because these neurotransmitter systems shape BLA activity and
output, it is reasonable to suggest that these systems can be altered by exposure to alcohol.

**Effects of Acute Alcohol on Amygdala Neurotransmitters**

Alcohol can produce intoxicating effects through inducing changes in neurotransmission. Specifically, decreased anxiety-like behavior following acute alcohol has been shown to involve altered synaptic transmission within the BLA (Zhu and Lovinger, 2006; Silberman et al., 2007; Lack et al., 2008) as well as the central amygdala (Roberto et al., 2003; Roberto et al., 2006). While these studies have all shown that these alterations are not permanent, they do demonstrate the significant effects that acute alcohol can have on the amygdala.

In addition, acute alcohol has been shown to alter glutamatergic synaptic transmission in the amygdala. Several studies have shown that NMDA and KA-Rs within the BLA and CeA are sensitive to the effects of acute ethanol. Previous studies have shown that acute alcohol can inhibit NMDARs in the BLA (Lack et al., 2008) and the CeA (Roberto et al., 2004b). This effect has been shown to be dependent on the Mg$^{2+}$-block, suggesting a molecular site of action for acute alcohol (Calton et al., 1998). The inhibitory effects of acute alcohol are not limited to NMDARs. Acute alcohol also dose-dependently inhibits evoked KA-R-mediated currents within the BLA (Lack et al., 2008). Interestingly, this same study found that AMPARs are insensitive to acute ethanol (Lack et al., 2008). In the CeA, the effects of acute alcohol on AMPA and KA-Rs have not been examined separately. Yet, evoked AMPA/KA-R (non-NMDAR) mediated miniature currents are inhibited by acute alcohol in the CeA by inhibition of postsynaptic non-
NMDARs and presynaptic glutamate release (Zhu et al., 2007). Given that acute ethanol only altered KA-R and not AMPAR function in the BLA, it is reasonable to suggest that the non-NMDA findings from the CeA were mediated by KA-Rs. These data indicate that acute alcohol can modulate glutamatergic transmission in the amygdala; however, the primary action of acute alcohol in the amygdala has been shown to be on GABAergic transmission.

The effects of acute alcohol on GABAergic transmission in the amygdala have been extensively studied. One of the known mechanisms of acute alcohol is to potentiate GABAergic transmission in the amygdala, suggesting a possible mechanism for its anxiolytic property. Several groups have characterized various aspects of acute alcohol’s actions on naïve GABAergic transmission in the amygdala to better understand the synaptic mechanisms. For example, 100mM acute alcohol potentiated GABAergic currents in transfected cells expressing GABA\(_\text{A}\)Rs similar to those found in the BLA (\(\alpha_1\beta_2\gamma_{2S}\) and \(\alpha_2\beta_2\gamma_{2S}\)) (McCool et al., 2003). Interestingly, this acute alcohol effect only occurred in cells expressing the \(\alpha_1\beta_2\gamma_{2S}\) receptors. This finding suggests a postsynaptic role for the effects of acute alcohol, as GABA\(_{\text{A}}\)Rs of any conformation are found postsynaptically. Importantly, this study also suggested that not every GABA\(_{\text{A}}\)R responds the same to acute alcohol, as the GABA currents from \(\alpha_2\beta_2\gamma_{2S}\)Rs were significantly inhibited by 100mM ethanol. Conversely, Zhu and Lovinger found no postsynaptic effects of acute alcohol in a postsynaptic neuron/synaptic bouton preparation extracted from the BLA (Zhu and Lovinger, 2006). Despite the contrasting results on the postsynaptic effects of acute alcohol (perhaps due to the difference in preparations), Zhu and Lovinger (2006) found an increase in the frequency of tetrodotoxin (TTX)-
insensitive spontaneous activity was, suggesting that acute alcohol’s effects are also on presynaptic-terminals via \(\text{GABA}_B\)Rs, and not on intrinsic cell properties (i.e. excitability). Although the effects of acute alcohol on TTX-sensitive versus -insensitive spontaneous activity within the BLA have not been investigated in other preparations (i.e. slices, \textit{in vivo}) the potentiating effects of acute alcohol do appear to be mediated via a presynaptic \(\text{GABA}_B\)R mechanism at local synapses, consistent with the latter study (Zhu and Lovinger, 2006), and by a postsynaptic mechanism at LPC synapses (Silberman et al., 2007). Acute alcohol’s actions on the GABAergic system of the CeA are very similar to those demonstrated in the BLA. GABAergic transmission in the CeA is potentiated by acute alcohol through pre- (increased mIPSC frequency) and post-synaptic (amplitude) mechanisms (Roberto et al., 2003). GABAergic spontaneous activity in the CeA was elevated with perfusion of acute alcohol (Roberto et al., 2003; Siggins et al., 2005), suggesting that, at least in the CeA, acute alcohol’s actions are to increase GABAergic tone. Interestingly, only a portion of the recorded cells in the Roberto et al (2003) study demonstrated the facilitatory effect of acute alcohol on evoked GABAergic transmission. This study and the study in the BLA (McCool et al., 2003) suggest that acute alcohol may have an inhibitory effect at specific GABAergic synapses, in contrast to the ‘typical’ effects of acute alcohol on GABAergic transmission (reviewed in (Weiner and Valenzuela, 2006)). Although the GABAergic system of the amygdala has received much attention in studying the effects of acute alcohol, its effects on amygdalar dopaminergic transmission have also been studied.

However, research on the effects of acute alcohol on the DA system in the amygdala is scarce. One study demonstrated that DA receptor blockade in the BLA, but
not the CeA, blocked ethanol conditioned place preference expression in mice (Gremel and Cunningham, 2008). These results demonstrate that the DA system is involved in alcohol seeking behavior. Another study found that an intraperitoneal injection of alcohol drastically increased extracellular DA levels to 270% above baseline levels in the rat amygdala (Yoshimoto et al., 2000). This suggests that the DA system is actively engaged in response to alcohol, making it an important target to examine. Given the numerous effects of acute alcohol on dopaminergic, glutamatergic, and GABAergic transmission in the amygdala, it is reasonable to suggest that chronic exposure to ethanol would lead to robust effects on these neurotransmitter systems.

**Chronic Alcohol on Amygdala Neurotransmitters**

Long-term exposure to alcohol (“chronic alcohol”) has been shown to have significant effects on anxiety-like behaviors in rodents. For example, mice chronically exposed to ethanol expressed decreased anxiety-like behavior in a light/dark box (Costall et al., 1990). Likewise, rats exposed to chronic alcohol, via a liquid diet, also demonstrated decreased anxiety-like behavior in a light/dark box (McCool et al., 2003). The changes in anxiety-like behavior following chronic ethanol exposure have been associated with alterations within the amygdala as microinjections of various drugs into the amygdala also alter anxiety-like behavior in the light/dark box. For example, decreases in anxiety-like behavior have been correlated with decreased levels of amygdalar activity as observed following microinjections of strychnine, a glycine receptor (a ligand-gated chloride channel) agonist, into the BLA (McCool et al., 2003; McCool and Chappell, 2007). In contrast, increased anxiety-like behavior has been
correlated with ATPA-induced (KA-R agonist) increase in amygdalar activity (Lack et al., 2008). While these studies demonstrate a parallel between anxiety-like behavior and BLA activity (↓ BLA activity and ↓ anxiety-like behavior; ↑ BLA activity and ↑ anxiety-like behavior), other research using animal models has shown that amygdala activity is elevated during chronic alcohol exposure (Floyd et al., 2003; Roberto et al., 2004b; Roberto et al., 2005; Lack et al., 2009) when anxiolysis occurs (Costall et al., 1990; McCool et al., 2003). Taken together, the discrepancies between function and behavior during exposure to chronic ethanol suggest that alterations in multiple neurotransmitter systems within the BLA may occur.

The effects of chronic ethanol exposure on amygdala glutamatergic transmission are consistent across the BLA and CeA. Increases in glutamate-receptor function from cortical inputs in animals exposed to chronic ethanol were shown to contribute to an increase in BLA activity as measured by local field excitatory postsynaptic potentials (fEPSPs) (Lack et al., 2009). This increase was independent of presynaptic changes, suggesting postsynaptic alterations within the BLA for this input pathway. Other data consistent with postsynaptic alterations shows NMDAR function to be elevated in the BLA of rats exposed to a chronic liquid ethanol (Floyd et al., 2003) and in the CeA of chronic ethanol treated (CET) animals (Roberto et al., 2004b; Roberto et al., 2005). Moreover, KA-R function in the BLA, presumably postsynaptic KA-Rs, is also facilitated following chronic ethanol exposure (Lack et al., 2009). Given that chronic ethanol-induced increases in amygdala glutamatergic function contrasts decreased anxiety-like behavior (Costall et al., 1990; McCool et al., 2003), it is reasonable to suggest a role for the GABA system in these findings.
Chronic alcohol exposure has been shown to significantly alter the GABAergic system in the amygdala. For example, BLA GABA\textsubscript{A}R expression was shown to be increased in rats treated with a chronic ethanol liquid diet (McCool et al., 2003). An increase in GABA\textsubscript{A}R expression would lead to an increase in GABAergic function. Furthermore, in a non-human primate model of ethanol self-administration, GABA\textsubscript{A} α2 and α3-subunit mRNAs were decreased in the BLA compared to control levels (Floyd et al., 2004). The suppressive effect of chronic ethanol exposure on GABA\textsubscript{A} subunit gene expression is not limited to the α-subunit. Other data collected from ethanol self-administering non-human primates showed a decrease in GABA\textsubscript{A}-β1 and γ2 mRNA levels compared to levels in control animals (Anderson et al., 2007). These decreases in GABA\textsubscript{A}-β1 and γ2-subunit message were found to result in decreased benzodiazepine sensitivity in BLA neurons. This suggests another postsynaptic alteration in the GABA system following chronic alcohol exposure. Interestingly, chronic ethanol can also induce presynaptic alterations within the amygdala. Presynaptic GABAergic transmission within the CeA is increased following chronic ethanol treatment (Roberto et al., 2004a), suggesting an overall increase in GABAergic function within the amygdala during exposure to chronic ethanol. Although gene expression has not been investigated in the BLA of rodents and function has not been investigated in the BLA of non-human primates, the afore mentioned reports suggest that the GABAergic system within the amygdala is susceptible to the effects of chronic alcohol exposure across species. Overall, these increases in GABAergic transmission in the amygdala following exposure to chronic ethanol may explain the observed decrease in anxiety-like behavior. However,
dopamine system modulation of anxiety-like behavior via the GABAergic system in the BLA may also contribute to the effects of chronic ethanol exposure.

Although there have been limited studies, chronic alcohol has been shown to impact the DA system in the amygdala. Interestingly, in type 2 alcoholics (type 2 is classified as early-onset with impulsive and aggressive temperament), there is an increase in amygdala D1R binding, suggesting a possible disturbance in the amygdala DA system relating to certain characteristics of alcoholism (i.e. impulsiveness) (Tupala and Tiihonen, 2008). Similarly, alcohol-preferring rats exposed to a repeated-alcohol deprivation exposure showed increases in D1R binding densities in the lateral and intercalated nuclei (between the basolateral and central nuclei) of the amygdala, compared to water-treated and continuous alcohol exposed rats (Sari et al., 2006). Another study found that chronic alcohol consumption increases dopamine transporter (DAT) levels in the BLA of Wistar and Wistar-Kyoto rats (Jiao et al., 2006). These studies provide strong evidence that the amygdala DA system is altered by chronic alcohol exposure. Although these studies have shown gross changes in receptor binding, one study has suggested that functional changes may lead to the above mentioned alterations in the amygdala DA system. Specifically, the effects of chronic alcohol exposure on DA receptor numbers are independent of increased DA terminals in the CeA, but may be due to a change in the efficiency of DA release (Zhou et al., 2006). The previous findings indicate that mass alterations occur across multiple neurotransmitter systems in the BLA following chronic alcohol administration. Interestingly, further alterations have been demonstrated during periods of alcohol withdrawal.
Alcohol Withdrawal Effects on Amygdala Neurotransmitters

Once alcohol withdrawal has occurred, many changes take place in the amygdala. One change, demonstrated in humans, shows that amygdala volume was reduced in abstaining alcohol dependent subjects compared to healthy controls (Wrase et al., 2008). These volume decreases were also associated with increased alcohol craving and alcohol intake as a significant number of these subjects relapsed during the abstinence period. Alterations during withdrawal have also been found to occur in animals, as a significant increase in anxiety-like behavior (Cagetti et al., 2003), suggestive of changes in amygdala activity. During withdrawal the amygdala has been shown to be in a ‘hyperexcitable’ state, as measured with an increase in expression of an immediate early gene, (c-fos) (Knapp et al., 1998). C-fos expression has been correlated as a marker of increased excitability/activity (Ferkany et al., 1984; Ben-Ari, 1985; Popovici et al., 1990; Smeyne et al., 1992). Conversely, it has been suggested that a highly active amygdala results in increased anxiety-like behaviors (Sajdyk and Shekhar, 1997). The parallel between increased anxiety-like behavior and a ‘hyperexcitable’ amygdala during withdrawal suggest that increased function following chronic ethanol exposure may persist into withdrawal.

Several studies have examined if glutamate is responsible for the hyperexcitable state seen during alcohol withdrawal in the amygdala. While glutamatergic excitability is elevated at 24 hours of withdrawal of chronic ethanol exposure, this increase is independent of a presynaptic effect at EC-BLA synapses (Lack et al., 2009). Therefore, glutamatergic excitability may be a result of increased presynaptic activity from another input into the BLA. This increase in glutamatergic function appears to be long-term given
that NMDAR composition, which may contribute to increased NMDAR function, is altered up to 2 weeks of withdrawal in the central amygdala (Roberto et al., 2005). Changes in behavior and glutamatergic function appear to parallel during withdrawal and indicate a ‘hyperexcitable’ amygdala; however, it is important to understand the contributions of the GABA system on these adaptations.

Unfortunately, the effects of alcohol withdrawal on GABAergic transmission in the amygdala have not been well characterized. One study found that feedback inhibition is disrupted in the BLA following alcohol withdrawal (Isoardi et al., 2007). This study suggests that the ‘hyperexcitability’ of the amygdala during withdrawal may be related to a loss of GABAergic inhibition. However, more detailed studies would be necessary to test this hypothesis. In addition, the effects of withdrawal on the GABAergic system of the CeA, have not been exclusively examined either. Interestingly, there are many reports on the effects of withdrawal on endogenous GABA-modulators, such as CRF. Microinjections of α-helical CRF, a CRF antagonist, into the central amygdala decreased alcohol withdrawal-induced anxiety as measured on an elevated plus maze (Rassnick et al., 1993). This suggests that during withdrawal, CRF functions to suppress GABAergic function. While these findings suggest a possible treatment for withdrawal-induced anxiety, the majority of the CRF literature suggests an important pathway in alcohol addiction is through the extended amygdala, specifically the CeA (Nie et al., 2004; Heilig and Koob, 2007; Bajo et al., 2008; Koob, 2008). Yet, a recent report showed that CRF1 receptors are significantly upregulated in the BLA following 3 weeks of withdrawal from a 7 week chronic ethanol exposure (Sommer et al., 2008). This study indicates that the CRF system within the BLA may also function to suppress GABAergic function, in turn
contributing to the effects of chronic ethanol withdrawal on anxiety-like behavior. Although the effects of withdrawal on the CRF system, a GABA-modulatory system, have been investigated, the effects of withdrawal on the amygdala DA system, another GABA modulatory system, remain unknown. Though there are a number of studies investigating the effects of withdrawal on GABAergic and glutamatergic transmission, much remains unknown about the changes that occur in the amygdala during withdrawal that ultimately lead to withdrawal-induced anxiety.

Summary

Alcohol can have diverse actions on many systems throughout the brain, while withdrawal from chronic alcohol exposure can profoundly impact the basolateral amygdala, contributing to withdrawal-induced anxiety. In particular, alterations in the flow of information within the BLA have been attributed to behavioral manifestations of withdrawal-induced anxiety. Given this relationship, understanding the changes that BLA neurotransmitter systems undergo during withdrawal can ultimately lead to developing better treatments for preventing alcohol relapse. The goal of the present studies was to characterize the effects of chronic ethanol exposure and withdrawal on glutamatergic (excitatory) and GABAergic (inhibitory) synaptic transmission within the BLA. Furthermore, these studies also examined the role of dopamine, a significant modulator of BLA-mediated anxiety-like behaviors. Changes in modulatory systems such as dopamine may underlie anxiety produced by alcohol withdrawal. In conclusion, these studies served to illustrate the neurophysiological adaptations that occur within the basolateral amygdala in response to chronic ethanol exposure and withdrawal.
References


2. Chronic Ethanol and Withdrawal Differentially Modulate Pre- and Post-synaptic Function at Glutamatergic Synapses in Rat Basolateral Amygdala

**Abbreviated Title:** Ethanol and withdrawal alter BLA glutamatergic transmission

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Abstract

Withdrawal anxiety is a significant factor contributing to continued alcohol abuse in alcoholics. This anxiety is long lasting, can manifest well after the overt physical symptoms of withdrawal, and is frequently associated with relapse in recovering alcoholics. The neurobiological mechanisms governing these withdrawal-associated increases in anxiety are currently unknown. The basolateral amygdala is a major emotional center in the brain and regulates the expression of both learned-fear and anxiety. Neurotransmitter system alterations within this brain region may therefore contribute to withdrawal-associated anxiety. Since evidence suggests that glutamate-gated neurotransmitter receptors are sensitive to acute ethanol exposure, we examined the effect of chronic intermittent ethanol (CIE) and withdrawal (WD) on glutamatergic synaptic transmission in the basolateral amygdala. We found that slices prepared from CIE and WD animals had significantly increased contributions by synaptic NMDA receptors. In addition, CIE increased the amplitude of AMPA receptor-mediated spontaneous excitatory postsynaptic currents (sEPSC), while only WD altered the amplitude and kinetics of tetrodotoxin-resistant spontaneous events (mEPSC). Similarly, the frequency of sEPSCs was increased in both CIE and WD neurons; but, only WD increased the frequency of mEPSCs. These data suggest that CIE and WD differentially alter both pre- and post-synaptic properties of BLA glutamatergic synapses. Finally, we show that microinjection of the AMPA receptor antagonist, DNQX, can attenuate withdrawal-related anxiety-like behavior. Together, our results suggest that increased glutamatergic function may contribute to anxiety expressed during withdrawal from chronic ethanol.
Introduction

The amygdala serves as the center for regulation of specific aspects of fear and anxiety behaviors. Highly processed sensory and cognitive information flows into the amygdala from the cortex and thalamus. After additional processing, the amygdala then projects to regions regulating risk assessment (e.g., medial prefrontal cortex, bed nucleus of the stria terminalis) and reward (e.g. nucleus accumbens). More recently, it has been demonstrated that various amygdala subdivisions also modulate drug-seeking behaviors in rodents (Alderson et al. 2000; Di Ciano and Everitt 2004; Fuchs and See 2002; McLaughlin and See 2003), drug cravings in humans (Childress et al. 1999), and the regulation of anxiety-like behaviors during withdrawal from chronic drug exposure (Menzaghi et al. 1994; Rodriguez de Fonseca et al. 1997; Watanabe et al. 2002).

In this context, the lateral and basolateral subdivisions within the amygdala are essential for the development of fear and anxiety-like behaviors. The basolateral amygdala (BLA) is the primary input region for cortical and thalamic afferents. Alterations of neurotransmitter systems within this amygdala region would therefore significantly impact the flow of information both within the amygdala as well as along BLA efferents, including the central amygdala. This point is emphasized by the ability to block fear learning (Walker and Davis 1997a), and the behavioral response to naturally anxiogenic unconditioned stimuli via injection of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor antagonist, CNQX, into the BLA (Sajdyk and Shekhar 1997; Walker and Davis 1997a). Additionally, ethanol self-administration increases glucose utilization in the BLA (Porrino et al. 1998); and, modulation of BLA neuronal activity can modulate operant ethanol self-administration
These data provide a clear rationale for examining the effects of chronic ethanol and withdrawal on BLA glutamatergic neurophysiology.

Given that classic fear-learning behaviors are associated with a N-methyl-D-aspartate (NMDA) receptor-dependent increase in glutamatergic synaptic transmission within the BLA (McKernan and Shinnick-Gallagher 1997), we hypothesize that chronic ethanol-related increases in BLA NMDA receptor function (Floyd et al. 2003) might mediate similar increases in glutamatergic synaptic transmission, ultimately driving increased anxiety during withdrawal. In rats, alcohol withdrawal leads to increased glutamate release in the hippocampus (Dahchour and De Witte 2003). While they are distinctly different regions, NMDA-dependent changes in synaptic plasticity within the BLA and hippocampus are thought to rely upon similar neurophysiological and cellular processes (Chapman et al. 2003). This suggests some parallels between these brain regions. Given that chronic ethanol facilitates NMDA receptor expression and function in isolated BLA neurons (Floyd et al. 2003), it is reasonable to suggest that molecular mechanisms associated with the long-term anxiogenic effects of alcohol exposure are related to alterations in these receptor systems. Indeed, chronic ethanol exposure increases glutamate neurotransmission in the neighboring central nucleus of the amygdala (Roberto et al. 2004b). However, less is known about the effects of chronic ethanol and withdrawal in the BLA, an amygdala subdivision that controls information flow throughout the entire amygdala.

In the present study, we have used a chronic intermittent ethanol inhalation model to examine the effects of chronic ethanol exposure and withdrawal on glutamatergic synaptic transmission in the BLA. Repeated withdrawal from ethanol has been shown to
enhance seizure severity in response to convulsants (Pinel 1980). Repeated cycles of exposure/withdrawal also appear to more dramatically alter several different neurotransmitters (Jarvis and Becker 1998; Kang et al. 1998). More importantly, recent findings suggest that the kindling-like effect of repeated ethanol exposure/withdrawal can occlude synaptic plasticity in the amygdala (Stephens et al. 2005). Since plastic synaptic processes in the BLA are induced by NMDA-type glutamate receptors and expressed as increased function of glutamatergic synapses (Huang and Kandel 1998), the current study therefore tested the hypothesis that repeated ethanol exposure/withdrawal cycles would enhance or alter similar glutamatergic mechanisms within the lateral/basolateral amygdala.

Methods

Animals: All animal procedures were performed in accordance with protocols approved by Wake Forest University School of Medicine Animal Care and Use Committee and were consistent with the NIH animal care and use policy. Male Sprague-Dawley rats were obtained from Harlan (Indianapolis IN) and were housed in an animal care facility at 23°C with a 12-hour light/dark cycle and given food and water ad libitum. Rats were weighed daily to ensure that at least 80% of their free-feeding weight was maintained during vapor chamber ethanol exposure.

Chronic Ethanol Exposure: Ethanol exposure was accomplished via ethanol inhalation using a method similar to that used in other studies (Becker and Hale 1993; Roberto et al. 2004b). All animals (100-120g) were housed four-six animals/cage in large, standard polycarbonate cages (Allentown Inc., Allentown NJ) containing wood-
chip bedding and with chow/water ad libitum. These home-cages were placed within large, custom-built plexiglass chambers (Triad Plastics, Winston-Salem NC) that were identical to those described elsewhere (Lopez and Becker 2005) at the beginning of the light-cycle (lights on at 9pm EST). Ethanol-vapor, produced by submerging an air-stone in 95% ethanol, was mixed with room air and was pumped into the chambers at a rate of 16L/min. Ethanol levels were checked daily and were maintained at 25-28mg/L within the chamber. Animals were exposed to the ethanol vapor or room air (CON group) for 12 hours/day over 10 consecutive days. Animals exposed ethanol vapor and sacrificed immediately after the 10th exposure (while still intoxicated) are referred to throughout the manuscript as the CIE (chronic intermittent ethanol) group. Those CIE animals removed from the ethanol exposure for 24hr prior to experimentation are defined here as the WD (withdrawal) group. No supplemental ethanol doses or alcohol dehydrogenase inhibitors were used at any time. Animals were weighed daily at the end of each exposure period (9am); and home-cages were changed every 2-3 days. Tail blood was taken at the end of some exposure periods and then trunk blood was collected on the day of sacrifice for subjects in the CIE group. Blood ethanol concentrations (BEC) were determined using a standard, commercially-available alcohol dehydrogenase/NADH enzymatic assay (Diagnostic Chemicals Limited, Oxford CT). At 3-5 days of exposure, BECs from tail blood were 265±12mg/deciliter. At the time of sacrifice (~30 minutes after removal from ethanol vapor), blood ethanol levels were 186±18 mg/deciliter.

Behavioral Assays: To confirm that the CIE exposure paradigm produced withdrawal anxiety, anxiety-like behaviors were assessed using a two-compartment light/dark box (McCool et al. 2003). Individuals were placed in the ‘light’ side of a
Plexiglass arena divided equally into ‘light’ and ‘dark’ sides by an opaque Plexiglass insert (Rat Truscan Activity Arena; Coulborn Inst., Allentown PA). The center of the animal ± 0.8 cm was followed for 300 seconds by two infrared sensor rings surrounding the entire apparatus, one in the floor plane and one located ~5 cm above the floor to measure rearing behavior. Data were collected and analyzed for general locomotor activity, time spent in the light and dark compartments, number of light-dark transitions, egress latency, re-entry latency, and number of vertical beam breaks. Data was analyzed using one-way ANOVA across the different treatment groups. All variables are reported as the mean±SEM.

Preparation of Brain Tissue: Animals were anesthetized with isoflurane and euthanized by decapitation. 400 µm coronal brain slices were prepared as described previously (Floyd et al. 2003). For in vitro slice preparations, 100 µM ketamine was added to a modified aCSF (in mM: 180 Sucrose, 30 NaCl, 4.5 KCl, 1 MgCl₂•6H₂O, 26 NaHCO₃, 1.2 NaH₂PO₄, 10 glucose) during preparation of slices to minimize excitotoxicity. Slices were transferred and stored in 0.5 liter of standard oxygenated aCSF solution (in mM: 126 NaCl, 3 KCl, 1.25 NaH₂PO₄, 2 MgSO₄, 26 NaHCO₃, 10 glucose, and 2 CaCl₂) at room temperature for at least 1 hour and up to 6 hours before performing electrophysiological analysis.

Electrophysiology: Methods for whole-cell blind patch-clamp recordings from BLA neurons within slices were similar to those reported previously (DuBois et al. 2006). Electrodes were filled with an intracellular pipette solution containing (in mM): 122 CsOH, 17.5 CsCl, 10 HEPES, 1 EGTA, 5 NaCl, 0.1 CaCl₂, 4 Mg-ATP, and 0.3 Na-GTP, 2 QX-314 (Cl), pH adjusted to 7.2 with gluconic acid, osmolality ranged from 280-
290 mmol/kg with sucrose. Excitatory postsynaptic currents (EPSCs) were electrically evoked every 20 sec by brief (0.2 msec) square-wave stimulations near the boundary between lateral/basolateral amygdala (Fig.2.1A) using platinum/iridium concentric bipolar stimulating electrodes (FHC, Bowdoinham, ME) with an inner pole diameter of 25 µm and resistance of 8-12MΩ. Stimulation intensities ranged from 5 to 50µA. This varied considerably across different experiments and was a function of the specific stimulating electrode used in that particular experiment. Unless stated otherwise, we used sub-maximal stimulations (just above threshold) that yielded consistent synaptic responses.

All glutamatergic events were pharmacologically isolated using 10µM bicuculline to inhibit fast GABAergic transmission. Recordings were acquired with an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA), and digitized with a Digidata 1200B (Axon Instruments). From a holding potential of -60mV, we included square-wave command hyperpolarizations (-5mV) in every sweep to constantly monitor input resistance and capacitance. Neurons with high initial input resistance (>50MΩ; presumptive interneurons (Rainnie 1999)) or whose input resistance increased more than 10% during data collection were excluded from the study.

NMDA Synaptic Responses: To record NMDA-mediated EPSCs, neurons were voltage-clamped at -60mV in the presence of low extracellular magnesium (0.2mM). For the ratio experiment, stimulus strength was adjusted until the amplitude of the compound (AMPA+NMDA) synaptic currents was approximately equivalent across all neurons; and stimulus strength was held constant throughout the remainder of the experiment. After recording a stable baseline of the resulting compound AMPA/NMDA EPSC, the AMPA-
component was inhibited with bath application of 20µM 6,7-dinitroquinoxaline-2,3-dione (DNQX). The relative contribution of each component within an individual neuron was calculated by dividing the amplitude of the remaining DNQX-resistant (NMDA receptor-mediated) current by the amplitude of the DNQX-sensitive (AMPA-mediated) component. These ratio data are presented as mean±SEM (averaged across cells) and were analyzed using one-way ANOVA across the different treatment groups.

For the NMDA input-output study, neurons from each treatment group were recorded at -60mV holding potential and incubated with low extracellular Mg$$^{2+}$$ and the AMPA receptor antagonist DNQX (20µM). Increasing stimulus intensities (10µA to 50µA) produced graded monosynaptic responses.

Spontaneous Glutamatergic Synaptic Events: Spontaneous excitatory postsynaptic currents (sEPSC) were acquired at 20kHz, and were filtered at 2kHz. For miniature EPSCs (mEPSC), 1µM tetrodotoxin (TTX) was bath applied for >5 minutes before recording spontaneous events. This concentration of TTX inhibited >99% of all electrically-evoked EPSCs (not shown). TTX-sensitive and –resistant spontaneous EPSCs were detected and analyzed using Mini Analysis Program 6.0.3 (Synaptosoft Inc., Fort Lee, NJ). Measures from individual cells were averaged across treatment groups (Van Sickle et al. 2004), reported as mean±SEM, and analyzed using standard one-way ANOVA. In some cases, sEPSC and mEPSC data are expressed as cumulative probability distributions from individual cells representing the median across a treatment group. These data were analyzed using the Kolmogorov-Smirnov (KS) test for different population distributions. Significance was determined at a KS statistic of 0.09 or greater
rather than using the P value; this cutoff makes the less-conservative KS test more stringent.

Noise Analysis: Root mean square current noise (I\textsubscript{rms}) was analyzed in the spontaneous event records. Noise was measured independently in four 50-msec epochs by comparing current amplitudes at each data point to the mean current amplitude across the epoch as previously described (Mtchedlishvili and Kapur 2006). Epochs containing obvious spontaneous EPSCs were avoided in the analysis. These data are reported as a mean±SEM and statistically analyzed using standard one-way ANOVA.

Paired pulse ratio: Paired-pulse facilitation (PP) was measured using pairs of electrical stimuli of equal intensity at 25, 50, or 250 msec interpulse intervals. Ratios of the amplitudes of the evoked EPSCs were calculated as the difference between the amplitude of the second event minus the amplitude of the first, with the result divided by the amplitude of the first synaptic response (Schulz et al. 1995). All values were expressed as mean±SEM, and data were subjected to a one way ANOVA, with P<0.05 considered statistically significant.

Microinjections: We accomplished microinjections into the BLA of male Sprague Dawley rats according to previously published procedures (McCool and Chappell 2007). Briefly, we deeply anesthetized animals with pentobarbital (90mg/kg) and affixed them to a stereotaxic instrument. Chronic guide cannulae were placed bilaterally into the dorsal aspect of the BLA and affixed to the skull with dental cement. We used sterile obturators to maintain the patency of the guide cannulae. During a five day recovery period, animals were handled extensively and habituated to the injection
procedure and sound of the syringe pump. On test days, 0.5µl drug in standard aCSF was directly infused into the BLA over a one minute period. Injection cannulae were left in place for 1min; and, animals were placed into the light/dark box 5min after the microinjection. Following the microinjections and behavioral measurements, guide cannulae placement was confirmed post-mortem.

Two separate microinjection experiments were performed in the current study. In the first experiment, adult male Sprague Dawley rats (n=39; 303.9±0.6g at the time of surgery) were used to test the effects of muscimol microinjection into the BLA on light/dark box anxiety-like behavior. For these experiments, guide cannulae were implanted according to the following stereotaxic coordinates (millimeters relative to Bregma, (Paxinos and Watson 1997): -2.8 anterior/posterior; ±5.05 medial/lateral; and -6.20 dorsal/ventral (measured from the top of the brain). Sham, 88nmol (10µg), or 264nmol (30µg) muscimol (in aCSF) was microinjected into separate animals; and, individual animals were exposed to the light/dark box only once. Across two separate cohorts, we misplaced guide cannulae in only three out of 39 animals used in the muscimol study; these are not included in the analysis (not shown, see text).

In the second experiment, male Sprague-Dawley rats (n=19, 138.0±2.6g at the time of surgery) similar to those used for the electrophysiology experiments were used to test the effects of DNQX on withdrawal-related anxiety-like behavior expressed in the light/dark box. For these studies, animals were surgerized 1hr following the fifth ethanol intermittent inhalation. This time point was selected for two reasons. First, animals appeared behaviorally tolerant to the effects of the ethanol inhalation by the fifth day; this may help reduce interactions between the anesthetic and the ethanol during the surgery.
Second, surgerizing animals prior to the ethanol exposure proved difficult because of changes in body weight and skull size during the long-term exposure. For the surgery, ethanol exposed animals were deeply anesthetized with pentobarbital (50mg/kg). In three separate cohorts (5-7 animals/cohort), guide cannulae were implanted according to the following stereotaxic coordinates (millimeters relative to Bregma) that were empirically determined in pilot studies on juvenile animals: -2.7 anterior/posterior; +4.5 lateral on the right, +4.75 lateral on the left; -5.8 dorsal/ventral from the top of the brain. Following the surgery, animals recovered for 10hr before being placed back into the inhalation chamber to complete the last 5 days of the chronic inhalation treatment. When not in the inhalation chamber, animals were handled during this period to habituate them to the injection procedure. Following 24hr withdrawal after the 10th ethanol exposure, individual animals were microinjected with either sham or 100pmol DNQX; and, individuals were exposed to the light/dark box only once. In three separate cohorts, we placed guide cannulae outside the BLA in only two out of nineteen animals (see Fig. 2.6A).

**Results**

*Withdrawal from chronic ethanol inhalation increases anxiety-like behaviors.*

We initially assessed whether our treatment paradigm was relevant for the anxiety-related effects of chronic ethanol and withdrawal. Fifty-seven animals were divided equally into three groups (n=19 each) with two groups receiving 10 days of 12hr/day ethanol vapor while the third control group received room air in an identical chamber. We then measured anxiety-like behavior using the “light/dark” box assay in
controls, immediately after the last ethanol exposure while individuals were still intoxicated (as evidenced by blood-ethanol levels; ‘CIE’ animals), or 24hr after withdrawal from ethanol vapors (‘WD’ animals). One CIE animal appeared sedated, was clearly an outlier with regard to low locomotor activity, and was excluded from the behavioral study. Similarly, four animals from the withdrawn group appeared to freeze when initially placed on the light-side of the behavioral apparatus (e.g. reduced locomotion with $\geq 60$sec to initially egress from the light- to the dark-side) and were not analyzed further.

For the remaining animals, 24 hours of withdrawal from 10 days chronic intermittent ethanol exposure caused a significant decrease in time spent in the light side, a significant decrease in the number of light/dark transitions, and a significant increase in the re-entry latency (time to re-enter the light following first egress into the dark; Table 2.1). Egress latency (initial time leave the light-side and move into the dark side) was not significantly different between the treatment groups. The alterations in most ‘anxiety-related’ variables are consistent with increased expression of anxiety-like behaviors in the WD animals. The total move time was not different between groups indicating that the treatment did not affect the locomotor activity in the animals. Exploratory behaviors, represented by vertical-plane entries (‘rears’), were significantly suppressed in both CIE and WD animals again suggesting that the anxiety-effects were specific to withdrawal.
Table 2.1 Increased Anxiety-Like Behavior in the Light/Dark Box Following Withdrawal from Chronic Intermittent Ethanol.

* – Anxiety-like behavior was assayed for 300sec immediately following 10days exposure to room air (‘Control’, n=18), ethanol vapor (‘CE’, n=17), or 24hr withdrawal from ethanol vapor (‘WD’, n=14).
Values are mean ± SEM. See text for details. † – P<0.05 vs. Control with Dunnett’s Multiple Comparisons posttest. ‡ – P<0.01 vs. Control

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>CE</th>
<th>WD</th>
<th>Statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Move Time (sec)</td>
<td>234.1±2.5</td>
<td>231.8±4.9</td>
<td>221.2±4.3</td>
<td>ns</td>
</tr>
<tr>
<td>Light Time (sec)</td>
<td>95.9±8.2</td>
<td>104.0±10.1</td>
<td>55.7±11.6†</td>
<td>P&lt;0.01</td>
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<tr>
<td>Transitions (#)</td>
<td>7.8±0.6</td>
<td>9.1±1.2</td>
<td>4.9±0.9†</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Egress Latency (sec)</td>
<td>21.5±3.0</td>
<td>22.4±4.9</td>
<td>16.4±3.2</td>
<td>ns</td>
</tr>
<tr>
<td>Re-entry Latency (sec)</td>
<td>72.2±15.4</td>
<td>65.5±19.6</td>
<td>171.4±30.8†</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Vertical-plane Entries (#)</td>
<td>32.0±2.8</td>
<td>21.2±2.6‡</td>
<td>21.3±1.9†</td>
<td>P&lt;0.01</td>
</tr>
</tbody>
</table>

Recent evidence suggests that anxiety-like behavior expressed in the light/dark box is dependent upon BLA neurotransmitter systems (Bueno et al. 2005; McCool and Chappell 2007). To test this, we microinjected 0 (n=12), 88nmol (10µg; n=14), or 264nmol (30µg; n=13) muscimol into the BLA and measured light/dark box behavior. For several of the anxiety-related dependent variables expressed in this assay, muscimol diminished anxiety-like behavior in a dose-dependent fashion. For example, 264nmol muscimol increased the time spent in the light-side of the apparatus (108±9sec; P<0.05, one-way ANOVA) compared to sham (73±13sec) and 88nmol muscimol (63±15sec). Likewise, the re-entry latency (time to re-enter the light-side following egress into the dark) was significantly less in animals microinjected with 264nmol muscimol (48±14sec; P<0.05, one-way ANOVA) compared to either sham (140±33sec) or 88nmol muscimol (111±22sec). The number of light-dark transitions and the initial egress latency (time to leave the light-side after placement) were not significantly affected by muscimol.

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however. Likewise, locomotor-related behaviors were not altered by the test doses of muscimol examined here. The total distance moved during the assay was 1343±42 cm in sham animals, 1241±72 cm in animals microinjected with 88 nmol muscimol, and 1377±38 with 264 nmol muscimol (P>0.05, one-way ANOVA). Similarly, the total time spent moving was 227.3±5.0 sec in sham, 209.7±6.6 sec with 88 nmol muscimol, and 229.8±3.3 sec with 264 nmol muscimol. Together, these data confirm that manipulation of BLA neural activity can alter anxiety-like behavior expressed in the light dark box.

_Chronic ethanol and withdrawal increase the contribution of NMDA-receptors at BLA glutamatergic synapses._

Previous studies in our lab have demonstrated that chronic ethanol can lead to increased NMDA receptor function measured in somatic compartments of acutely isolated basolateral amygdala neurons (Floyd et al. 2003). To test whether these alterations are expressed within synaptic compartments, we investigated the effects of CIE and WD on NMDA receptor function at glutamatergic synapses using whole-cell in vitro slice electrophysiology. We first established a stable excitatory synaptic response using both low extracellular magnesium as well as the most minimal stimulation required to produce consistent monosynaptic responses. For these studies, response amplitudes in the low extracellular magnesium were 127±23 pA in CON neurons, 114±15 pA in CIE neurons, and 114±13 pA in WD neurons; these values were not statistically different from one another (P>>0.05, one-way ANOVA). Within each neuron, we next perfused slices with the AMPA receptor antagonist DNQX (20 µM; Fig. 2.1B) to pharmacologically isolate NMDA receptor-mediated synaptic currents. When expressed as a ratio of
NMDA receptor-mediated current amplitude to the DNQX-sensitive, AMPA receptor-specific component, we found significant increases in the DNQX-insensitive NMDA receptor-mediated current (Fig. 2.1C) in both CIE- (P<0.05, n=6) and WD- (P<0.05, n=7) neurons compared to control (n=7). In a separate set of experiments, greater than 95% of DNQX-insensitive synaptic current in BLA neurons was inhibited by 50µM of the NMDA-receptor antagonist 2-amino-5-phosphonovaleric acid (APV; not shown). This inhibition did not differ between the three treatment groups.

We also examined the effects of CON, CIE, and WD treatments on NMDA-mediated synaptic currents by measuring responses to increasing stimulation intensities (input-output relationship). In all three treatment groups, local electrical stimuli elicited DNQX-insensitive synaptic currents whose amplitude increased in an intensity-dependent fashion (Fig. 2.1D). At the highest stimulation intensities, the amplitude of the DNQX-insensitive, NMDA-mediated synaptic current was significantly greater in both CIE and WD neurons compared to control (P<0.05, one-way ANOVA). Along with the ratio data, these input-output results clearly suggest that the contributions by synaptic NMDA receptors are increased by chronic ethanol exposure and are maintained during 24hr of withdrawal.
Figure 2.1 Chronic ethanol and withdrawal increase the NMDA/AMPA current ratio in the BLA measured with low extracellular Mg2+.

A, Slices were stimulated locally near the boundary of the lateral/basolateral amygdala and the recording electrode was placed in the basolateral amygdala as shown. B, Average synaptic responses for control and 24hr WD cells, in the presence of 10µM bicuculline, show the compound current consisting of the DNQX-sensitive and –resistant components and the current resistant to 20µM DNQX. The DNQX-resistant currents from these neurons are also enlarged below the compound current traces to aid with visual comparisons. The NMDA receptor antagonist APV inhibited >95% of the DNQX-resistant current (see text). C, The NMDA/AMPA ratio, calculated from the amplitude of the DNQX-resistant component (NMDA) divided by the amplitude of the DNQX-sensitive component (AMPA), show a significant increase of 405% in CIE neurons and 342% following 24hr WD (**, P<0.01 and *, P<0.05 compared to CON, one-way ANOVA with Bonferroni post-test). D, NMDA input-output relationships were measured from CON (D1, top), CIE (D1, middle), and WD (D1, bottom) neurons. Note that the amplitude NMDA-mediated (DNQX-resistant) synaptic responses were dependent on the intensity of the stimulation (in □A) and were significantly larger at the greatest intensities for CIE (n=7 neurons from 3 individuals) and WD neurons (n=6 neurons from three individuals) compared to CON neurons (D2, n=6 neurons from three individuals; *, CIE vs. CON P<0.05, #, WD vs. CON P<0.05, one-way ANOVA with Bonferroni post-test).
**Chronic ethanol and withdrawal increase the amplitude and frequency of spontaneous EPSCs.**

Since the NMDA/AMPA ratio is also dependent upon the relative contribution by AMPA receptors, a decrease in AMPA receptor-mediated synaptic currents might increase the NMDA/AMPA ratio in slices prepared from CIE and WD animals. We attempted to test this by measuring the AMPA input-output relationship. However, under the current recording conditions (local stimulation and standard aCSF containing 10µM bicuculline), AMPA-mediated synaptic responses had an extremely steep stimulus-response relationship with polysynaptic responses occurring at stimulation intensities >20µA (responses >200pA). This prevented a comparison of AMPA input-output across the treatment groups.

Alternatively, we examined spontaneous excitatory postsynaptic currents (sEPSCs; Fig. 2.2B). Under standard recording conditions (2mM extracellular magnesium and -60mV holding potential), spontaneous synaptic currents were entirely sensitive to the AMPA receptor-specific antagonist GYKI 53655 (Fig.2.2A). GYKI 53655 also inhibited electrically-evoked responses by 96±1% in CON neurons (n=4), 98±1% in CIE neurons (n=6), and 96±1% in WD neurons (n=5; P>0.05, one-way ANOVA). These data suggest that AMPA-type glutamate receptors mediate the spontaneous synaptic currents. With respect to the treatment groups, we found the sEPSC amplitude in CIE-, but not WD-, neurons was significantly increased relative to control (Fig. 2.2B & C). However, the charge carried by these spontaneous events was not significantly different between the treatment groups (Fig. 2.2D). Analysis of the background current (I_{rms}) was also not significantly different between groups (control,
1.2pA±.01; CIE 1.7pA±0.3; WD 1.3pA±0.1). These results indicate that our ability to detect sEPSCs was approximately equivalent across the treatment groups. In contrast to the modest effects of chronic ethanol and withdrawal on sEPSC amplitude, the frequency of spontaneous events was significantly greater in both treatment groups relative to control. For example, the inter-event interval of the sEPSCs was significantly decreased for CIE- (P<0.01, one-way ANOVA) and WD-neurons (P<0.01) compared to control (Fig. 2.2E).

*Chronic ethanol inhalation and withdrawal decreases the paired pulse ratio.*

The increased frequency of sEPSCs without substantial changes in amplitude might suggest increased presynaptic function following CIE and WD. To test this, we measured synaptic responses to two closely-juxtaposed electrical stimuli (paired pulses; Fig. 2.3A). At short interpulse intervals, the ratio of the second synaptic response relative to the first is believed to be inversely related to the probability of release at synapses (Andreasen and Hablitz 1994; Katz et al. 1993). At inter-pulse intervals of 25 and 50 msec (Fig. 2.3A), CIE and WD both significantly decreased the paired pulse ratios (Fig. 2.3B). In support of the sEPSC frequency data, the paired-pulse findings suggest that CIE and WD increase presynaptic glutamatergic function, potentially by increasing release probability at the terminal.
Figure 2.2 Chronic ethanol and withdrawal increase the frequency of spontaneous excitatory postsynaptic currents (sEPSCs).

A, Sample traces illustrate that, under standard conditions (10µM bicuculline; holding potential, -60mV), sEPSCs are mediated by GYKI 53655-sensitive AMPA receptors. B, Sample traces of sEPSCs in the presence of 10µM bicuculline (holding potential, -60mV) are shown for each treatment group. C, The sEPSC amplitude in CIE neurons was significantly larger than CON (*, P<0.05, one-way ANOVA with Bonferroni post-test). Mean±SEM are shown across all cells in a particular treatment group. For these experiments, n=8 neurons for CON (from five rats), n=11 neurons for CIE slices (from four rats), and n=11 for WD neurons (from four animals). D, The charge carried by sEPSCs (amplitude X decay time, expressed in femtoCoulombs) was not significantly affected by CIE or WD. E, The sEPSC inter-event interval was dramatically and significantly decreased (increased frequency) for both CIE and WD neurons (**, P<0.01 compared to CON, one-way ANOVA). F, A cumulative probability plot comparing the inter-event interval from individual CON, CIE, and WD neurons, each neuron represents median values from each treatment group. KS=0.39 for CIE neurons and KS=0.36 for WD neurons (P<0.0001 relative to CON).
Figure 2.3 Chronic ethanol and withdrawal decrease paired pulse ratio in the BLA.
A, Sample traces of paired pulse EPSCs at 50msec inter-pulse interval in the presence of 10µM bicuculline. The amplitude of the second synaptic response has been normalized across treatment groups to emphasize the relative differences between the first synaptic response and the second. B, Paired pulse ratio is significantly decreased at both the 25 and 50msec interval (*P<0.05, **P<0.01 vs. control, one-way ANOVA with Bonferroni’s post-test). These decreased ratios indicate that chronic ethanol and withdrawal may increase presynaptic release of glutamate.

Withdrawal increases miniature EPSC amplitude, decay, and frequency.

To confirm the possible alterations of presynaptic terminal function, we measured the effects of CIE and WD on the amplitude and frequency of TTX-resistant miniature
EPSCs under standard recording conditions (2mM extracellular magnesium and -60mV holding potential; Fig. 2.4A&B). WD robustly increased the frequency of mEPSCs (decreased inter-event interval) compared to control (P<0.01, one-way ANOVA) and CIE (P<0.05; Fig. 2.4E&F). Importantly, CIE did not alter mEPSC frequency, in contrast to the sEPSC and paired-pulse findings. Because TTX blocks action potentials, these data in the CIE neurons may imply that the presynaptic alterations evident in this treatment group are action potential-dependent. Furthermore, comparisons between the sEPSC and mEPSC data suggest that CIE and WD may alter presynaptic glutamatergic function via distinct mechanisms.

In addition to these apparent presynaptic changes, WD also increased the amplitude of the mEPSCs (Fig. 2.4C). However, the charge carried by mEPSCs was not significantly altered by any treatment (Fig. 2.4D). Together these amplitude and charge data suggest some change in the kinetics of glutamatergic responses in WD neurons. We therefore examined the onset and decay kinetics of the miniature EPSCs (Fig. 2.5A). Average mEPSC traces were generated by aligning the rising phase of each event in each cell and scaling the amplitude. The resulting average mEPSC from each cell was fit to a 1st-order exponential equation. Rise-times were not significantly different from each other (one-way ANOVA, Fig. 2.5B). However, the decay of mEPSCs was significantly faster in WD neurons compared to both CIE and CON (P<0.05, one-way ANOVA; Fig. 2.5C). These data help explain the significant up-regulation of mEPSC amplitude in WD neurons in the absence of any substantial alterations in the amount of charge carried by events in this treatment group.
Figure 2.4 Withdrawal increases the frequency and amplitude of mEPSCs.

A, Sample traces demonstrating the efficacy of 1µM tetrodotoxin used in these studies. B, Sample traces of mEPSCs in the presence of 10µM bicuculline and 1µM TTX (holding potential, -60mV) are shown for each treatment group. C, The mEPSC amplitude from WD neurons was significantly greater than CON (*, P<0.05) and CIE neurons (#, P<0.05; one-way ANOVA with Bonferroni’s post-test). D, The charge carried by mEPSCs (amplitude in pA X decay time in milliseconds, expressed as femtoCoulombs) was not significantly different from CON in the CIE and WD treatment groups. E, The inter-event interval of WD mEPSCs was significantly smaller than both control (**, P<0.01) and CIE neurons (#, P<0.05; one-way ANOVA with Bonferroni’s post-test). F, Cumulative probability plot of inter-event interval from individual CON, CIE, and WD neurons; each cell is representative of the median from that particular treatment group. The inter-event interval distribution of the WD neuron (KS=0.19, P<0.0001), but not the CIE neuron (KS=0.06, P>>0.05), was significantly different from the CON neuron.
Figure 2.5 Withdrawal from chronic ethanol decreases the current-decay time constant of miniature events. A, Average traces from each treatment group illustrate the faster decay kinetics of miniature events in withdrawal neurons. B, Rise-times were not significantly different from each other (one-way ANOVA). C, Using a single-exponential fit of miniature EPSCs, the decay time was significantly smaller than CON for WD events (*, P<0.05, one-way ANOVA) but not CIE events. These results suggest possible postsynaptic alterations of the receptor during withdrawal and provide a mechanism for increased amplitude, but not charge, in these neurons.
BLA glutamatergic system and withdrawal-related anxiety-like behavior.

Given that the focus of the current work on glutamatergic function in the BLA, we examined the effects of microinjection with the AMPA/kainate receptor antagonist DNQX (100pmol) on anxiety-like behavior in withdrawn (WD) animals (Fig. 2.6A). Importantly, DNQX substantially diminished the anxiety-like behavior expressed by WD animals in the light/dark box. Both the time spent in the light side (Fig. 2.6B2) and the number of light-to-dark transitions (Fig. 2.6B3) were significantly increased (P<0.05 and P<0.01, respectively; t-test) by DNQX microinjection. Likewise, DNQX significantly (P<0.01, t-test) decreased the re-entry latency in WD animals (Fig. 2.6B4). These effects on anxiety-like behavior appear to be specific to delivery of DNQX to the BLA. In two animals where guide cannulae were placed outside the BLA (open circles, Fig. 2.6A), neither the time spent in the light-side (6.5±3.5sec; mean ± standard deviation), nor the number of light-dark transitions (3.0±2.8; mean ± standard deviation), nor the re-entry latency (293.5±3.5sec; mean ± standard deviation) were significantly affected by the DNQX microinjections. Likewise, microinjection of DNQX into the BLA did not significantly alter locomotor-related behaviors like the total time spent moving (Fig. 2.6B1) or the total distance moved (934.8±50.4cm in WD animals, 1102.3±72.9cm in DNQX-injected animals; P>0.05, t-test). Together, these data indicate that delivery of the AMPA/kainate receptor antagonist DNQX into the BLA of WD animals can diminish anxiety-like behavior expressed in the light/dark box.
Figure 2.6 Microinjection of the AMPA-type glutamate receptor antagonist, DNQX, into the lateral/basolateral amygdala attenuates anxiety-like behavior expressed during withdrawal.

A, Figure (modified from Paxinos and Watson 1997) illustrating the approximate locations for the guide cannulae tips (correct placements=closed circles). Note that some cannulae were placed outside the BLA (open circles, see text).  B, Summary of light/dark box behaviors expressed by sham- (WD) and DNQX-injected animals with guide cannulae place within the BLA. While variables like the time spent moving during the assay were not affected (B1), 100pmol DNQX significantly increased both the total time spent in the light side (B2) and the number of light/dark transitions (B3). Intra-BLA DNQX also significantly decreased the latency to re-enter the light-side following initial egress to the dark side (B4). *P<0.05, **P<0.01, t-test
Discussion

Our results suggest that the increased anxiety-like behavior observed during withdrawal may be due to increases in glutamatergic function seen during CIE and WD. This effect appears to have a presynaptic origin, as evidenced by the decreased paired-pulse ratio and increased frequency of mEPSCs during WD. We also found increases in the NMDA/AMPA ratio as well as increases in amplitude and frequency of spontaneous events during CIE and WD and during WD for mEPSCs; these latter data suggest that post-synaptic alterations may result from these treatments as well. Our NMDA findings parallel previous evidence showing an increase in the function and expression of NMDA receptors in isolated BLA neurons following chronic ethanol liquid-diet exposure (Floyd et al. 2003). The most reasonable interpretation of the changes seen during CIE and WD include increased excitability of glutamatergic afferents in the BLA, increased presynaptic terminal function/number, and more modest postsynaptic increases in receptor function.

It should be noted that our findings must be interpreted in the context of the current exposure paradigm. Our intermittent exposure produces robust blood ethanol concentrations during the ten day exposure period. Regardless, CIE rats with ~190mg/dL have little apparent motor in-coordination, at least as it is represented in the light/dark box (Table 1). None-the-less, this same exposure paradigm produces elevated anxiety-like behavior during subsequent withdrawal. Chronic human alcoholics can also have remarkable functional tolerance despite blood levels that would produce coma and death in naïve individuals (Davis and Lipson 1986; Hammond et al. 1973). It is clear then that
the intermittent exposure pattern and severity of exposure employed in the current study may model this type of profound ‘tolerance-dependence’.

*Chronic Ethanol facilitates synaptic-NMDA and -AMPA receptors in the basolateral amygdala.*

Our previous work has shown that chronic exposure to an ethanol liquid diet increased NMDA receptor function in the somatic compartments of acutely isolated BLA neurons (Floyd et al. 2003). Here we show that chronic intermittent ethanol inhalation also significantly increased NMDA receptor function, exemplified by the NMDA/AMPA ratio, at BLA glutamatergic synapses. This is similar to findings in the central nucleus after chronic ethanol (Roberto et al. 2006). Together, these results strongly support the hypothesis that CIE up-regulates post-synaptic NMDA receptor number or function in the amygdala. The amygdala therefore joins a growing list of brain regions responding to chronic ethanol by increased expression/function of NMDA receptors.

Chronic ethanol also increases AMPA receptor protein in primary cortical cultures (Chandler et al. 1999) as well as AMPA receptor dependent calcium signaling in cerebellar Purkinje neurons (Netzeband et al. 1999). However, we found no evidence that CIE produces any alteration in AMPA receptor postsynaptic function. Preliminary evidence (J. Weiner, personal communication) suggests that acute ethanol robustly inhibits BLA NMDA-mediated synaptic responses while AMPA-mediated currents are relatively insensitive. This differential acute sensitivity might explain the robust up-regulation of NMDA synaptic currents to chronic ethanol while AMPA-mediated events are more modestly affected.
Chronic ethanol facilitates TTX-sensitive pre-synaptic function in the basolateral amygdala.

Our paired-pulse data, specifically the decrease in facilitation, clearly indicate increased presynaptic function with CIE. This is supported by an increased frequency of spontaneous events in these neurons. Our data are further supported by increased presynaptic glutamate release in both the central nucleus of the amygdala using a similar exposure paradigm (Roberto et al. 2004b) as well as in the hippocampus during ethanol consumption (Sabria et al. 2003). However, the increase in BLA sEPSC frequency was entirely TTX-sensitive; CIE did not alter the frequency of miniature, TTX-resistant EPSCs. These data may imply that CIE facilitates action potential-dependent mechanisms within the BLA glutamatergic afferents themselves. In support of this, chronic ethanol can alter a number of action potential-related processes (Scott and Edwards 1981) including decreased calcium-activated potassium channels (Pietrzykowski et al. 2004) and increased TTX-sensitive voltage-gated sodium channels (Brodie and Sampson 1990) or L-type calcium channels (Watson and Little 1999). In fact, dihydropyridine-sensitive voltage-gated calcium channels appear to be recruited to BLA glutamatergic synapses following fear-learning (Shinnick-Gallagher et al. 2003). Changes within the context of action potential-related, TTX-sensitive cellular processes could therefore lead to increased neurotransmitter release at BLA glutamatergic synapses.

Finally, it is worth noting that slices prepared from CIE animals were incubated at room temperature in aCSF for as long as six hours during the data collection. Since we did not include ethanol in the slice incubation media, it is possible that CIE slices were experiencing ‘acute in vitro’ withdrawal during this period. Our data do not support this
hypothesis. First, all CIE animals were sacrificed within 30 minutes following removal from the inhalation chamber. Since we know precisely when each data file was collected, we performed a correlation analysis between the various sEPSC and mEPSC parameters and the time elapsed between collection of these data and preparation of the slices. For all CIE neurons, there was no significant correlation between these event-related dependent variables and the time the slice had been stored in vitro. This argues against a significant contribution by ‘acute in vitro’ withdrawal to CIE-related alterations in glutamatergic transmission.

Withdrawal maintains postsynaptic changes seen during chronic exposure.

After 24 hours of withdrawal from chronic ethanol, we saw an increase in NMDA/AMPA ratio and the NMDA input-output relationship in BLA neurons. Given that sEPSC data suggests that AMPA receptor function is not down-regulated during WD, the NMDA data indicate that the increased receptor function seen with CIE is maintained for at least twenty-four hours after the exposure. There are conflicting reports concerning the effects of ethanol withdrawal, relative to chronic ethanol, on NMDA receptors in other brain regions. NMDA receptor function appears to increase during ethanol withdrawal in the mouse hippocampus (Whittington et al. 1995). However, in the central amygdala, neither chronic ethanol nor withdrawal alter NMDA receptor synaptic function (Roberto et al. 2006). These data indicate that WD-related changes in NMDA receptor function are clearly brain region-dependent, even within the amygdala.

Withdrawal also induced postsynaptic facilitation of AMPA-mediated glutamatergic spontaneous EPSCs in the BLA. Specifically, we found a large increase in
the amplitude of TTX-resistant spontaneous EPSCs. This effect appears to share similarities with other drugs of abuse. For example, the AMPA receptor subunit GluR1 has been shown to be up-regulated after 1 and 30 days of withdrawal from cocaine in the BLA of rats (Lu et al. 2005). Similarly, TTX-resistant EPSCs from WD neurons decayed faster than those measured in both control and CIE. Increased AMPA-mediated EPSC decay is associated with increased delivery of GluR1 homomeric channels to glutamatergic synapses in response to sensory-stimulation of barrel cortex (Clem and Barth 2006) and pharmacologic blockade of AMPA receptors in hippocampal neuron cultures (Thiagarajan et al. 2005). The faster decay (Atassi and Glavinovic 1999; Hirasawa et al. 2001; Veruki et al. 2003), along with increased amplitude (Carroll et al. 1998; Thio et al. 1992), may indicate postsynaptic alterations within the AMPA receptor complex expressed by WD neurons. One possible mechanism explaining increased EPSC amplitude/alterted kinetics following WD would be increased delivery of specific AMPA-receptor subtypes to BLA glutamatergic synapses. However, this remains to be directly investigated. Regardless, the increase in mEPSC amplitude was not associated with substantial increases in charge carried by individual events. The contribution of postsynaptic alterations to BLA-dependent, WD-related behaviors may therefore be subtle.

Withrawal Produces Presynaptic, Terminal-Specific Alterations

There is a general lack of data on the effects of ethanol withdrawal on presynaptic mechanisms. In the central nucleus of the amygdala, glutamate release remains elevated during both chronic ethanol exposure and withdrawal (Roberto et al. 2006). The results
of our mEPSC and paired pulse data in the BLA complement these findings and suggest that WD specifically alters presynaptic terminal function. Potential mechanisms include altered release machinery (Bacci et al. 2001; Capogna et al. 1997; Herreros et al. 1995; Pang et al. 2006), increased resting calcium levels in the terminal (Cummings et al. 1996; Levesque and Atchison 1988; Li et al. 1998; Nishimura et al. 1990), or increased numbers of synapses (Lauri et al. 2003) following WD. Chronic blockade of glutamate receptors in hippocampal cultures selectively increases AMPA-mediated mEPSC frequency without altering synapse number (Bacci et al. 2001). Conversely, withdrawal from repeated cocaine exposures increases the number of glutamate-containing terminals in the shell of the nucleus accumbens (Kozell and Meshul 2004). Ultimately, the mechanisms regulating increased mEPSC frequency during WD from CIE are likely to be both brain region- and treatment-specific.

Behavioral Implications

Given the association between the BLA glutamatergic system and anxiety (Sajdyk and Shekhar 1997), increases in glutamatergic function like those observed in the current work may contribute to the anxiety-like behavior evident during withdrawal from chronic ethanol. Additional studies that follow a withdrawal time course would be necessary to more robustly illustrate this. However, we have used juvenile animals to facilitate the whole-cell electrophysiology recordings; and this creates confounds between developmental issues and a protracted time-course. Studies in adult animals would perhaps be more appropriate in this respect but are also substantially more challenging.
Regardless, our microinjection results with DNQX clearly suggest that BLA mechanisms associated with AMPA/kainate-type glutamate receptors help regulate the expression of anxiety-like behavior during withdrawal. These findings parallel studies showing that amygdala AMPA receptors are important for the expression of learned fear-potentiated startle (Walker and Davis 1997b) and learned avoidance tasks (Mesches et al. 1996). Importantly, the expression of learned-fear is coincident with increased AMPA receptor-mediated synaptic function at BLA glutamatergic synapses (McKernan and Shinnick-Gallagher 1997) and with increased delivery of AMPA receptor subunits to the cell surface (Yeh et al. 2006). The coincidental increase in BLA glutamatergic synaptic function during withdrawal, the increase in withdrawal-related anxiety-like behavior, and the apparent contribution of BLA AMPA-type glutamate receptors to withdrawal-associated anxiety-like behavior together suggest an intimate relationship between these parameters.

Paradoxically, we found that glutamate function was elevated during the CIE exposure, despite an absence of elevated anxiety-like behavior measured in the light/dark box. One potential explanation is that the BLA contributes minimally to CIE-related behaviors expressed in the light/dark box. Most evidence does not support this hypothesis. Several recent studies (de la Mora et al. 2005; McCool and Chappell 2007; Perez de la Mora et al. 2006; Salome et al. 2006) as well as the muscimol study reported in the current work have demonstrated the contribution of BLA-dependent processes to light/dark box anxiety-like behavior in naïve animals. Therefore, compensatory changes in other BLA/downstream neurotransmitter systems or continued sensitivity of these systems to acute ethanol are more likely to explain the paradoxical increase in BLA
glutamatergic function in the absence of significant increases in anxiety-like behavior. This latter ‘continued sensitivity’ hypothesis is particularly relevant since anxiety-like behaviors in CIE animals were measured while the individuals were still intoxicated (based on BEC at sacrifice).

The potential neurotransmitter systems potentially contributing to CIE-related behaviors are quite numerous. Within the amygdala fear/anxiety-circuit, recent reports suggest that GABAergic function is increased (Roberto et al. 2004a) while glutamatergic function is decreased (Roberto et al. 2004b) in the neighboring central amygdala in ethanol-dependent rats. Since the projections from BLA to central amygdala are critical for the expression of learned-fear (Davis 2006), it is possible these central amygdala alterations might offset increased glutamatergic function in the BLA during CIE treatment, ultimately masking anxiety-related behavioral manifestations in these animals. Within the BLA itself, alcohol has well known effects on numerous neurotransmitter systems. For example, acute and chronic ethanol alter GABAergic function in BLA neurons (Floyd et al. 2004; McCool et al. 2003; Zhu and Lovinger 2006). In addition, BLA serotonergic (Gonzalez et al. 1996), dopaminergic (de la Mora et al. 2005), noradrenergic (Schroeder et al. 2003), and various neuropeptide systems (Rupniak et al. 2003; Sajdyk et al. 1999a; Sajdyk et al. 1999b; Wunderlich et al. 2002) all regulate anxiety-like behavior. Disruption or alteration of any of these systems might also contribute to anxiety-related behavior observed in intoxicated rats exposed to chronic ethanol.
Conclusions

In conclusion, we have shown that chronic intermittent ethanol exposure and withdrawal lead to significant increases in glutamatergic synaptic transmission in the basolateral amygdala. Our working hypothesis for the sequence of events that result from this treatment is that chronic ethanol produces both increased postsynaptic NMDA receptor function as well as increased glutamatergic afferent excitability. At some point during the withdrawal process, these alterations trigger significant changes in presynaptic terminal function and more modest alterations of postsynaptic AMPA receptor function. This increased glutamatergic system in the basolateral amygdala was not observed behaviorally until withdrawal because we hypothesize that the additional ethanol-sensitive systems may be engaged while the animal is still intoxicated.

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3. Chronic Ethanol and Withdrawal Differentially Modulate Local Feedback and Lateral Paracapsular Feedforward Interneurons of the Basolateral Amygdala

Abbreviated Title: Chronic ethanol and withdrawal alter BLA GABAergic synaptic transmission

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Summary

The effects of alcohol withdrawal on anxiety are frequently cited as a major contributor to relapse in recovering alcoholics. Many of the neurobiological processes that lead to the expression of withdrawal-induced anxiety occur in the amygdala, an emotional center of the brain. Specifically, changes in basolateral amygdala (BLA) neurotransmitter systems are thought to underlie changes in anxiety-like behaviors following chronic intermittent ethanol (CIE) exposure and withdrawal (WD). We have recently shown that CIE and WD enhance the BLA glutamate system. However, enhanced anxiety occurs only during WD; and, this suggests additional neuro-adaptations are important. The effects of these exposures on the GABAergic system remain largely unknown. Interestingly, the GABA system of the BLA is comprised of at least two populations of interneurons – local feed-back interneurons and feed-forward interneurons situated in the lateral/paracapsular region of the BLA (LPCs). In the present study, we characterized the effects of CIE and WD on these two GABAergic populations in the BLA. Although we found that CIE and WD did not alter presynaptic function at local GABAergic synapses, release probability at LPC synapses is decreased during WD. At the post-synapse, there is a decrease in GABA\(_A\) \(\alpha_1\)-subunit function at LPC synapses and a coincidental change in \(\alpha_1\)- and \(\gamma_2\)-subunit expression during CIE and WD. Importantly, neither local nor LPC synapses develop tolerance to the effects of acute ethanol or altered sensitivity to the benzodiazepine midazolam following exposure to CIE and WD. These data together suggest that CIE and WD differentially affect pre- and post-synaptic mechanisms at local and LPC synapses. More importantly, these alterations
compliment and extend our understanding of the neurobiological mechanisms governing changes in anxiety-like behavior observed during chronic ethanol and withdrawal.

Keywords: Anxiety, Chronic Ethanol, Withdrawal, Electrophysiology, Slice Recording, lateral paracapsular cells

Introduction

Alcohol withdrawal is known to severely increase anxiety in alcoholics. This withdrawal-induced anxiety has also been shown to occur in alcohol dependent rodents (Lack et al., 2007; Santucci et al., 2007). Although the effects of alcohol are widespread throughout the brain, the amygdala has been shown to play an important role in mediating alcohol-withdrawal in humans (Duka et al., 2003; Little et al., 2005; Stephens et al., 2005; Townshend and Duka, 2003) and rodents (Lack et al., 2007). As the amygdala is responsible for initiating and processing anxiety-like behaviors, it is a major target for studying the effects of withdrawal-induced anxiety. In particular, the basolateral amygdala (BLA) functions as the primary input into the anxiety circuit and has been shown to be modulated by chronic intermittent ethanol (CIE) and withdrawal from CIE (Floyd et al., 2003; Lack et al., 2009; Lack et al., 2007).

The balance between the GABAergic and glutamatergic systems in the BLA controls the expression of anxiety-like behaviors. We have previously shown large increases in pre- and post-synaptic glutamatergic function, specifically AMPA, kainate, and NMDA, during CIE and withdrawal (Lack et al., 2009; Lack et al., 2007). These
findings are likely to be mechanistically related to the increased anxiety-like behavior observed during withdrawal (Breese et al., 2005; File et al., 1989; Moy et al., 1997). However, CIE does not produce a change in anxiety-like behavior, despite the increases in glutamatergic function (Lack et al., 2007). Therefore, changes in the BLA GABAergic system in response to chronic ethanol and withdrawal can potentially modulate the ultimate influence of alcohol-related alterations in glutamate signaling.

The amygdalar GABAergic system is significantly altered by exposure to chronic alcohol. GABAergic function increases in the central amygdala (CeA) in animals exposed to chronic ethanol (Lack et al., 2005). In the BLA, a study using a non-human primate model of long-term ethanol self-administration found altered lateral amygdala GABA<sub>A</sub>-pharmacology and mRNA expression (Floyd et al., 2004). Similarly, chronic exposure to a liquid ethanol diet increases the functional expression of GABA<sub>A</sub>-receptors in acutely isolated rat BLA neurons (McCool et al., 2003). These data together suggest an increase in GABAergic function following a chronic ethanol exposure. During withdrawal however, data suggests a deficit in BLA GABAergic function. For example, a recent study showed that three days of withdrawal from an liquid ethanol diet suppressed BLA GABAergic interneuron excitability (Isoardi et al., 2007). Likewise, decreased BLA GABA<sub>A</sub>-α1 subunit and GAD67 mRNA were found at 60 days of withdrawal from a liquid ethanol diet (Falco et al., 2008). Although these studies appear internally consistent, they have employed different ethanol exposures as well as different aged animals. Therefore, the effects of chronic ethanol exposure and 24 hours of withdrawal from the same ethanol exposure on the GABAergic system in the BLA are not fully understood.
In the BLA, the GABAergic system is comprised of at least two anatomically and functionally distinct populations of interneurons. Lateral paracapsular interneurons (LPCs) receive cortical input arriving to the BLA via the external capsule (EC) and have been shown to synapse onto the distal dendrites of principal neurons in the BLA to provide feed-forward inhibition (Marowsky et al., 2005). In contrast, local interneurons, scattered throughout the subdivision, make peri-somatic synapses on principal neurons and are locally activated through feedback loops, in turn providing feedback inhibition to principal neurons (Muller et al., 2003, 2005, 2006; Woodruff et al., 2006; Woodruff and Sah, 2007). The GABAergic synapses arising from these anatomically distinct interneuron populations can be functionally separated using specific placement of stimulating electrodes used during in vitro electrophysiological recordings (Silberman et al., 2007). Using this technique in the current study, we show that these two populations differentially adapt during CIE and withdrawal.

Methods

Animals All animal procedures were performed in accordance with protocols approved by Wake Forest University School of Medicine Animal Care and Use Committee and were consistent with the National Institutes of Health animal care and use policy. Male Sprague–Dawley rats were obtained from Harlan (Indianapolis, IN) and were housed in an animal care facility at 23°C with a 12-h light/dark cycle and given food and water without restriction. Rats were weighed daily to ensure that ≥80% of their free-feeding weight was maintained during vapor chamber ethanol exposure.
**Chronic Ethanol Exposure** Ethanol exposure was accomplished via an ethanol vapor chamber as previously described (Lack et al., 2009; Lack et al., 2007). Briefly, male Sprague Dawley rats (~120-150 grams) were housed four to a cage in large, standard polycarbonate cages. Cages were placed in large, custom-built Plexiglas chambers (Triad Plastics, Winston-Salem, NC) that were identical to those previously described (Lopez and Becker, 2005). At the beginning of the light cycle (lights on at 9 pm EST), animals were exposed to either ethanol vapor or control air (CON group) for 12 hours during the light cycle for 10 days. To produce ethanol vapor, an air pump sent room air into an airstone submerged in a one liter flask containing 95% ethanol. Using calibrated pressure gauges, we mixed ethanol vapor with room air to achieve the desired vapor concentration (~45mg EtOH/L air) in the ethanol chamber. During the dark cycle the chambers were opened. The concentration of vapor in the ethanol chamber was tested daily. Blood was collected on the day of sacrifice for subjects in the chronic intermittent ethanol (CIE) group and blood ethanol levels were 194±11mg/dl. Withdrawal (WD group) animals remained in the ethanol chamber (without ethanol vapor) for 24 hours after the last ethanol exposure.

**Slice Preparation** Amygdala slices were prepared from CON, CIE, and WD animals. Animals were anesthetized with isoflurane (3%) and decapitated according to a protocol approved by an Institutional Animal Care and Use Committee. The brains were quickly removed and incubated in ice-cold sucrose/artificial CSF (aCSF) equilibrated with 95% O₂ and 5% CO₂ containing (in mM): 180 sucrose, 30 NaCl, 4.5 KCl, 1 MgCl₂·6H₂O, 26 NaHCO₃, 1.2 NaH₂PO₄, 10 glucose, and 0.1 ketamine. Coronal brain
slices (400µm) were prepared using a Vibratome Series 3000 (Vibratome, St. Louis, MO) and submerged in room-temperature (~25°C), oxygenated standard aCSF containing (in mM): 126 NaCl, 3 KCl, 1.25 NaH2PO4, 2 MgSO4, 26 NaHCO3, 10 glucose, and 2 CaCl2·2H2O. Slices were maintained in aCSF for ~1 hour before recording. All experiments were performed 1-4 hours after preparation of the BLA slices. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Whole-cell patch-clamp electrophysiology Methods used for whole-cell patch-clamp electrophysiology were similar to those previously described (Lack et al., 2007). Slices were placed in a recording chamber and perfused with room temperature aCSF at a rate of 2mL/min. Electrodes were filled with an internal solution containing (in mM): 122 Cs-gluconate, 10 CsCl, 10 HEPES, 1 EGTA, 5 NaCl, 0.1 CaCl2, 4 Mg-ATP, 0.3 Na-GTP, and 2 QX314-(Cl) and had an open-tip of 8-12 MΩ. Data were acquired with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) and analyzed using Clampex software (Axon Instruments, Foster City, CA). Electrically evoked GABA-IPSCs were pharmacologically isolated with 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX, 20µM), an AMPA/kainate receptor antagonist, and DL-2-amino-5-phosphono-pentanoic acid (APV, 50µM), an NMDA receptor antagonist, and were recorded from principal neurons. Principal neurons were identified as having an initial membrane resistance of <50 MΩ (Lack et al., 2007), and were only included in the study if tip resistance and baseline holding currents changed <20%.

GABA synaptic release was electrically stimulated using a platinum/iridium concentric bipolar stimulating electrodes (FHC, Bowdoinham, ME) with an inner pole diameter of 25µm. Stimulating electrodes were placed in the EC to stimulate distal LPC
synapses and locally within the BLA to stimulate proximal local synapses (Silberman et al., 2007). Minimal stimulation intensities were used at both stimulation sites that ranged in order to achieve a GABAergic response of ~100pA, less than <20% of electrically-evoked maximum responses (data not shown). Neurons were maintained at a holding potential between 0 and -10mV.

**Paired-Pulse Ratios** Paired-pulses were given to each stimulation site at interpulse intervals of 25, 50, and 250 msec. These intervals were chosen to target changes in release probability (25 and 50 msec) and presynaptic autoreceptor function. A normalized ratio of amplitudes of the inhibitory postsynaptic currents (IPSCs) for each stimulation site was taken ((amplitude of response 2 - amplitude of response 1)/amplitude of response 1) (Andreasen and Hablitz, 1994; Bonci and Williams, 1997)). All values were expressed as means ± SEM, and data were subjected to a one-way ANOVA, with P<0.05 considered statistically significant.

**Spontaneous GABAergic Synaptic Events** The same internal solution mentioned above was used for these experiments. Spontaneous inhibitory postsynaptic currents (sIPSCs) were acquired at 20 kHz, and were filtered at 2 kHz. For miniature (m)IPSCs 1µM tetrodotoxin (TTX) was bath-applied for >5 minutes before recording spontaneous activity. sIPSCs and mIPSCs were recorded for 1 minute following a baseline period (~5min). Event amplitude and frequency were measured using MiniAnalysis (SynaptSoft, Inc.). Medians of measures from individual cells were averaged across treatment groups (Lack et al., 2007), reported as mean ± SEM, and analyzed using one-way ANOVA, with Newman-Keuls post-hoc test and P<0.05 considered statistically significant.
Zolpidem, Midazolam, and Ethanol Pharmacology  Upon patching a neuron, neurons were allowed to dialyze for ~5min. After collecting a baseline of evoked GABA-IPSCs, 100nM zolpidem, a GABA_A-α1-specific modulator, or 1μM midazolam, a GABA_A-benzodiazepine modulator, or 80mM ethanol (made in aCSF with 10mM HEPES to prevent precipitants) was perfused onto slices until the effect reached steady state (~5-10min in all cases). All values expressed as mean percent change from baseline±SEM, and data were subjected to a one-way ANOVA, with Bonferroni’s post-hoc test and P < 0.05 considered statistically significant.

Western Blots  Lysis buffer (50mM Tris pH 7.4, 0.5% sodium dodecyl sulfate, 1mM EDTA pH 8, and protease inhibitors for mammalian tissue [Sigma, St. Louis, MO]) was added to BLA dissected from CON, CIE, and WD coronal brain slices at 5µl/mg tissue, disrupted by brief sonication, and incubated at 4°C on a rotisserie mixer for 2 hours. Protein yield was quantified using the BCA assay (Pierce Chemical, Rockford, IL). Fifteen micrograms of total protein was loaded on to 4-20% sodium dodecyl sulfate precast polyacrylamide gels (Pierce Chemical, Rockford, IL), separated, and transferred to a nitrocellulose membrane (Hybond N; Amersham, Piscataway, NJ). The membrane was blocked with Tris buffered saline (TBS)-T (150mM NaCl, 5.2 mM Na2HPO4, 1.7mM KH2PO4, 0.05% Tween-20) containing 10% nonfat dry milk (NFM). Subsequently, blots were incubated overnight at 4°C in TBS-T/0.5% NFM containing a rabbit polyclonal primary antibody that recognizes GABA_A subunits: α1, 1:3000 dilution (Millipore, CITY, ST); α4-N terminus, 1:650 dilution (PhosphoSolutions, CITY, ST); or γ2, 1:500 dilution (Sigma). Following extensive washing with TBS-T, the blots were exposed to a goat anti-rabbit, HRP-labeled secondary antibody (1:3000 dilution; Sigma)
for one hour at room temperature with agitation. Detection of bound secondary antibody was performed using enhanced chemiluminescence (Pierce). To normalize expression between experiments, the blots were probed with mouse monoclonal antibody directed against β-actin, 1:50,000 to 1:100,000 dilution (Chemicon) followed by peroxidase-labeled goat anti-mouse secondary antibody, 1:10,000 dilution (Sigma). Band intensity was quantified from digital images of x-ray film utilizing Un-Scan It Software (Silk Scientific Corporation, Orem, Utah).

Results

Differential changes in Probability of Release from Distal and Local Synapses

In order to functionally separate distal LPC proximal local synapses using electrical stimulation, we and others have used a dual stimulation protocol that has been shown to separately activate the two interneurons populations independently of each other (Diaz, Submitted; Silberman et al., 2009; Silberman et al., 2007). To measure presynaptic changes from both distal and local synapses we employed this dual stimulation protocol and measured probability of release using paired-pulse ratios (PPR). We found no changes in PPR at local synapses, suggesting that release probability is not altered during CIE or WD at these synapses (Fig. 3.1A, B: PP25: CON= 0.43±0.08, CIE=0.32±0.07, WD=0.27±0.07; PP50: CON=0.12±0.07, CIE=0.10±0.05, WD=0.13±0.05; PP250: CON= -0.28±0.05; CIE= -0.21±0.05; WD= -0.23±0.05; n=21, 17, and 11, respectively; P>0.05, one-way ANOVA). Conversely, the release probability from distal LPC synapses is significantly altered during at 24 hours of withdrawal.
Specifically, PPRs with 25msec (CON=0.29±0.06, n=21; CIE=0.53±0.06, n=17; WD=0.95±0.12, n=11), 50msec CIE = -0.42±0.04; WD= -0.21±0.08) stimulus interval are significantly increased with (CON=0.04±0.07; CIE=0.22±0.08; WD=0.46±0.14), and 250msec (CON= -0.42±0.05; CIE and/or WD compared to controls (Fig. 3.1A,C: ** -- P<0.001 compared to CON, # -- P<0.01 compared to CIE, two-way ANOVA with Bonferroni’s post-test). These data suggest that 24 hours of withdrawal decreases the probability of GABA release from distal LPC synapses.
Figure 3.1 Chronic ethanol and withdrawal differentially affect LPC and local interneuron release probability.

A) Sample paired-pulse (PP) traces from local and LPC neurons across treatment groups. P₁ is the first response and P₂ is the second response. Dotted line used to compare peak of the first response to the second. B) Graphical representation of PP ratios from local interneurons across the treatments at 25, 50 and 250msec interpulse intervals (IPI). PP ratios did not change in CIE or WD treated neurons. C) Graphical representation of PP ratios from LPC interneurons across the treatment groups at 25, 50, and 250msec IPI. PP ratios were significantly increased during CIE at 25msec IPI and doubled in WD treated neurons. At 50msec IPI, a trend toward an increase in PP ratio was apparent in CIE treated neurons that significantly increased in WD neurons. Similarly, at 250 IPI PP ratios significantly increased in WD treated neurons.
To confirm that presynaptic transmitter release from local synapses was not affected by CIE or WD we examined action-potential-dependent spontaneous activity, given that the detectable events under similar recording conditions predominantly arise from local synapses (Silberman et al., 2009). Consistent with the paired-pulse data, we found that neither CIE nor WD altered sIPSC inter-event-interval (IEI) (CON: 86.03±11.72msec, n=17; CIE: 64.65±4.80msec, n=17; WD: 74.25±6.85msec, n=19; P>0.05). sIPSC amplitude was also unaffected by CIE or WD (CON: 21.08±1.71pA, n=17; CIE: 21.25±1.83pA, n=17; WD: 19.19±1.37pA, n=19; P>0.05). Likewise, rise times, charge, and decay times were unaffected by treatment groups (data not shown). These data suggest and confirm that neither CIE nor WD alter pre- or post-synaptic characteristics of spontaneous IPSCs.

**GABA\_A Subunit Changes during CIE and WD**

Several studies have shown decreases in GABA\_A-α1 subunit expression and function following chronic ethanol exposure in various brain regions, such as hippocampus (Cagetti et al., 2003; Olsen et al., 2005), cortex, and ventral tegmental area (VTA) (Charlton et al., 1997). We found that zolpidem, a GABA\_A α1-containing receptor agonist, produced very little facilitation of the electrically-evoked GABAergic response from local synapses, and this was unchanged in CIE and WD slices (Fig. 3.2A, % effect
from baseline: CON=8.21±14.93%, n=5; CIE=-3.92±7.28%, n=13; WD=9.39±12.88%, n=10; P>0.05, one-way ANOVA). Conversely, in simultaneous recordings from LPC synapses, zolpidem robustly increased electrically-evoked GABAergic responses from lateral paracapsular GABA neurons. Importantly, CIE and WD significantly decreased LPC sensitivity to zolpidem (Fig. 3.2B, % effect from baseline: CON=75.18±27.75%, n=5; CIE=8.5±10.43%, n=13; WD=13.12±10.48, n=10; * -- P<0.05, one-way-ANOVA with Bonferroni’s post-test). Importantly, these data suggest that α1-containing receptors appear to be localized primarily to GABAergic synapses arising from the feed-forward paracapsular interneurons. Furthermore, chronic ethanol exposure and withdrawal substantially decrease BLA α1-subunit function at these synapses.

Given that GABA_A-α1 functional contributions were decreased in the BLA of CIE and WD animals, we tested whether CIE or WD also altered GABA_A subunit pharmacology (Cagetti et al., 2003; Olsen et al., 2005). We found that midazolam sensitivity was not affected at either local or LPC synapses (% effect from baseline; local –  CON: 89.00±16.19% n=6; CIE: 70.64±21.64%, n=6; WD: 68.10±13.73%, n=6, P>0.05, one-way ANOVA; LPC – CON: 76.28±15.26%, n=6; CIE: 58.97±22.30%, n=6; WD: 59.98±21.95%, n=5, P>0.05, one-way ANOVA). These data contrast with the diminished benzodiazepine sensitivity evident in the hippocampus following chronic ethanol and withdrawal (Cagetti et al., 2003; Liang et al., 2004; Liang et al., 2007; Liang et al., 2006) and suggest brain region-specific alterations in specific GABAergic subunits.
To examine potential molecular mechanisms governing changes in BLA GABA<sub>A</sub> zolpidem sensitivity, we measured protein expression of α1-4, and γ2 subunits. Several of
these subunits have been shown to be altered during chronic ethanol exposure (Cagetti et al., 2003; Mehta and Ticku, 2005; Olsen et al., 2005). Consistent with the zolpidem data (Fig. 3.2), we found that α1-subunit expression was significantly decreased 38.03±6.41% in CIE (P<0.05) and 53.45±6.37% in WD (P<0.01) compared to controls (Fig. 3.3A: n=4, one-way-ANOVA with Newman-Keuls post-test). In contrast, we did not observe changes in α4-subunit expression during CIE or WD (Fig. 3.3B: CIE = 114.21±7.31% and WD = 106.95±5.15% of 100% CON; n=4, P>0.05). Likewise, CIE and WD did not alter α2 (CIE = 79.08±4.40% and WD = 88.04±15.92% of 100% CON, n=4, P>0.05) or α3 (CIE = 90.65±4.73% and WD = 92.76±11.44% of 100% CON, n=4, P>0.05) protein expression. Finally, γ2-subunit expression was significantly increased during CIE (172.57±18.81%) and 24 hours of withdrawal (184.39±28.54%) (Fig. 3.3C: of 100% CON, n = 4, P<0.05, one-way ANOVA with Newman-Keuls post-test). The changes in α1 and γ2 are similar to those described in hippocampal neurons following a chronic intermittent oral ethanol treatment (Cagetti et al., 2003; Olsen et al., 2005). Importantly, these data indicate that significant postsynaptic changes in the BLA GABAergic system occur during CIE and WD.
Figure 3.3 Chronic ethanol and withdrawal differentially affect GABAA-subunit expression. Graphical representations and sample western blots show that GABAA-α1-subunit expression. (A) is significantly decreased in the BLA of CIE treated animals, with a larger decrease following WD. In contrast, GABAA-α4-subunit expression (B) is not affected by CIE or WD. Furthermore, BLA GABA A-γ2-subunit expression (C) is significantly increased by CIE and WD.
Lack of Tolerance to the Effects of Acute Ethanol during CIE and WD

Although the primary focus of this study is to characterize the effects of CIE and withdrawal on the BLA GABAergic system, recent evidence suggests that acute ethanol can potentiate GABAergic transmission in the central amygdala of ethanol-dependent animals (Roberto et al., 2004). To determine whether similar adaptations occur in the BLA of CIE and WD animals we examined the sensitivity of LPC and local GABAergic synapses to acute application of 80mM ethanol. We found neither CIE (Fig. 3.4A; local: 30.03±11.13%, n=10; Fig. 3.4B; LPC: 36.14±9.28%, n=10) nor WD (local: 33.26±18.22%, n=11; LPC: 23.55±13.48%, n=11) altered this sensitivity relative to alcohol-naïve controls (local: 47.08±11.28%, n=9; LPC: 45.03±12.66%, n=11; P>0.05, one-way ANOVA). These data suggest that there is a lack of tolerance to the facilitatory effect of acute ethanol on GABAergic transmission in the BLA of CIE and WD animals.
Figure 3.4 CIE and WD-treated BLA GABAergic synapses do not become tolerant to the effects of acute ethanol. Graphs show that the effect of 80mM acute ethanol on electrically evoked GABAergic transmission from local (A) and LPC (B) synapses are not altered by CIE or WD. Sample traces correspond to the bar graph above.
Discussion

In the amygdala, the GABAergic system is a major target for alcohol. The current study shows that GABAergic function within the BLA is altered during chronic intermittent ethanol exposure and withdrawal. Both pre- and post-synaptic changes occur as evidenced by decreases in GABA release probability and protein expression, respectively. Furthermore, remnant sensitivity of acute ethanol and midazolam on GABAergic transmission during withdrawal provides evidence for the ameliorating effect of acute ethanol and the clinical efficacy of benzodiazepines on withdrawal symptoms. Together with the large increase in glutamatergic function during WD (Lack et al., 2007), these factors likely contribute to the increase in withdrawal-induced anxiety-like behavior (Lack et al., 2007).

Effects of CIE and WD on presynaptic GABA in the BLA

Importantly, our data clearly show that local interneurons and LPCs are differentially modulated during WD. Specifically, LPC-mediated feed-forward inhibition is decreased only during withdrawal and not CIE. In contrast, local interneurons are unaltered by either treatment. Given the decrease in GABA release probability from LPCs following WD, our data suggests this cortically-driven feed-forward inhibition into the BLA is suppressed during WD. Furthermore, because increased cortical activity drives LPC firing (Marowsky et al., 2005; Rosenkranz and Grace, 2001), decreased feed-forward inhibition from distal LPC synapses might suggest a loss of cortical input into the BLA. Because feed-forward inhibition decreases glutamatergic information from exiting the BLA, this decrease in feed-forward inhibition may account for the increase in
BLA excitability (Lack et al., 2009; Lack et al., 2007). Despite the suppression of feed-forward GABAergic inhibition that occurs with withdrawal, local GABAergic feed-back inhibition is maintained. Although feed-back inhibition results in partial blockage of glutamatergic information leaving the BLA, these interneurons create synchronous networks that work together (Loretan et al., 2004; Woodruff and Sah, 2007) and appear to be sufficient in maintaining control over the BLA during CIE.

The pre-synaptic findings from the current study contrast previous work in the central amygdala and hippocampus. A recent study showed that chronic ethanol exposure increases GABAergic transmission in the central amygdala (Roberto et al., 2003). Although our current findings in CIE animals do not parallel those of the central amygdala, they do suggest that the increase in GABA levels and GABAergic transmission in the CeA of CIE rats (Lack et al., 2005) may function as a compensatory mechanism to inhibit the increase in BLA output (Lack et al., 2009; Lack et al., 2007). In addition, work within the hippocampus showed a decrease in both frequency and amplitude of mIPSCs two days following 60 days of chronic ethanol exposure (Cagetti et al., 2003), indicative of a robust down-regulation of GABAergic transmission in this brain region. Yet, we found that neither CIE nor WD had an effect on BLA spontaneous GABAergic transmission. One possible reason for these inconsistent findings is that the local BLA GABAergic system may not be able to compensate for increases in principal neuron excitability in CIE-treated animals (Lack et al., 2009; Lack et al., 2007). Another reason is the difference in exposure time. While our 10 day exposure was sufficient in producing anxiety-like behavior following withdrawal (Lack et al., 2007), it may not be sufficient to alter spontaneous GABAergic transmission, as did the 60 day exposure in
the hippocampus (Cagetti et al., 2003). Lastly, and more likely, is that the spontaneous activity that we detect under our recording conditions arises from local synapses (Silberman et al., 2009) that are unchanged during WD, as suggested by the lack of change in paired-pulse ratios. In fact, given the decrease in GABA release from feed-forward LPC synapses at 24 hours of withdrawal, spontaneous activity at these synapses may potentially be decreased during WD similar to the hippocampus. Unfortunately, we do not yet have the tools to measure spontaneous activity from LPC synapses.

Spontaneous GABAergic events may arise from multiple neuronal populations. For example, both local feed-back interneurons and paracapsular feed-forward interneurons potentially contribute to these spontaneous synaptic events. However, the GABA release from local synapses, as measured by synaptic responses to paired electrical stimuli, was not altered during CIE or WD while GABA release from LPCs was decreased in WD groups. Since CIE and WD did not change the frequency of spontaneous IPSCs, these data suggest that the majority of sIPSCs arise from local interneurons. Similarly, another study using similar techniques as in the current study also suggest that the majority of sIPSCs recorded in the BLA arise from local interneurons (Silberman et al., 2009). These conclusions are consistent with anatomical evidence showing that local interneurons form peri-somatic synapses onto glutamatergic pyramidal neurons (McDonald et al., 2005). In contrast, LPCs synapse onto distal dendrites of glutamatergic pyramidal neurons (Muller et al., 2007), and spontaneous IPSCs arising from these distal LPC synapses may not be readily detected relative to the peri-somatic local GABAergic synapses due to substantial cable filtering by the distal dendrites (Marowsky et al., 2005; Muller et al., 2007).
The sensitivity of local GABAergic transmission in the BLA to chronic exposure to ethanol and withdrawal may potentially be age-dependent. In relatively young animals (7-8 weeks), we found that local GABAergic transmission is unaltered by exposure to chronic ethanol and withdrawal. Similarly, mRNA levels of GAD67, correlated with levels of extracellular GABA, were unchanged 60 days after the last ethanol dose in adolescent rats (4 weeks) (Falco et al., 2008). In contrast, a previous study showed a decrease in feed-back GABAergic postsynaptic potentials in adult animals (~12 weeks) withdrawn from a liquid ethanol diet (Isoardi et al., 2007). One explanation for these discrepancies is that different routes of ethanol administration were used, vapor exposure vs. liquid ethanol diet. Another possible explanation for these discrepancies is the age of the animals during the exposure. While the Falco et al. study and the current study showed consistent results when using adolescent animals, Falco et al. also showed that GAD67 mRNA levels were significantly decreased after 60 days of withdrawal when animals began the ethanol exposure as adults (11-12 weeks), consistent with the reports by Isoardi et al.. Together these data suggest an age-dependent effect of chronic ethanol in the BLA.

*Postsynaptic Effects of CIE and WD on the BLA GABAergic System*

While there are major alterations in GABA release at LPC synapses during withdrawal, postsynaptic alterations are also apparent. We specifically found that CIE and WD decreased zolpidem-sensitivity at LPC synapses and decreased GABA$_A$ $\alpha_1$ subunit-like immunoreactivity. While the Western approach does not directly measure changes in GABA$_A$ receptor subunits at specific subcellular sites, the lack of a substantial
effect of zolpidem at local GABAergic synapses suggest the α1-subunits measured in the western data are localized to LPC synapses. In other brain regions like hippocampus, cortex, and VTA, chronic ethanol exposure decreases GABA_A α1-subunit function and/or expression (Cagetti et al., 2003; Charlton et al., 1997; Olsen et al., 2005). Likewise, we have recently shown decreases in BLA α1-mRNA in non-human primates following long-term ethanol self-administration (Floyd et al., 2004). α1-mRNA levels are also decreased in rats following 60 days of withdrawal from a restricted ethanol drinking paradigm (Falco et al., 2008). Thus, diminished α1 expression/function during CIE appears to be a consistent finding across several brain regions and experimental systems. Interestingly, α1-subunit contribution at local feed-back synapses is practically non-existent and is not affected by CIE or WD. One possible explanation for the difference between LPC and local synapse zolpidem sensitivity is the high levels of α1 expression in the BLA region adjacent to the EC (Marowsky et al., 2004), presumably where LPCs synapse onto glutamatergic pyramidal neurons. Given the difference in α1-expression, it would be reasonable to suggest that an area with a higher expression of α1 (LPC synapses) would perhaps be more sensitive to an exposure (i.e. ethanol) than an area with practically no expression (local synapses). Nevertheless, changes in α1-subunit may significantly contribute to the overall BLA output observed in CIE and WD treated animals.

The changes in subunit expression and function are not consistent across all alpha subunits or brain regions. While α4-expression/contribution has been shown to increase in the hippocampus in animals exposed to CIE (Cagetti et al., 2003; Olsen et al., 2005), we did not find a change in α4 protein expression in the BLA of CIE or WD exposed
animals. Similarly, there was no decrease in midazolam sensitivity suggesting that this indirect indication of \( \alpha 4 \) contributions to the \( \text{GABA}_A \) receptor was not altered by these treatments. These findings are similar to previous results describing a consistent effect of benzodiazepine on \( \text{GABA} \)-gated currents in isolated BLA neurons from control rats and rats chronically exposed to a liquid ethanol diet (McCool et al., 2003). \( \alpha 4 \)-mRNA levels in BLA of chronic ethanol exposed cynomolgus macaques have also been shown to be unchanged (Floyd et al., 2004). This difference between \( \text{GABA}_A \) subunit alterations in hippocampus and BLA could be related to the relatively low levels of \( \alpha 4 \) in the amygdala (Benke et al., 1997; Wisden et al., 1992). We also found that neither \( \alpha 2 \)- or \( \alpha 3 \)-subunit expression was affected by CIE or WD within the BLA. Contrasting findings in cultured cortical synaptoneurosomes have shown that \( \alpha 2 \)- and \( \alpha 3 \)-subunit protein levels are reduced following chronic exposure to ethanol (Mhatre et al., 1993). Likewise, \( \alpha 2 \)- and \( \alpha 3 \)-subunit mRNA is significantly reduced in the BLA of chronic ethanol exposed cynomolgus macaques (Floyd et al., 2004). These discrepancies could be related the tissue used (cultured vs. native), species (rat vs. non-human primate), and route or length of ethanol exposure. Regardless, further investigation would be required to clarify these inconsistencies.

Interestingly, other \( \text{GABA}_A \)-subunits were also altered by CIE and WD. Specifically, we found increased levels of \( \gamma 2 \)-subunit expression during CIE and WD, similar to those seen in hippocampus (Cagetti et al., 2003). This increase in \( \gamma 2 \) levels might suggest an increase in \( \text{GABA}_A \) receptor clustering at the synapse (Essrich et al., 1998; Schweizer et al., 2003). Although we did not measure changes in clustering during CIE or WD in the current study (i.e. changes in mIPSC frequency (Cagetti et al., 2003) or
changes in gephyrin expression that has been shown to co-localize with γ2 (Essrich et al., 1998; Kneussel et al., 1999)), increased receptor localization at the plasma membrane would be consistent with the increased GABA_A R density previously found following a liquid ethanol diet (McCool et al., 2003). Nonetheless, it would be important to further investigate the changes in γ2-expression using our measures of synaptic transmission.

Effects of CIE and WD on Acute Ethanol Sensitivity

The effects of acute ethanol on GABAergic transmission have been widely studied in the alcohol field. This is because many of the intoxicating behavioral effects of ethanol are believed to be manifested by modulation of GABAergic inhibition. In particular, acute ethanol has been shown to increase presynaptic GABAergic release in the BLA (Roberto et al., 2006; Silberman et al., 2007) and CeA (Lack et al., 2005; Roberto et al., 2003) of alcohol-naïve animals, presumably contributing to the anxiolytic properties of alcohol. Yet, the effects of acute ethanol on BLA GABAergic transmission in alcohol dependent and alcohol withdrawing animals have not been studied. Any changes in the sensitivity of the BLA GABAergic system to acute ethanol could be important given the association between changes in BLA excitability and anxiety-like behavior (Lack et al., 2009; Lack et al., 2007). Interestingly, the current study found no change in the sensitivity to acute ethanol at either LPC or local GABAergic synapses in CIE and WD animals. This compliments previous results showing a lack of tolerance to acute ethanol in the central amygdala of alcohol-dependent animals (Roberto et al., 2004). These data ultimately support the hypothesis that relapse may function as a form of self-medication (Breese et al., 2005). In this case, ethanol would continue to increase
GABAergic transmission in the amygdala during withdrawal, ultimately decreasing withdrawal-related behaviors like anxiety.

**Effects of CIE and WD on the anxiety circuit**

Information in the anxiety circuit flows into the BLA via glutamatergic projections, while the GABAergic system functions to locally regulate the flow of this information. We previously showed that during CIE pre- and post-synaptic glutamatergic function is increased in the BLA while anxiety-like behavior is increased only during withdrawal, not immediately following CIE (Lack et al., 2007). Based on the results of the current study, we hypothesize that the continued sensitivity of the BLA GABAergic system to acute ethanol helps control increased glutamatergic function in animals during the chronic ethanol exposure. In fact, LPCs make up a large network of inhibitory cells that envelope the BLA, with several LPCs synapsing onto an individual glutamatergic pyramidal neuron (Marowsky et al., 2005; Muller et al., 2007). Likewise, local feed-back interneurons are chemically and electrically interconnected, allowing for synchronous interneuron network activation and cyclic self-propagation (Woodruff et al., 2006; Woodruff and Sah, 2007). The effects of acute ethanol on these synapses are therefore likely to make significant contributions to the overall regulation of BLA principal neuron activity by the GABAergic system in CIE animals. Thus, it is the continued presence of ethanol in CIE animals that helps to maintain the balance between the glutamate and GABA systems in the face of substantial, potentially deleterious alterations in both systems. In fact, the potential disruption in this balance is likely related to the increased anxiety-like behavior expressed during withdrawal.
By 24 hours of withdrawal, GABAergic function at LPC synapses becomes suppressed within the BLA. This is coincident with persistent increases in glutamatergic function (Lack et al., 2007). Nonetheless, local feed-back GABAergic inhibition remains resistant to any large-scale alterations at 24 hours of WD and is perhaps still functioning to help maintain some control of the brain region. Importantly, both LPC and local synapses remain sensitive to the facilitatory effects of acute ethanol, suggesting that the withdrawal-related ‘excitability’ of BLA principal neurons can be diminished by the re-introduction of ethanol. Interestingly, by 3 days of withdrawal from a liquid ethanol diet, local GABAergic transmission is decreased (Isoardi et al., 2007). This suggests that additional, more long-lasting changes in the GABAergic system may be evident at later withdrawal times. Taken together, the changes in neurotransmitter function seen during WD in addition to the removal of the one thing that enhances GABAergic transmission, ‘acute alcohol’, are consistent with the persistent increases in anxiety-like behaviors.

**Behavioral implications**

In the BLA, the GABA and glutamate systems work intimately together to regulate BLA-mediated behaviors. Although glutamatergic function is elevated during CIE, the continuous local feed-back GABAergic transmission in the BLA and the absence of any significant tolerance to the acute effects of ethanol appears to be sufficient to maintain BLA-mediated behaviors. However, during WD, the suppressed GABA system, due to decreased feed-forward inhibition, the absence of any acute effect of ethanol, and enhanced glutamatergic function contributes to the observed increases in anxiety-like behaviors (Lack et al., 2007). These processes may be further accentuated
by cortical glutamatergic inputs through the EC that synapse on LPCs (Marowsky et al., 2005; Rosenkranz and Grace, 2001). Taken together, this study emphasizes the importance of the GABA system in regulating and maintaining control over the BLA during chronic alcohol and withdrawal.

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4. Dopamine D3 Receptors Modulate Anxiety-like Behavior and Regulate GABAergic Transmission in the Rat Lateral/Basolateral Amygdala

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Abstract

Fear and anxiety are complex behaviors governed by multiple brain regions. Central among these is the lateral/basolateral amygdala (BLA). BLA modulation of fear/anxiety is tightly controlled by the relative activity of two populations of inhibitory GABAergic interneurons, local feed-back cells distributed throughout the nucleus and feed-forward cells found along the lateral paracapsular border of this subdivision. Dopaminergic inputs from the ventral tegmental area form a potential link between fear/anxiety-states and mesolimbic reward/attentional processes. And, recent studies suggest that dopamine can modulate the BLA GABAergic system. However, the dopaminergic mechanisms regulating the activity of the two BLA GABAergic neuron populations have not been fully explored. We therefore examined the effects of dopamine D3 receptors on BLA-dependent anxiety-like behavior and on GABA \textsubscript{A}-mediated inhibitory postsynaptic currents. We found that microinjection of a D3-selective antagonist into the BLA decreased anxiety-like behavior expressed in both the light/dark transition test and the elevated plus maze. Consistent with this, D3 receptors inhibit synaptic transmission at both BLA feed-back and feed-forward GABAergic interneuron populations. At lateral paracapsular GABAergic synapses, this inhibitory effect of D3 receptors is synergistic with D1-mediated inhibition when using dopamine as an agonist. Conversely, at local GABAergic synapses, D3-mediated inhibitory effects are masked by a previously-identified D1-mediated facilitation in a concentration-dependent manner. Because environmental cues alter both dopamine release and relative activity states of the BLA, our data suggest that dopamine, acting through distinct receptors, shifts the relative contribution by inhibitory processes to modify the expression of BLA-related behaviors.
Introduction

The rat amygdala, a central regulatory component of the limbic system, contains five major nuclei that coordinate a multitude of brain regions responsible for different aspects of affective behaviors like fear/anxiety. The lateral and basolateral subdivisions (BLA) represent the primary ‘input’ nuclei of the amygdala’s anxiety circuit (Pitkanen et al., 1997). These nuclei are composed primarily of large, pyramidal-shaped glutamatergic projection neurons that receive excitatory projections from diverse cortical and thalamic inputs. BLA projection neuron activity is tightly regulated by at least two populations of GABAergic interneurons: the lateral paracapsular cells (LPCs) are concentrated within ‘islands’ found along the lateral border of the BLA and provide feed-forward inhibition in response to cortical input (Marowsky et al., 2005). Conversely, ‘locally’ distributed interneurons provide synchronous feedback inhibition in response to glutamatergic collaterals arising from the principle BLA neurons (Woodruff et al., 2006). GABAergic inhibition within the amygdala controls the acquisition and expression of different measures of conditioned fear (Davis, 2000). And, inhibition of LPC activity is believed to disinhibit the BLA in situations requiring an ‘emotional’ behavioral response to environmental cues (Marowsky et al., 2005). Thus, the relative activity of feed-forward LPC and feed-back local GABAergic neurons can modulate the flow of information through the BLA and ultimately influence the expression of innate anxiety or learned fear-like behaviors.

Recent evidence suggests that dopamine (DA) in the BLA may act as a switch between cortically and locally controlled states (Bissiere et al., 2003; Marowsky et al., 2005). In this context, manipulation of the BLA DA system produces significant effects
on affective conditioning (Lamont & Kokkinidis, 1998; Guarraci et al., 1999; Nader & LeDoux, 1999) and innate anxiety-like behavior (de la Mora et al., 2005). These findings suggest interactions between DA and emotional states mediated in part by amygdala-dependent processes. In support of this, recent evidence suggests that DA modulation of amygdala-associated behavior appears to involve the inhibitory GABAergic circuits within the lateral/basolateral amygdala. For example, activation of D2-like receptors (D2R) dis-inhibits the lateral amygdala in mice (Bissiere et al., 2003) while BLA D1 receptor (D1R) activation suppresses LPC-mediated GABAergic inhibition (Marowsky et al., 2005) while simultaneously enhancing local BLA interneuron excitability (Kroner et al., 2005).

In addition to the expression of D1- and D2-like receptors in the BLA (Gurevich & Joyce, 1999; Fuxe et al., 2003), there is also marked expression of D3-like receptor binding sites within this brain region (Camacho-Ochoa et al., 1995). However, the physiological role of D3 receptors in the BLA has not been extensively explored. Phillips et al. (Phillips et al., 2002b) has previously shown that microinjection of a selective D3 receptor (D3R) antagonist into the BLA can attenuate taste-related associative learning. These data suggest a functional role for D3R in this brain region. In the current study, we show that BLA D3 receptors also modulate innate anxiety-like behavior, potentially by modulating GABAergic synaptic transmission. Furthermore, DA itself can differentially modulate GABAergic synaptic transmission arising from distinct interneuron populations within the BLA via activation of D1R and D3R.
Materials and Methods

*Animals:* Male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) were used for all experiments described in this manuscript. All experimental procedures were reviewed and approved by the WFUSM Animal Care and Use Committee and are consistent with the NIH Guidelines for the Care and Use of Laboratory Animals.

*Surgical Procedure and Microinjections:* Microinjection of the D3 dopamine receptor antagonist into the BLA was accomplished using previously published procedures (Anderson *et al.*, 2007; Isoardi *et al.*, 2007). Rats (300g) were anesthetized with pentobarbital and implanted with guide cannulae directed at the dorsal aspect of the lateral amygdala according to the following stereotaxic coordinates (millimeters relative to bregma (Paxinos & Watson, 1997): -2.80 anterior/posterior, +5.05 lateral on the right, +5.25 lateral on the left, -6.20 dorsal/ventral from the top of the brain. During a five day recovery period, animals were extensively handled and repeatedly exposed to the manipulations associated with the microinjection procedure. On injection days, injection cannulae were placed such that their tips extended 1mm below the ventral aspect of the guide cannulae; and, animals received 0.5µl of the D3 receptor antagonist, U99194, delivered over a 30 second period. Injection cannulae were left in place for an additional 1min before being removed. Animals receiving the sham injection were subjected to the same procedure, but no drug was injected. Animals received three microinjections, once a week, for three consecutive weeks. Each week, animals were exposed to a different behavioral apparatus (see below). Animals were counter-balanced across injection days such that they never received the same dose of U99194 (or sham) across the three
injections. Placement of the guide cannulae was confirmed post-mortem by preparing coronal slices from fixed tissue (see Fig. 4.1A).

**Behavioral Assays:** To assess the effects of U99194 into the basolateral amygdala, two different assays were employed to measure anxiety-like behaviors and a separate apparatus was used to assess locomotion. To measure anxiety with the light/dark box (McCool *et al.*, 2003; Lack *et al.*, 2007), individuals were placed in the ‘light’ side of a 40 cm × 40 cm plexiglass arena divided equally into ‘light’ and ‘dark’ sides by an opaque plexiglass insert (Rat Truscan Activity Arena, Coulbourn Instruments, Allentown, PA). The arena was illuminated by ambient room lighting to ~400 lux at the center of the ‘light’ side. The geometric center of the animal ±1.3 cm was followed for 5 min by two infrared sensor rings surrounding the entire apparatus, one in the floor plane and one located ~15 cm above the floor to measure rearing behavior. Data were collected at 0.25 Hz and stored on a personal computer. Dependent variables included the total number of moves, time to leave the light side following initial placement (egress latency), time to return to the light side after initial entry to the dark side (re-entry latency), total time spent in the light and dark compartments, number of light–dark transitions, and number of vertical beam breaks. Between animals, the apparatus was cleaned with warm water, 70% ethanol, again with warm water, and thoroughly dried.

In the second microinjection week, sessions were performed using the elevated plus maze (Pellow *et al.*, 1985) with incandescent lighting (~40 lux on the open arms). The apparatus sets atop a 28.5 in. (72.4 cm)-high aluminum frame (Med Associates, St. Albans, VT). Each runway is 4.0 in. wide (10.2 cm) and 20.0 in. long (50.8 cm). Open runways have lips that are 0.5 in. high (1.3 cm), while closed runways have black
polypropylene walls 16.0 in. high (40.6 cm). Infrared sensors were attached at the opening to each arm to score an animal's entry or exit. Experimental control and data acquisition was achieved with a personal computer interfaced with control units and programmed using MED-PC (Med Associates). Dependent variables included the total amount of time an animal spent in each arm, the total amount of time the animal spent at the central junction, and the number of transitions between arms. At the beginning of each 5 min session, individuals were placed at the junction of the open and closed arms facing an open arm. Between animals, the apparatus was cleaned with warm water, 70% ethanol, again with warm water, and thoroughly dried.

In the final assay, locomotor activity was measured in an open-field (Steiner et al., 1997). Animals were placed in the center of a 40 cm × 40 cm plexiglass arena (Rat Truscan Activity Arena) and locomotor activity was recorded in the dark for 5 minutes using infrared sensor rings. Dependent variables included total distance moved, total number of moves, and move time.

Slice Preparation: For electrophysiology experiments, coronal amygdala slices were prepared from male Sprague Dawley rats (100-150 gm, 3-5 weeks of age). Animals were anesthetized with isoflurane (3%) and decapitated according to an institutional IACUC-approved protocol. The brains were quickly removed and incubated in ice-cold sucrose/artificial CSF (aCSF) equilibrated with 95% O$_2$ and 5% CO$_2$ containing (in mM): 180 sucrose, 30 NaCl, 4.5 KCl, 1 MgCl$_2$·6H$_2$O, 26 NaHCO$_3$, 1.2 NaH$_2$PO$_4$, 100 ketamine, and 10 glucose. Brains were sliced (400µm) on a Vibratome Series 3000 (Vibratome, St. Louis, MO) and submerged in room-temperature (~25°C), oxygenated standard aCSF containing (in mM): 126 NaCl, 3 KCl, 1.25 NaH$_2$PO$_4$, 2 MgSO$_4$, 26
NaHCO₃, 10 glucose, and 2 CaCl₂·2H₂O. Slices were maintained in ACSF for ~1 hour before recording. All experiments were performed 1-4 hours after preparation of the BLA slices. All chemicals obtained from Sigma-Aldrich (St. Louis, MO).

**Whole-cell patch-clamp recording:** Methods for whole-cell voltage clamp recordings from BLA neurons within slices were similar to those reported previously (Lack *et al.*, 2007). For recording, slices were placed in a recording chamber that was continuously perfused with room-temperature aCSF at a rate of 2ml/min. Input resistance was 8-12 MΩ when electrodes were filled with an internal solution containing (in mM): 122 Cs-gluconate, 10 CsCl, 10 HEPES, 1 EGTA, 5 NaCl, 0.1 CaCl₂, 4 Mg-ATP, 0.3 Na-GTP, and 2 QX314-(Cl). Data were acquired with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) and stored for later analysis using ClampEx software (Axon Instruments, Foster City, CA). GABAₐ-mediated synaptic currents were pharmacologically isolated using 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX, 20µM, an AMPA/Kainate receptor antagonist; Sigma-Aldrich, St. Louis, MO, USA) and DL-2-amino-5-phosphono-pentanoic acid (APV, 50µM, a NMDA receptor antagonist; Sigma-Aldrich). Only neurons with a high membrane capacitance (>100pF) and low input resistance (<30MΩ) were considered presumptive principal neurons (Washburn & Moises, 1992; Faber *et al.*, 2001; Kroner *et al.*, 2005) and included in the current analysis.

GABA synaptic release was electrically stimulated using a range of intensities to achieve an average synaptic response of ~100pA. Minimal stimulation intensities were
used at both stimulation sites that ranged in order to achieve a GABAergic response of ~100pA, less than <20% of electrically-evoked maximum responses (data not shown).

We placed the stimulating electrode either in the external capsule (to activate LPCs) or medial to the recording electrode (to activate local interneurons) as specified within each experiment. For specific DA receptor activation, slices were perfused with D1- and D3-like receptor antagonists SCH23390 (Sigma-Aldrich) and U99194 (Tocris Bioscience, Ellisville, MO, USA) respectively, prior to application of DA. For some studies, we used the selective D3R agonists PD128907 (Tocris Bioscience).

**Paired-pulse ratio:** Paired-pulse ratios were measured with two equal-intensity electrical stimulations with a 25, 50, or 250 msec inter-pulse interval. A ratio of the amplitudes of the evoked IPSCs was taken (second amplitude divided by the first). All values are expressed as mean ± SEM.

**Spontaneous GABAergic Synaptic Events:** Spontaneous inhibitory postsynaptic currents (sIPSC) were acquired at 20kHz, and were filtered at 2kHz. The same internal solution previously mentioned was used for these experiments. sIPSCs were recorded for 1 minute following a brief period after rupture of the seal (~5min), and for 1 minute at the maximum drug effect (~5min after application). Event amplitude and frequency were measured using MiniAnalysis (SynaptSoft, Inc.). Median values from individual cells were averaged across treatment groups (Lack et al., 2007) and reported as mean±SEM.

**Statistics:** Behavioral data were analyzed using standard one-way ANOVA. Electrically-evoked IPSC data were analyzed with paired t-tests and one-way ANOVA as appropriate within each experiment. sIPSC data was analyzed using t-tests by comparing
median amplitude and frequency in each cell before and during drug application. P<0.05 was considered statistically significant.

Results

Anxiolytic effects of BLA D3 receptor blockade

Recent reports of D3R modulation of BLA-dependent learned behaviors (Phillips et al., 2002b) and altered anxiety-like behavior in D3R knock-out mice (Steiner et al., 1997) led us to examine D3R involvement in anxiety-like behaviors. Animals were bilaterally microinjected with 1.5pmol and 5pmol of U99194, a selective D3R antagonist (Audinot et al., 1998), into the BLA (Fig. 4.1A). In the light/dark box, U99194 had no effects on total number of moves (Fig. 4.1B). However, U99194 microinjection caused a significant dose-dependent increase both in the time spent on the light side of the apparatus (Fig. 4.1C, *--P<0.05, **--P<0.01) and in the total number of transitions between the light and dark sides (Fig. 4.1D, *--P<0.05 compared to vehicle, ***-P<0.001 compared to vehicle, ##--P<0.01 compared to 1.5pmol U99194). U99194 microinjection also decreased the re-entry latency (Fig. 4.1E, ***--P<0.001) compared to vehicle. Egress latency was modestly increased at both the 1.5pmol (33.8±10.6sec) and 5pmol doses (36.2±5.0sec) relative to sham (15.7±5.8sec) but this did not reach statistical significance. Likewise, the number of vertical rears was not altered by U99194 microinjection into the BLA (not shown).
Figure 4.1 D3R-blockade reduces anxiety-like behavior in a light/dark box.
A) The approximate location of guide cannulae tips are indicated by the open circles. Anterior-to-posterior stereotaxic coordinates of representative coronal brain slices are shown at left (Paxinos & Watson, 1997). B) U99194 did not significantly affect the number of movements in the light/dark arena (P>0.05, One-way ANOVA). C) U99194 increased time spent in light side compared to vehicle injected (aCSF – 15.42±5.58sec, 1.5pmol U99194 – 86.44±24.82sec, 5pmol U99194 – 142.3±16.80sec, *--P<0.05, **--P<0.01 relative to aCSF; One-way ANOVA, Newman-Keuls post-hoc). D) U99194 dose-dependently increased number of transitions from dark to light side compared to vehicle injected (aCSF – 1.0±0.26, 1.5pmol U99194 –3.62±0.75, 5pmol U99194 – 6.83±0.87, *--P<0.05, ***--P<0.001 compared to aCSF; One-way ANOVA, Newman-Keuls post-hoc). E) U99194 decreased re-entry latency to the dark side compared to vehicle injected (aCSF – 284.3±5.79sec, 1.5pmol U99194 – 87.38±38sec, 5pmol U99194 – 33.50±5.32sec, ***--P<0.001 relative to aCSF; One-way ANOVA, Newman-Keuls post-hoc).

In the elevated plus maze (Table 4.1), animals microinjected with U99194 spent significantly more time on the open-arms (F=13.91, P<0.001, one-way ANOVA) and significantly less time on the closed arms (F=10.16, P<0.01, one-way ANOVA) than
sham animals. Likewise, U99194-injected animals had significantly more open-arm entries (F=13.49, P<0.001, one-way ANOVA) and a greater percentage of open-arm entries (open arm entries/total arm entries; F=10.89, P<0.001, one-way ANOVA). Neuman-Kuels post-tests indicated that these effects were all dose-dependent (see Table 4.1). There were no significant effects of U99194 microinjection into the BLA on closed arm entries (F=0.16, P>0.05, one-way ANOVA), the total number of arm entries (F=2.4, P>0.05, one-way ANOVA), or on total time spent in the junction (F=0.21, P>0.05, one-way ANOVA) between open and closed arms.

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<th>Sham</th>
<th>1.5pmol</th>
<th>5.0pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Open-Arm Time (sec)</strong></td>
<td>7.47±3.72</td>
<td>46.09±8.3</td>
<td>104.8±16.86***,##</td>
</tr>
<tr>
<td>Closed-Arm Time (sec)</td>
<td>208.3±22.82</td>
<td>175.4±10.28</td>
<td>106.6±16.4***,##</td>
</tr>
<tr>
<td>Junction Time (sec)</td>
<td>84.3±23.3</td>
<td>78.6±5.5</td>
<td>88.5±7.5</td>
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<tr>
<td># Open-Arm Entries</td>
<td>0.8±0.4</td>
<td>2.7±0.8</td>
<td>6.9±0.9***,##</td>
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<tr>
<td>% Open-Arm Entries</td>
<td>7.2±3.0</td>
<td>31.4±8.1*</td>
<td>50.1±5.1***,##</td>
</tr>
<tr>
<td># Closed-Arm Entries</td>
<td>6.20±2.65</td>
<td>7.57±1.92</td>
<td>6.62±0.65</td>
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</table>

Table 4.1 Anxiolytic Effects of U99194 Microinjection in the Elevated-Plus Maze.

— Anxiety-like behavior was assayed for 300 seconds 5 minutes following a microinjection of either sham (aCSF, n=5), 1.5pmol (n=7), or 5.0pmol U99194 (n=8). Data are expressed as mean±SEM and were analyzed with a standard one-way ANOVA and Neuman-Kuels post-tests. #, ## – P<0.05, P<0.01 versus the 1.5pmol dose. *, **, *** – P<0.05, P<0.01, P<0.001 versus sham.

To examine the effects of U99194 microinjection on locomotor behavior, animals were tested in an open-field assay (Table 4.2). U99194 BLA did not alter the total number of moves (F=0.06, P>0.05, one-way ANOVA), the total movement distance (F=1.00, P>0.05), the amount of time spent moving (F=0.33, P>0.05), or the number of rears (F=1.53, P>0.05). Together, these data suggest that blockade of D3 receptors in the
BLA via a selective D3R antagonist can produce anxiolytic effects without significant alterations of locomotor-related behaviors.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>1.5pmol(^b)</th>
<th>5.0pmol U99194(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong># Moves</strong></td>
<td>26.0±3.4</td>
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<td>26.6±2.5</td>
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<tr>
<td><strong>Movement Time (sec)</strong></td>
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<td>254.0±9.5</td>
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<td><strong>Total Distance (cm)</strong></td>
<td>1171±114</td>
<td>1220±101</td>
<td>1345±57</td>
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<tr>
<td><strong>Rears</strong></td>
<td>21.4±5.0</td>
<td>19.8±2.6</td>
<td>29.1±3.4</td>
</tr>
</tbody>
</table>

Table 4.2 Locomotor Effects of U99194 Microinjection in an Open-field.

\(^a\) Locomotor behavior was measured for 300 seconds 5 minutes following a microinjection of either sham (aCSF, n=5), 1.5pmol (n=7), or 5.0pmol U99194 (n=8). Data are expressed as mean±SEM and were analyzed with a standard one-way ANOVA and Neuman-Kuels post-tests. \(^a\) – \(^b\) – No significant differences between groups.

**LPC and local GABAergic synapses can be functionally distinguished**

Recent data demonstrate that local feedback interneurons synapse on the soma and proximal dendritic compartments (McDonald & Betette, 2001; McDonald & Mascagni, 2001; Silberman et al., 2007) while LPC feed-forward interneurons synapse onto distal dendritic processes (Marowsky et al., 2005; Muller et al., 2007). To test whether these distinct anatomical arrangements are also functionally distinct, we preformed a collision experiment, taking advantage of the paired-pulse depression that occurs when paired-stimuli are delivered to GABAergic inputs using a long interpulse-interval (250msec in this case) (Silberman et al., 2007). When stimulating either local or LPC GABAergic synapses individually (Fig. 4.2A), we found a 20-30% inhibition of the second synaptic response relative to the first at both the local and LPC GABAergic synapses (Fig. 4.2B\(_1\) & 2C). Conversely, when stimulation of one GABAergic input was
Figure 4.2 BLA interneuron populations can be functionally distinguished. 
A) Schematic diagram of the BLA indicating the approximate anatomical relationships between stimulating (black bars) and recording electrodes (hollow pipette). B1) Here, L= local and EC = external capsule (LPC cell bodies). Sample traces illustrating paired-pulse depression (PPD) of IPSC amplitude (L2/L1 and EC2/EC1) during a paired stimulation of the same anatomical site using an inter-stimulus interval of 250 msec. Both local and LPC synapses were characterized by pronounced paired-pulse depression. B2) Sample traces showing the absence of any significant paired-pulse depression when both anatomical sites are stimulated consecutively with an inter-stimulus interval of 250 msec. Traces are from the same cell and exemplify the experimental approach of alternating stimulation sites to calculate the PPD ratio. C) Graphical summary of the percent suppression of the second response compared to the first response during the PP250 when a single anatomical site is stimulated (“Single-Site”) compared to sequential stimulation of the two different stimulation sites (“Mixed”). (% suppression of second response compared to first response: single-stimulations: EC2/EC1=34.58±5.73%, n=23; L2/L1=21.43±4.93%, n=18; mixed-stimulations: EC2/EC1=3.19±1.99%, n=3; L2/L1=-0.77±4.21%, n=3).

followed 250msec later by stimulation of the other input, we found no such paired-pulse depression (Fig. 4.2B2 and C). These data suggest that the mechanisms responsible for paired-pulse depression at each synapse are not engaged by sequential stimulation of the local and LPC GABAergic inputs. Thus, GABAergic synapses from the two interneuron
populations can therefore be functionally separated by placement of the stimulating electrode.

_D3 receptor mediated suppression of LPCs and local synapses_

Given the anxiolytic effects of U99194 when injected into the BLA, we examined the effects of D3R activation at local and LPC GABAergic synapses in the BLA. We specifically targeted GABAergic transmission because D3R activation reduces GABA-mediated currents in the nucleus accumbens (Chen _et al._, 2006) and in the hippocampus (Hammad & Wagner, 2006; Swant _et al._, 2008). Because we could functionally separate LPC and local synapses by stimulating in the EC and locally (see Fig. 4.3), we used the two stimulation sites for the remainder of the experiments. Electrically-evoked IPSCs from both synapses were significantly inhibited using PD128907 (10µM), a D3R-selective agonist (Audinot _et al._, 1998) (Fig. 4.3A and B – LPC; Fig. 4.3C and D – Local). Importantly, the effects of PD128907 at both synapses were blocked with 10µM U99149, the same D3-selective antagonist used in the behavioral assays (Fig. 4.3A and B – LPC, *--P<0.03, n=5; Fig. 4.3C and D – Local, *--P<0.04, n=7). These data confirm the presence of D3R in the BLA and suggest that tonic dopaminergic modulation of GABAergic transmission from LPCs and local interneurons is a possible physiological mechanism for the anxiolytic effects of U99194 microinjection.
Figure 4.3 D3R activation suppresses LPC and local interneuron GABA transmission. A) Sample traces showing the effects of the D3R-selective agonist PD128907 on LPC GABA-IPSCs (top). The D3R-selective antagonist U99194 fully blocked the inhibitory effect of PD128907 (bottom). B) Summary of PD128907 (65.98±16.85%, n=6) and PD128907+U99194 (92.10±6.54%, n=6) at LPC GABAergic IPSCs (*--P<0.05, t-test). C) Sample traces illustrating the effects of PD128907 on local GABA-IPSCs (top). U99194 completely suppressed the inhibitory effect of PD128907 (bottom). D) Summary of PD128907 (82.33%±4.79, n=6) and PD128907+U99194 (100.4±5.63%, n=7) at local GABAergic synapses (*-- P<0.05, t-test).
Dopamine modulation of local and LPC GABAergic synapses

The D3-mediated modulation of BLA GABAergic inputs raises the issue of how the native neurotransmitter might act at these synapses. At LPC synapses, 30nM DA significantly inhibited electrically-evoked GABAergic IPSCs (Fig. 4.4A, n=4). 10µM SCH23390, a D1-selective antagonist (n=9), did not reverse this DA-mediated suppression of LPC GABA-IPSCs. Similarly, the D3-selective antagonist U99194 (10µM, n=4) had no effect on the DA-mediated suppression of LPC GABAergic IPSCs. Interestingly, simultaneous D1 and D3R blockade by combining both SCH23390 and U99194 in the perfusate significantly attenuated the inhibitory effect of 30nM DA at LPC synapses (n=7; F=5.8, P<0.01, one-way ANOVA), suggesting that either D1-like or D3-like receptor activation by 30nM dopamine is sufficient to suppress LPC GABA-IPSCs.

At local GABAergic synapses, 30nM DA also suppressed evoked GABAergic IPSCs (Fig. 4.4B, n=6). Neither D1R-blockade with SCH23390 alone (10µM; n=5) nor D3R blockade with U99194 alone (10µM; n=7) had any significant effect on the DA-mediated inhibition of local GABA-IPSCs. Similar to LPC synapses, simultaneous blockade of both D1R and D3R attenuated the inhibitory effect of 30nM DA (n=6; F=3.2, P<0.05, one-way ANOVA), suggesting that either D1R or D3R activation by 30nM DA is sufficient to inhibit local GABAergic synapses.
Figure 4.4 D1R and D3R mediate the inhibition of evoked GABAergic transmission by low concentrations of DA.

A) Graphical representation of the effects of 30nM DA and various D1R and D3R antagonists on evoked GABAergic IPSCs from LPC synapses. 30nM DA substantially inhibits LPC GABA-IPSC amplitude (-28.29±5.02%, n=4). Neither D1R blockade alone (10µM SCH23390, -28.30±4.14%, n=9) nor D3R blockade alone (10µM U99194, -26.13±7.39%, n=4) could alter the inhibitory effects of 30nM DA. However, simultaneous D1R and D3R blockade (-6.66±2.95%, n=7) significantly attenuated the effects of 30nM DA on LPC GABA-IPSCs (*--P<0.01, One-way ANOVA, n=7).

B) Graphical representation of the effects of 30nM DA and various DA receptor antagonists on local interneuron evoked GABA-IPSCs. Neither D1R blockade alone (-41.73±7.95%, n=5) nor D3R blockade alone (-32.60±9.86%, n=7) could reduce the inhibitory effects of 30nM DA (-28.21±4.6%, n=6). Simultaneous D1R and D3R blockade significantly attenuated the inhibiting effects of 30nM DA on local GABA-IPSCs (-3.33±7.55%, n=5; *--P<0.05, One-way ANOVA).
To determine the synaptic location of the differential effects of DA on the GABAergic system, we examined action potential-dependent spontaneous GABAergic activity (sIPSCs). 30nM DA application significantly suppressed both sIPSC amplitude and inter-event interval (IEI, Fig. 4.5A). These data suggest that low DA concentrations inhibit spontaneous interneuron synaptic activity, potentially via a postsynaptic mechanism.

The effects of 30nM DA on sIPSCs and evoked IPSCs contrast with previous studies that have shown that higher concentrations of DA increase spontaneous activity at the local interneurons (Bissiere et al., 2003; Kroner et al., 2005; Marowsky et al., 2005). To confirm this, we measured the effects of 50μM DA on sIPSCs and also found a significant increase in sIPSC frequency (decreased inter-event interval) along with no effect on sIPSC amplitude (Fig. 4.5B). These data confirm that high concentrations of DA increase intrinsic interneuron activity. These data also suggest that DA can have qualitatively distinct effects on BLA GABAergic transmission that depend upon DA concentration.

To test whether the concentration-dependent effects of DA involve the differential activation of D1-like and D3 receptors, we measured sIPSCs in the presence of 30nM DA either with or without the D1R antagonist SCH23390 or the D3R antagonist U99194. While blocking D1R, we found that 30nM DA still reduced sIPSC frequency and
amplitude, similar to the effects seen with the low DA concentration alone (Fig. 4.6A).

In contrast, blockade of D3Rs with U99194 alone attenuated the 30nM DA-dependent

Figure 4.5 Concentration-dependent DA effects on spontaneous GABAergic transmission.
A) Low concentrations of DA inhibit spontaneous GABAergic IPSCs. Sample traces (A1) illustrate the effects of 30nM DA on GABAergic sIPSCs. 30nM DA significantly increased sIPSC inter-event interval (IEI, A2) and decreased sIPSC amplitude (A3; baseline IEI = 177.9±7.01msec, 30nM DA IEI = 208.4±11.95msec, baseline amplitude = 27.01±2.14pA, 30nM DA amplitude = 19.05±1.48pA, n=6, * -- P<0.05, ** -- P<0.01, t-test). B) High concentrations of DA facilitate spontaneous GABAergic IPSCs (B1). 50μM DA significantly decreased sIPSC inter-event-interval (B2; baseline IEI = 167.4±8.67msec, 50μM DA IEI = 150.7±10.73msec, B2, *--P<0.01, t-test, n=6) but had no effect on sIPSC amplitude (B3; baseline amplitude = 25.69±4.75pA, 50μM DA amplitude = 25.96±2.45pA, P>0.05).
Figure 4.6 DA facilitatory effects on spontaneous GABAergic transmission are D1R mediated.
A) D1R antagonism with SCH23390 during 30nM DA (A1) did not alter the increase in sIPSC inter-event interval (A2; baseline IEI = 96.62±14.61msec, SCH23390+30nM DA = 147.0±24.56msec, * -- P<0.05, t-test, n=5) or the decrease in sIPSC amplitude (A3; baseline amplitude = 12.90±0.94pA, SCH23390+30nM DA = 10.44±0.96pA, ** -- P<0.01, t-test). B) D1 antagonism during 50µM DA reverses the normal facilitation seen with this concentration (see Fig. 5) and produces profound inhibition of both sIPSC frequency (B2; baseline IEI = 61.84±5.15msec, SCH23390+50µM DA IEI = 100.5±10.70msec, n=6, * -- P<0.05, t-test) and amplitude (B3; baseline amplitude = 18.06±2.16pA, SCH23390+50µM DA amplitude = 12.70±1.51pA, * -- P<0.05, t-test).
decreases in sIPSC frequency and amplitude (Fig. 4.7A). This suggests that D3R, but not D1R, are preferentially activated by low DA concentrations and reduce GABAergic spontaneous transmission. In support of this, D3R activation with PD128907 also decreased the frequency and amplitude of sIPSCs (Fig. 4.7B).

Figure 4.7 DA inhibitory effects on spontaneous GABAergic transmission are D3R mediated.
A) The competitive D3R antagonist U99194 (A1) attenuates the inhibiting effect of 30nM DA on sIPSC frequency (A2; baseline = 66.39± 5.38msec, U99194+30nM DA = 62.45± 6.75, n=5, P>0.05) and amplitude (A3; baseline = 17.24± 3.74pA, U99194+30nM DA = 13.64± 0.89pA, P>0.05). B) The D3R-selective agonist PD128907 (B1) reduces sIPSC frequency (increased inter-event interval, B2; baseline = 92.7±14.00msec, PD128907 = 153.8±30.34msec, *--P<0.05, n=7) and reduced sIPSC amplitude (B3; baseline = 16.20±1.66pA, PD128907 = 12.09±1.19pA, *--P<0.02).
Using the same experimental strategy with 50μM DA, we found that D1R blockade with SCH23390 completely suppressed the facilitation of sIPSC frequency and amplitude (Fig. 4.6B) that is typical for this much higher concentration of DA (compare with Fig. 5B and (Kroner et al., 2005; Marowsky et al., 2005)). In fact, D1R blockade in conjunction with these higher dopamine concentrations caused a robust inhibition of sIPSC frequency and amplitude, similar to the low DA concentrations. Therefore, these data demonstrate that the concentration-dependent shift from inhibition of sIPSC at low DA concentrations to facilitation at higher DA concentrations is mediated by the differential activation of D3R and D1R, respectively.

Discussion

Our current work shows that BLA dopaminergic transmission plays a prominent role in modulating anxiety-like behaviors. Importantly, our electrophysiologic findings illustrate the potential physiologic mechanism by which dopamine influences these behaviors. Specifically, we show that microinjection of a D3R antagonist into the BLA decreases anxiety-like behavior measured in both the light/dark box and in the elevated plus maze. We subsequently show that D3R activation in vitro suppresses both feed-forward inhibition (LPCs) and feed-back inhibition (local interneurons) in the BLA. In addition to confirming previous findings showing D1R-mediated facilitation of local interneuron GABAergic function at high dopamine concentrations, we also found that
lower concentrations of DA can suppress GABAergic inhibition partially via a D3R-dependent mechanism.

D3Rs have been previously shown to modulate anxiety-related behaviors in other models. For example, Steiner et al. demonstrated that D3 knockout mice were less ‘anxious’ than wildtype controls and spent significantly more time in the open arms of an elevated plus maze and in the center of an open field apparatus (Steiner et al., 1997). In addition, Phillips and colleagues (Phillips et al., 2002a) showed that microinjections of the D3R antagonist nafadotride into the rat amygdala reduced taste-related associative learning in isolation reared animals. Our behavioral data complement and extend these findings specifically to BLA-mediated innate anxiety-like behaviors. Specifically, rats microinjected with low doses of U99194 spent significantly more time on the light side of a light/dark box compared with vehicle injected animals. Likewise, U99194-injected animals spent significantly more time on the open arms of an elevated plus maze than the closed arms compared with vehicle injected animals. These effects were dose-dependent in both assays. Importantly, the effects of U99194 on anxiety-like behavior were not due to significant alterations in locomotion, as there were no significant differences in movement time or distance measured in an open-field. Since U99194 has 14-fold higher affinity for D3R over D2R (Audinot et al., 1998), our data strongly suggests that BLA D3Rs play a significant role in modulating innate anxiety-like behaviors expressed in the light/dark box and the elevated plus maze.

Our data also suggests that modulation of BLA GABAergic transmission by D3Rs may be a primary mechanism by which dopamine exerts anxiety-related effects. The GABA system in the BLA is comprised of at least two populations of interneurons.
that differ with respect to their forms of inhibition as well as their neuroanatomical placements (McDonald & Betette, 2001; Marowsky et al., 2005; McDonald et al., 2005; Muller et al., 2007). We and others have now shown that DAergic innervation controls the function of each population. In particular, D3R activation robustly inhibits GABAergic transmission in BLA from both feedforward LPC and feedback local interneurons, consistent with the anxiolytic-like effects of the D3R antagonist U99194 microinjected into the BLA. Interestingly, D3R can also suppress GABAergic inhibition in the nucleus accumbens (Chen et al., 2006) and in the hippocampus (Hammad & Wagner, 2006; Swant et al., 2008), suggesting a commonality for D3Rs in the brain regions associated with reward or emotional processes. Our data also suggest that the DA projections to the BLA are active during behavioral tests like the light/dark box and elevated plus maze. Therefore, these findings suggest that targeting D3Rs might be extremely beneficial in treating anxiety-related disorders.

Although D3Rs play a major role in suppressing GABAergic transmission in the BLA, D1Rs also modulate GABAergic transmission in this brain region. Previous results have shown that D1Rs can facilitate GABAergic transmission in the BLA (Rosenkranz & Grace, 1999; Kroner et al., 2005; Marowsky et al., 2005). Interestingly, as we have now shown in the current study, this facilitation occurs at high concentrations of DA. In the current study, low concentrations of DA appear to have the opposite effect and inhibit evoked BLA GABAergic transmission via a mechanism that requires either D1- or D3-receptors. The precise mechanism by which different concentrations of DA cause qualitatively different D1-dependent effects on BLA GABAergic transmission is not completely clear. One possible explanation is that different DA concentrations may
activate distinct types of D1-like, SCH23390-sensitive receptors. For example, D5 receptors (D5R) are also expressed within the rat BLA (Ciliax et al., 2000). These receptors have a higher affinity for DA compared to D1Rs (Tupala & Tiihonen, 2004). Therefore, D5R may be preferentially activated at low DA concentrations while high concentrations activate a more dominant D1R-mediated effect. Interestingly, it has been shown that microinjections of SCH23390 into the BLA elicit anxiolytic-like effects in rat (de la Mora et al., 2005). Since increased BLA GABA function is associated with decreased anxiety-like behavior (Davis et al., 1994), these SCH23390 behavioral data suggest that the D1R-like suppression of the GABA system at low DA concentrations may be more behaviorally relevant than the facilitation of GABAergic transmission evident at higher DA concentrations.

Although the GABA system is a primary target for DA in the BLA, the precise cellular mechanism of action of these effects remains unclear. The facilitation of GABAergic function at higher DA concentrations does not involve a change in GABA release probability (data not shown (Kroner et al., 2005). However, we found that a high DA concentration increased the frequency of action potential-dependent spontaneous activity specifically through D1-like receptors, similar to previous reports (Kroner et al., 2005). Thus, high DA concentrations may increase the intrinsic excitability of GABAergic interneurons. In the prefrontal cortex, D1-like receptors enhance IPSCs via increased cAMP and subsequent PKA activation (Trantham-Davidson et al., 2004). It has also been reported that intracellular Ca^{2+} levels change with DA receptor activation (Bergson et al., 2003; Hernandez-Lopez et al., 2000; Lee et al., 2004; Trantham-Davidson et al., 2004). Additional studies have shown that D1 receptor-mediated signaling in the
amygdala is independent of cAMP (Leonard et al., 2003), PLC (Undie et al., 1994) and calcyon activation (Bergson et al., 2003), but can involve activation of Src-like protein tyrosine kinases (Loretan et al., 2004). However, we did not investigate any other possible cellular mechanisms. Thus, both cAMP/PKA- and tyrosine kinase-dependent signaling remain potential candidates for mechanisms increasing spontaneous activity of GABAergic interneurons during exposure to high DA concentrations.

The cellular mechanisms mediating the inhibitory effects of low DA concentrations and a D3-selective agonist on BLA GABAergic transmission are also not clear. In the nucleus accumbens (NAcc), D3 receptor signaling involves a PKA-dependent mechanism resulting in increased endocytosis of GABA receptors and suppressed GABAergic function (Chen et al., 2006). In a separate study using a heterologous cell expression system, D3 receptor activation modulated adenylyl cyclase and induced mitogenesis possibly via changes in tyrosine phosphorylation (Griffon et al., 1997). Thus, both the PKA- and tyrosine kinase-pathways are possible mechanisms related to the D3R-dependent inhibition of BLA GABAergic transmission. However, we found that D3R activation altered sIPSC amplitude as well as frequency. This supports the hypothesis that BLA D3Rs may function postsynaptically.

It is also important to note that both interneuron populations can synapse onto the same principal neuron. This makes it difficult to distinguish spontaneous IPSC events arising from these distinct GABAergic synapses. However, in the current study we observed that 50μM DA increased spontaneous GABAergic activity. Consistent with our data, previous studies have shown that high concentrations of DA also increase local interneuron firing (Kroner et al., 2005; Marowsky et al., 2005). These data suggests that
spontaneous GABAergic activity detected under our recording conditions arises primarily from local synapses within the BLA.

Studies have shown that stressful or predictive environmental stimuli can dynamically regulate the concentration of DA in both the BLA (Herman et al., 1982; Coco et al., 1992; Harmer & Phillips, 1999; Inglis & Moghaddam, 1999) and in medial prefrontal cortex (Diamond & Weinberger, 1986; Rosenkranz & Grace, 2001; 2003). These fluxes in DA concentration can alter the expression of specific DA receptor-mediated behaviors as DA receptors demonstrate varying affinities for the endogenous agonist. In support of this hypothesis, there is also a concentration-dependent DA modulation of the inhibitory GABA system in the prefrontal cortex (Trantham-Davidson et al., 2004). Within the context of the BLA, cortical projections synapse onto LPCs; and GABAergic inputs arising from these cells are suppressed by both D1R-like and D3R activation. This DA-dependent suppression of LPCs would presumably reduce feed-forward inhibition of the BLA. Based on our findings and recent neuroanatomical evidence (Pinto & Sesack, 2008), D3 receptors may contribute to these processes. This robust, multi-faceted suppression of feed-forward inhibition paired with a very tightly controlled feedback system via D1R-like facilitation/inhibition (our study and (Kroner et al., 2005; Marowsky et al., 2005)) may ultimately modulate the spatial and temporal character of BLA output during an environmental stimulus. Furthermore, changes in the relative expression of these receptors may contribute to the pathophysiology of different disorders, such as drug abuse (Follesa et al., 2005; Guitart-Masip et al., 2006; Vengeliene et al., 2006; Di Ciano, 2008).
In summary, we show that the relative activity of the DA receptors at local feed-back and feed-forward LPC GABAergic synapses can regulate the inhibitory control over BLA principal neurons. Overall, this dopaminergic modulation has the potential to tightly regulate the output from the BLA to the down-stream targets and ultimately alter the expression of anxiety-like behaviors.

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References


5. Chronic Ethanol and Withdrawal Differentially Alter Dopaminergic Modulation of GABAergic Transmission in the Rat Basolateral Amygdala

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The following manuscript encompasses preliminary findings that are not yet ready for publication. Stylistic variations represent requirements for the European Journal of Neuroscience. M. Diaz preformed all experiments. M. Diaz prepared the manuscript. Dr. Brian McCool acted in an advisory and editorial capacity.
Abstract

Chronic ethanol exposure and withdrawal have been shown to significantly alter synaptic transmission within the basolateral amygdala (BLA). In particular, decreases in GABAergic function and increases in glutamatergic function can play a significant role in withdrawal-induced anxiety-like behavior. Previous findings have shown that the DA system within the BLA differentially modulates GABAergic transmission from two interneuron populations (local feedback and feedforward LPC interneurons) in young, alcohol-naïve rats, ultimately regulating BLA-mediated behaviors. Therefore, in this study we examined the effects chronic intermittent ethanol (CIE) and withdrawal on DA modulation of GABAergic transmission in adolescent/young adult rats. Using whole-cell patch clamp electrophysiology, we found an age-dependent difference in this DAergic-GABAergic interaction at local synapses, with dopamine facilitating GABAergic function in younger animals and inhibiting GABAergic function in BLA slices from older individuals. Chronic ethanol inhalation reversed DA-mediated GABAergic inhibition in older animals, leading to a D1R-mediated facilitation of local GABAergic transmission. Interestingly, following 24 hours of withdrawal, this ethanol-dependent reversal of DA modulation appears to be returning toward control levels. Therefore, this study demonstrates that the dopamine system undergoes developmental changes between juvenile (3-5 weeks) to adolescent (7-8 weeks) age. Furthermore, these changes are dynamically sensitive to chronic ethanol exposure, as the removal of ethanol during withdrawal leads to a re-establishment of the native function of this system. Taken together, this study reveals the effects of CIE and WD on the interaction between the DA
and GABA system within the BLA that may ultimately contribute to chronic ethanol- and withdrawal-related anxiety-like behavior.

**Introduction**

Alcoholism is a major issue in society that affects many people. These societal issues are cost-related, can ruin families, and can have detrimental effects on health (Grant, 2004). In addition, abstaining from chronic alcohol use can lead to severe withdrawal-induced anxiety that often results in relapse (Becker, 2000; Breese et al., 2005). Interestingly, increases in anxiety-like behavior can also occur in rodents exposed to chronic ethanol and subsequent withdrawal (Becker & Hale, 1993; Becker, 2000; Cagetti et al., 2003), providing an animal model with good predictive validity for the symptoms. Therefore, due to the negative effects of chronic alcohol and withdrawal in rodents, as well as humans (Koob & Le Moal, 2001; Koob, 2008), it is important to understand the brain region associated with anxiety.

The amygdala is the emotional center of the brain that has been shown to be associated with the anxiety experienced during withdrawal (WD) from chronic alcohol use. Specifically, the inhibitory GABAergic system within the basolateral amygdala (BLA), the primary input into the anxiety circuit, undergoes significant adaptations following chronic exposure to ethanol and subsequent WD. Interestingly, GABAergic transmission in the BLA consists of both feed-back and feed-forward inhibitory mechanisms via paracapsular GABAergic interneurons (LPCs) and local interneurons, respectively (Marowsky et al., 2005; Silberman et al., 2007). It has been shown that feedback GABAergic inhibition is reduced at 3 days of WD from chronic ethanol.
exposure (Isoardi et al., 2007), suggesting that chronic ethanol and withdrawal differentially alter GABAergic transmission within the BLA. More recently, we have found that chronic intermittent ethanol (CIE) and 24 hours of withdrawal reduces release probability from feed-forward LPCs. CIE and WD did not alter local synaptic release (Diaz, In preparation). Based on these findings it is clear that much remains to be known about the effects of chronic ethanol and WD on these two GABAergic populations. Yet, it is possible that changes in modulatory systems may be associated with changes of GABAergic transmission.

Dopamine (DA) can modulate GABAergic transmission within the BLA. Specifically, DA can facilitate local interneuron excitability (Rosenkranz & Grace, 1999; Kroner et al., 2005) and suppress GABAergic transmission from LPCs via D1R activation (Marowsky et al., 2005). This bi-directional modulation of GABAergic transmission by DA has been suggested to result in changes in anxiety-like behavior, given that DA antagonists can block the expression of fear conditioning (Lamont & Kokkinidis, 1998; Greba & Kokkinidis, 2000) as well as reduce anxiety-like behavior (de la Mora et al., 2005). Given the role of DA in shaping BLA-mediated behaviors inhibitory processes and the chronic ethanol exposure and WD alteration of GABAergic transmission, we hypothesize that chronic intermittent ethanol (CIE) and WD will alter the DA system resulting in the observed changes in GABAergic transmission.

We previously showed DA modulation of GABAergic transmission from LPCs and local interneurons occurred in a concentration-dependent manner in young animals (Diaz, Submitted). Specifically, DA suppresses LPC GABAergic transmission via D3R and D1R activation in a concentration-independent manner. In contrast, low
concentrations of DA also suppress local GABAergic transmission via D3 and D1Rs while high concentrations of DA potentiate these same interneurons exclusively via D1Rs. This latter finding is consistent with previous reports using higher DA concentrations (Rosenkranz & Grace, 1999; Kroner et al., 2005). In the current study, we examine the effects of CIE and WD on the bi-directional modulation of dopamine on GABAergic transmission at LPC and local synapses.

**Methods**

*Animals:* All animal procedures were performed in accordance with protocols approved by Wake Forest University School of Medicine Animal Care and Use Committee and were consistent with the National Institutes of Health animal care and use policy. Male Sprague–Dawley rats were obtained from Harlan (Indianapolis, IN) and were housed in an animal care facility at 23°C with a 12-h light/dark cycle and given food and water without restriction. Rats were weighed daily to ensure that ≥80% of their free-feeding weight was maintained during vapor chamber ethanol exposure.

*Chronic Ethanol Exposure:* Ethanol exposure was accomplished via an ethanol vapor chamber as previously described (Lack et al., 2007; Lack et al., 2009). Briefly, male Sprague Dawley rats (~120-150 grams) were housed four to a cage in large, standard polycarbonate cages. Cages were placed in large, custom-built Plexiglas chambers (Triad Plastics, Winston-Salem, NC) that were identical to those previously described (Lopez & Becker, 2005) at the beginning of the light cycle (lights on at 9 pm EST). Animals were exposed to either ethanol vapor or room air for 12 hours during the
light cycle for 10 days. During the dark cycle the chambers were opened and aired out. The concentration of vapor in the ethanol chamber was tested daily. Blood was collected on the day of sacrifice for subjects in the chronic intermittent ethanol (CIE) group and blood ethanol levels were 194±11mg/dl. Withdrawal (WD) animals remained in the ethanol chamber for 24 hours after the last ethanol exposure.

Slice Preparation: Amygdala slices were prepared from control, CIE, and WD animals. Animals were anesthetized with isoflurane (3%) and decapitated. The brains were quickly removed and incubated in ice-cold sucrose/artificial CSF (aCSF) equilibrated with 95% O₂ and 5% CO₂ containing (in mM): 180 sucrose, 30 NaCl, 4.5 KCl, 1 MgCl₂·6H₂O, 26 NaHCO₃, 1.2 NaH₂PO₄, 0.1 ketamine, and 10 glucose. Coronal brain slices (400µm) were prepared using a Vibratome Series 3000 (Vibratome, St. Louis, MO) and submerged in room-temperature (~25°C), oxygenated standard aCSF containing (in mM): 126 NaCl, 3 KCl, 1.25 NaH₂PO₄, 2 MgSO₄, 26 NaHCO₃, 10 glucose, and 2 CaCl₂·2H₂O. Slices were maintained in aCSF for ~1 hour before recording. All experiments were performed 1-4 hours after preparation of the BLA slices. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Whole-cell patch-clamp electrophysiology: Methods used for whole-cell patch-clamp electrophysiology were similar to those previously described (Lack et al., 2007). Slices were placed in a recording chamber that was perfused with room temperature aCSF at a rate of 2mL/min. Electrodes were filled with an internal solution containing (in mM): 122 Cs-gluconate, 10 CsCl, 10 HEPES, 1 EGTA, 5 NaCl, 0.1 CaCl₂, 4 Mg-ATP, 0.3 Na-GTP, and 2 QX314-(Cl) and had input-resistances of 8-12 MΩ. Data were acquired with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) and
analyzed using Clampex software (Axon Instruments, Foster City, CA). Evoked GABA-IPSCs were pharmacologically isolated with 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX, 20μM), a non-NMDA receptor antagonist, and DL-2-amino-5-phosphono-pentanoic acid (APV, 50μM), an NMDA receptor antagonist and recorded from principal neurons. Principal neurons were identified as having an initial access resistance of <50 MΩ (Lack et al., 2007), and were only included in the study if access resistances and baseline holding currents changed <20%.

GABA synaptic release was electrically stimulated using a range of intensities to achieve an average synaptic response of ~100pA. Minimal stimulation intensities were used at both stimulation sites that ranged in order to achieve a GABAergic response of ~100pA, less than <20% of electrically-evoked maximum responses (data not shown). We placed the stimulating electrode either in the external capsule (to activate LPCs) or medial to the recording electrode (to activate local interneurons) as specified within each experiment. We have shown previously that GABAergic IPSCs evoked from these two locations are functionally distinct from one another (Diaz, Submitted). For specific DA receptor activation, slices were perfused with the D1-like receptor antagonist SCH23390 (Sigma-Aldrich) prior to application of DA. For some studies, we used the selective D3R agonist PD128907 (Tocris Bioscience).

Statistics: Electrically-evoked IPSC data were analyzed with paired t-tests and one-way ANOVA as appropriate within each experiment with a P<0.05 considered statistically significant. Dunnett’s post-hoc analysis was used with P<0.05.
Results

*Dopamine modulation of GABAergic transmission is age-dependent*

It has been shown that the development of the DA system within the BLA of rodents is not complete until postnatal day (P) 30 (Brummelte & Teuchert-Noodt, 2006). Given that our previous characterization of the effects of DA on GABAergic transmission was during this period (P28-35 or 4-5 weeks old), we investigated whether effects of DA on GABAergic transmission elicited similar effects in alcohol-naïve, CON young adult rats (P49-56 or 7-8 weeks old). In P28-35 animals, a high concentration of DA (50µM) potentiated local GABA-IPSCs and suppressed GABA-IPSCs from LPCs as previously reported (Diaz, Submitted). In contrast, in P49-56 animals DA (50µM) no longer elicits a potentiation of local GABA-IPSCs (Fig. 5.1A) but suppressed GABA-IPSCs from both local (78.11±5.16% from baseline, n=5) and LPC synapses (50.06±9.21% from baseline, n=3). This inhibitory effect of 50µM DA on local GABAergic transmission is similar to the effect of a low DA concentration (30nM) in young (P28-35) animals. These data suggest that the DA system within the BLA, in particular DA modulation of GABAergic transmission, undergoes age-dependent changes between juvenile (3-5 weeks old) and adolescence/young adulthood (7-8 weeks old).
Figure 5.1 Dopamine modulation of GABAergic transmission is age-dependent. DA (50µM) bi-directionally modulates GABAergic transmission within the BLA of young (juvenile) alcohol-naïve animals (suppression of LPC GABA-IPSCs and facilitation of local GABA-IPSCs). In contrast, DA (50µM) suppresses GABAergic transmission at both LPC and local synapses in adolescent alcohol-naïve animals. Exemplar traces represent effects of DA (50µM) at both synapses (LPC and local) in young and adolescent animals.
In our previous characterization of DA modulation of GABAergic transmission, we determined that D3Rs predominantly mediate the inhibitory effect of DA. Therefore, we examined whether D3Rs also mediated the inhibitory effect of DA in adolescent/young adult rats. Similar to what we found in young animals, the selective D3R agonist PD128907 (Audinot et al., 1998) significantly suppressed GABAergic transmission from both local interneuron (-16.58± 9.69%, n=3) and LPC synapses (-17.13%, n=1). These data may suggest that D3Rs help mediate the inhibitory effect of DA at both GABAergic populations in adolescent/young adult rats.

**Chronic Ethanol and Withdrawal Reverse Age-related Changes in DA Modulation of Local Synapses**

After establishing the age-dependent shift of the DA-GABA interaction within the BLA, we examined the effects of chronic ethanol exposure and withdrawal on the DA modulation of GABAergic transmission in P49-56 rats. After 10 days of CIE, perfusion of 50µM DA on BLA slices potentiated GABA-IPSCs from local synapses (Fig. 5.2: CON = -17.42±6.14%, n=6; CIE = +17.62±10.86%, n=10, *--P<0.05, one-way-ANOVA with Dunnett’s post-hoc test). In contrast, CIE did not alter the inhibitory effect of 50µM DA on GABA-IPSCs from LPCs (Fig. 5.2: CON = -45.96±6.98%, n=5; CIE = -32.94±4.75%, n=6, P>0.05). These CIE-induced adaptations in DA signaling at local GABAergic synapses were unexpected given that the majority of the direct effects of CIE on GABAergic transmission occur at LPC synapses (Diaz, In preparation). Interestingly, the effects of CIE on DA modulation of local GABAergic transmission appear to begin to diminish by 24 hours of withdrawal (+8.06±9.41%, n=7). Furthermore, withdrawal had
Figure 5.2 Chronic ethanol and withdrawal reverse the effect of DA at local synapses in adolescent animals.

The inhibitory effect of DA on local GABA-IPSCs is reversed to a facilitatory effect in slices from CIE-treated animals (*--P<0.05). This reversal appears to be returning to CON levels by 24 hours of withdrawal (no significance). The effect of DA on LPC GABA-IPSCs remains unaffected in slices from CIE- and withdrawal-treated animals. Exemplar traces demonstrate the effect of DA (50µM) at both synapses across the three treatment groups (CON, CIE, and WD).
no additional effect on DA modulation of LPC GABAergic-IPSCs (-29.29±6.68%, n=9, P>0.05). These data show that CIE reverses modulation of GABAergic transmission at local synapses and suggest that bi-directional modulation of GABAergic transmission by DA may be re-acquired during chronic ethanol exposure.

D1R-mediated Effect of DA at Local Synapses during Chronic Ethanol

As we previously characterized the pharmacology of the effects of DA on GABAergic transmission, we wanted to determine which DA receptor was mediating the facilitatory effect of a high DA concentration on GABAergic transmission from local interneurons in P49-56 rats. We (Diaz, Submitted), and others (Rosenkranz & Grace, 2001; Kroner et al., 2005; Marowsky et al., 2005), have shown that D1Rs can increase local interneuron excitability in much younger/juvenile animals (P21-35) resulting in a potentiation of GABA-IPSCs. Consistent with these previous findings, D1R blockade attenuated the facilitatory effect of DA (50µM) at local synapses from CIE-treated slices (Fig. 5.3A). Although we did not measure whether SCH23390 alone altered basal GABA-IPSCs, in CIE slices pre-treated with the D1R antagonist SCH23390, 50µM DA suppressed GABA-IPSCs from local synapses (-28.53±10.77%, n=3, **-- P<0.05 compared to DA alone, 2-way ANOVA) to the same extent as 50µM DA suppressed GABA-IPSCs in SCH23390-treated CON slices (-41.87± 8.85, n=4). Likewise, after 24 hours of withdrawal, the slight facilitatory effect of 50µM DA at local synapses was completely blocked by the D1R antagonist (DA = +8.06±9.41%, n=7; DA+SCH = -31.80±7.36%, n=5, **--P<0.05, 2-way ANOVA). In contrast, the inhibitory effect of
50µM DA on LPC GABA-IPSCs was not altered by D1R blockade at either treatment

Figure 5.3 D1Rs mediate the facilitatory effect of DA during CIE and WD at local synapses.  
(A) D1R blockade reveals an inhibitory effect of DA at local synapses in slices from CIE and WD animals.  
(B) D1R blockade does not alter the inhibitory effect of DA at LPC synapses in any of the treatment groups.
group (Fig. 5.3B: CON = -36.18±10.75%, n=4; CIE = -62.17±5.98%, n=5; WD = -46.90±6.26%, n=3; P<0.05). Taken together, these data suggest that the exposure to chronic ethanol reverses the effect of DA on GABAergic transmission only at local synapses via increases in facilitatory D1R-function or decreases in an inhibitory DA receptor.

Discussion

Dopamine has been shown to regulate the gating of information flow through the BLA by modulating GABAergic transmission. Given that chronic exposure to ethanol and withdrawal alter BLA excitability (Lack et al., 2009), resulting in withdrawal-induced anxiety-like behavior (McCool et al., 2003; Lack et al., 2007), we hypothesized that CIE and WD would alter DA modulation of GABAergic transmission. We have previously shown in juvenile animals that DA can either suppress or facilitate GABAergic transmission in a concentration-dependent manner. This bi-directional effect is likely mediated by differential activation of D3Rs (inhibitory) and D1-like receptors (facilitatory and inhibitory) (Diaz, Submitted). In the current study, we found that the facilitatory effect of DA on local GABAergic synapses does not exist in adolescent/young adult rats. Furthermore, this age-dependent shift in the BLA DA system is reversed by CIE and appears to return toward control levels by 24 hours of withdrawal. Finally, the change in DA modulation of GABAergic transmission at local synapses is D1R-mediated suggesting that exposure to chronic ethanol alters D1R-function within the BLA.
The DA system within the BLA undergoes age-dependent changes from juvenile animals (P28-35) to adolescence/young adulthood (P49-56). This “delayed” maturation of the DA system within the BLA switches a bi-directional effect of DA on different GABAergic synapses into a uni-directional effect across all interneuron populations. Interestingly, only DA modulation of local feed-back GABAergic transmission is altered during development. In young/juvenile rats, high concentrations of DA potentiate local interneuron excitability (Rosenkranz & Grace, 1999; 2001; Kroner et al., 2005; Marowsky et al., 2005; Diaz, Submitted) and suppress LPC GABAergic transmission (Marowsky et al., 2005). The facilitatory effect of DA on local interneurons is replaced by an inhibitory response in adolescent/young adult rats. Thus, DA appears to only suppress GABAergic transmission within the BLA in these older animals regardless of the interneuron population. These findings were particularly interesting given that the difference in age between young alcohol-naïve rats and older control (CON) rats was only 2-3 weeks. Previous reports have shown that the DA system within the BLA undergoes significant development between P14 and P30 (Brummelte & Teuchert-Noodt, 2006). Specifically, there is a significant increase in the quantity of DA fibers during this time. Therefore, it is possible that the increase in DA fibers may result in changes in receptor expression resulting in the age-dependent switch.

The dopamine system can modulate plasticity processes within the amygdala. It has been shown that DA suppression of feed-forward GABAergic transmission can facilitate the induction of long-term potentiation (LTP) within the lateral amygdala via D2R activation (Bissiere et al., 2003). In addition, that same study found that DA, via D1Rs, can facilitate spontaneous GABAergic transmission at the same synapses without
influencing LTP induction. Importantly, this study demonstrated a bi-directional effect of DA similar to that found in the current study. This DA-gating of LTP may be associated with DA-mediated modulation of fear conditioning, an in vivo model that has been shown to involve plastic changes within the amygdala. Specifically, DA antagonists alter the acquisition and expression of fear conditioning across several different behavioral paradigms (Guarraci et al., 1999; Greba & Kokkinidis, 2000; Guarraci et al., 2000; Greba et al., 2001; Rosenkranz & Grace, 2002). Based on the data from the current study we suggest that this DA-dependent plasticity may be necessary at a young age when new fear and anxiety behaviors are learned for survival purposes. Our data also suggest that the BLA may again become plastic under certain exposures (this case chronic ethanol exposure) that lead to changes in DAergic modulation of GABAergic transmission.

Chronic exposure to ethanol reverses the age-related DA modulation of GABA-IPSCs at local synapses. Specifically, following CIE, a high concentration of DA potentiates local GABA-IPSCs. This effect is similar to the effect of high DA concentration in young animals (Diaz, Submitted). Interestingly, by 24 hours of withdrawal, this facilitatory effect of DA on local GABAergic transmission is diminished. In contrast, the DA modulation of LPC GABAergic transmission is not affected by CIE or withdrawal. The current study demonstrated a bi-directional effect of DA at the two populations in juvenile animals that was re-established following chronic ethanol exposure in young adult animals. A similar bi-directional effect has been shown to mediate the induction of LTP within the amygdala (Bissiere et al., 2003). Therefore, it is possible that the re-occurrence of this bi-directional effect during CIE may be associated with a DA-dependent induction of LTP within the amygdala. It is important to
note that this facilitatory effect may not be the same as in young animals, but may in fact be an effect of CIE via a different signaling pathway. Interestingly, changes in the glutamate system during CIE and WD suggest that processes similar to those involved with the initiation and expression of LTP (increased NMDA receptor and AMPA receptor function (McKernan & Shinnick-Gallagher, 1997)) occur in the BLA (Lack et al., 2007; Lack et al., 2009). Given this significant CIE-induced alteration of DA-mediated modulation of GABAergic transmission within the BLA, determining the receptors that are involved in these processes may be beneficial in treatment development for alcoholism.

D1 receptors mediate the facilitatory effect of DA at local synapses. Specifically, we found that D1R blockade attenuated the DA-mediated potentiation of local GABA-IPSCs in CIE-treated slices. In fact, while D1Rs were blocked, DA suppressed local GABA-IPSCs in CIE and WD slices to the same extent as CON slices. In contrast, D1R blockade had no effect on DA modulation of LPCs. These data suggest an increase in D1R function during CIE and WD solely in association with local GABAergic synapses. However, it has been shown that chronic ethanol exposure increases D1R binding densities in the amygdala (Sari et al., 2006). In addition, the affinity (K_d) of SCH23390 for the D1-like receptor increases during withdrawal from chronic ethanol exposure in the striatum (Bailey et al., 2001). Studies of other drugs of abuse (i.e. cocaine) have also shown that D1Rs within the BLA play a major role in mediating the responding behavior for cocaine (See et al., 2001). Importantly, we have previously demonstrated that the D1R-mediated facilitatory effect on local GABAergic transmission was dependent on high concentrations of DA (Diaz, Submitted). Ethanol exposure has been shown to
significantly increase DA levels, while withdrawal from chronic ethanol decreases DA levels (reviewed in (Grace, 2000)), suggesting that during chronic ethanol exposure DA levels would be elevated. Therefore, it is reasonable to suggest that DA release was increased at local synapses during CIE in turn facilitating the activation of D1Rs at these synapses. Based on these previous reports and the findings from the current study, it is reasonable to suggest that D1Rs play a significant role in altering BLA GABAergic transmission, leading to withdrawal-induced anxiety-like behavior (Cagetti et al., 2003; Lack et al., 2007). Furthermore, these findings suggest a potential role for the use of D1-selective agonists as treatments for withdrawal-induced anxiety. Although D1R function changes in response to CIE and WD, a DA-mediated suppression of GABAergic transmission is maintained at LPC synapses.

We found that DA inhibits GABA-IPSCs from feed-forward LPCs during CIE and WD. Although we did not pharmacologically examine whether this suppression was mediated by D3Rs (i.e. using a D3R antagonist), we did find that this suppression was D3R-mediated in CON animals, similar to juvenile animals. Furthermore, we confirmed that this inhibitory effect was not D1R-dependent, given that D1R blockade did not alter the DA-mediated suppression of GABA-IPSCs. These data may suggest that D3Rs continue to reduce feed-forward inhibition during CIE and subsequent WD. Given that D3R activation reduces feed-forward inhibition in alcohol-naïve animals, which likely leads to increases in anxiety-like behavior (Diaz, Submitted), the current study suggests that D3R function may be enhanced in older animals. This would ultimately result in the decrease in feed-forward inhibition that takes place during withdrawal (Diaz, In preparation). Interestingly, D3Rs have recently received much interest in the alcohol field.
(reviewed in: (Le Foll et al., 2005; Vengeliene et al., 2006)). Specifically, D3Rs have been shown to be involved in conditioned-place preference for ethanol (Boyce & Risinger, 2000; 2002), operant self-administration and re-instatement of alcohol seeking (Heidbreder et al., 2007), and alcohol craving (Vengeliene et al., 2006). Based on the important role of D3Rs in various aspects of alcoholism, D3R antagonists could potentially be a powerful tool for treating withdrawal-induced anxiety following chronic ethanol exposure.

This study demonstrates that the development of the DA system within the BLA alters the role of this DA system. Furthermore, exposure to chronic ethanol and subsequent withdrawal significantly alter the mechanisms by which DA modulates GABAergic transmission within the BLA. These neuroadaptations ultimately influence the BLA output, resulting in alcohol-withdrawal induced anxiety.
References


Chapter 2 Summary

In Chapter 2 we demonstrated that withdrawal-induced anxiety-like behavior was dependent on the BLA glutamate system. Specifically, microinjections of an AMPA/kainate receptor antagonist into the BLA significantly attenuated increases in anxiety-like behavior in withdrawal animals. These studies demonstrate a role for the BLA in alcohol withdrawal; in particular anxiety-like behavior experienced upon removal of alcohol implicated the glutamatergic system as a key mediator for the observed changes in behavior.

Despite unaltered anxiety-like behavior observed in CIE animals, we found pre- and post-synaptic increases in glutamatergic function following exposure to chronic ethanol. First, NMDAR function was significantly elevated in CIE-treated slices across several measures. These data were consistent with increases in NMDAR function previously shown in our lab in rats given a liquid ethanol diet (McCool et al., 2003). Furthermore, this increase in NMDAR function was paired with an increase in AMPAR function, indicated by altered spontaneous EPSCs. In fact, both amplitude and frequency of sEPSCs were potentiated in the BLA of CIE animals. While it is possible that these changes were a result of postsynaptic AMPAR alterations, it is possible that the increases in amplitude could be an additive result of a higher frequency. This summation effect
would indicate a presynaptic phenomenon. Consistent with a presynaptic effect, we found an increase in release probability from glutamatergic principal neurons. However, we did not find a change in TTX-insensitive spontaneous activity (mEPSCs), ruling out a terminal-specific change. This increase in presynaptic glutamate release ultimately increased glutamatergic function in the BLA of CIE animals.

Similar to CIE, animals also expressed increased glutamatergic function at 24 hours of withdrawal. NMDAR function remained elevated during withdrawal. In contrast to CIE, AMPAR function was directly potentiated during withdrawal evidenced by a robust increase in mEPSC amplitude and decay. Although these data do not necessarily represent increases in receptor numbers, they do suggest that adaptations of AMPARs have taken place, which resulted in an increase in glutamatergic function. Moreover, glutamate release continued to be facilitated during withdrawal, as measured by an increase in release probability. Interestingly, unlike with CIE, mEPSC frequency increased with withdrawal suggesting terminal-specific adaptations at glutamatergic synapses.

Regardless of the exact mechanisms responsible for facilitation of glutamate function, our electrophysiological findings during CIE were not consistent with a lack of change in anxiety-like behavior observed in CIE-treated animals. There may be several reasons for these inconsistencies, such as CIE-induced changes in other neurotransmitter systems (i.e. GABA) that may function to buffer the increases in glutamate. Therefore, it will be important to determine the effects of CIE and WD on the GABA system within the BLA.
Chapter 3 Summary

Chapter 3 described the neuroadaptations that occurred in two of the BLA GABAergic interneuron populations during chronic ethanol exposure and withdrawal. Using electrophysiology we found presynaptic decreases in GABAergic function in slices from WD-treated animals. In particular, there was a significant decrease in release probability at lateral paracapsular cells (LPC) following 24 hours of withdrawal from chronic ethanol, which suggests a marked suppression of GABA release from LPCs. These data also indicate that feed-forward inhibition is reduced during withdrawal. This suggests a potential contribution to the observed increases in glutamatergic transmission in addition to changes in anxiety-like behavior found in chapter 2. Consistent with a lack of anxiety-like behavior in chronic ethanol treated animals, we found that neither feed-forward LPC nor local feed-back GABAergic inhibition were affected by CIE. Importantly, these data suggest that the GABAergic system within the BLA can sufficiently suppress the elevated glutamatergic system reported in chapter 2.

As common in other systems, presynaptic changes ultimately result in postsynaptic modifications. Our studies showed that GABA$_A$ $\alpha_1$-function is reduced from LPCs in CIE and WD treated slices. Consistent with a decrease in function, $\alpha_1$ subunit protein expression was also decreased in the BLA during CIE and WD. In contrast, $\alpha_2$, $\alpha_3$, and $\alpha_4$-expression as well as $\alpha_4$-contribution are unaffected by CIE and WD. In addition to the effects of CIE and WD on $\alpha$-subunits, $\gamma_2$-subunit protein level was increased following CIE and WD. Given that certain components of the BLA GABA system are unaffected by CIE and WD, it is reasonable to suggest that the sensitivity of GABAergic transmission to acute ethanol following CIE and WD does not change. In
The findings from chapter 3 are suggestive of mechanisms that support the observed changes in glutamatergic transmission. Furthermore, as the GABA system is regulated by other neurotransmitter systems, it is important to investigate those systems that may ultimately contribute to the alterations in GABAergic transmission seen following CIE and WD.

**Chapter 4 Summary**

In chapter 4 we examined the dopamine system, specifically D3Rs, within the BLA of alcohol-naïve animals. We targeted D3Rs because several *in vivo* studies have suggested a role for D3Rs in modulating BLA-mediated behaviors (Steiner et al., 1997; Phillips et al., 2002a, b; Phillips and Hitchcott, 2009). Interestingly, we found that microinjections of a D3R antagonist dose-dependently decreased anxiety-like behaviors as measured in a light/dark box and an elevated-plus maze. Furthermore, we found functional data that were consistent with these changes in behavior. Specifically, D3R activation suppressed GABAergic transmission at LPC and local synapses. These findings support the hypothesis that D3Rs play a major role in BLA-mediated behaviors, and poses the question of how DA modulates GABAergic transmission in the BLA.

To examine the mechanisms by which DA modulated GABAergic transmission more closely, we investigated the effects of high (50µM) and low (30nM) concentrations of exogenous dopamine at local and LPC synapses. We found that high and low
concentrations of DA inhibited GABAergic transmission from LPCs via D1 and D3Rs. In contrast, while low concentrations of DA inhibited local GABAergic transmission via D1 and D3Rs, high concentrations of DA potentiated local GABAergic transmission. Furthermore, this previously reported D1R-mediated facilitation of local interneuron excitability (Rosenkranz and Grace, 1999; Kroner et al., 2005; Marowsky et al., 2005) masked a simultaneous D3R-mediated inhibitory effect on GABAergic transmission. Based on these findings, it is reasonable to suggest that stimuli that alter DA levels within the BLA could potentially be associated with anxiety-like behaviors. Our data suggest that concentration-dependent DA modulation of GABAergic transmission in the BLA can alter innate anxiety-like behaviors, indicating that the BLA DA system may be involved in withdrawal-induced anxiety.

Chapter 5 Summary

Chapter 5 encompassed preliminary findings describing how chronic ethanol and withdrawal altered the dopamine system in the BLA. First, we found that in adolescent/young adult (7-8 weeks) alcohol-naïve animals, high concentrations of DA did not elicit the same effect on local GABAergic transmission as it did in juvenile animals (3-5 weeks). In fact, in older animals, DA’s effect at both synapses (local and LPC) was to suppress GABAergic transmission. Interestingly, a recent study showed that the DA fibers within the BLA do not finish developing until postnatal day (P) 30 (Brummelte and Teuchert-Noodt, 2006). Given that our young and old groups fall just before and after this important cut-off, this study suggests that the differences may represent additional
development of DA-R signaling. Despite these age-dependent changes, we found that DA served to modulate GABAergic transmission within the BLA. Given that the GABA system does in fact adapt to CIE and WD (chapter 3), it is reasonable to suggest that the dopamine system may also be vulnerable to the effects of chronic alcohol and withdrawal.

Although we only examined the effects of CIE and WD on older (CON) animals (7-8 weeks), these exposures appeared to differentially affect DA modulation of GABAergic transmission in the BLA. While the effects of DA on GABAergic transmission at LPC synapses were not influenced by CIE and WD, the DA effect on local GABAergic transmission was reversed by CIE. Specifically, we found a D1R-mediated potentiation of local GABAergic transmission in CIE-treated slices that begins to return to control levels by 24 hours of withdrawal. These data suggest that the presence of alcohol in CIE animals activates a D1R-mediated facilitation of local GABAergic transmission. Furthermore, this D1R effect masked an underlying D3R-mediated suppression of these same synapses, evident with the use of a D3R agonist. Although this study only represents preliminary findings, it supports a hypothesis that the shift in balance between glutamate and GABA that occurs with CIE and WD may be attributed to changes in modulatory systems within the BLA.

**Alcohol Naïve-State**

_Neuroanatomy of the BLA_

Rats that have never been exposed to drugs, specifically alcohol, presumably maintain a balance between glutamatergic excitation and GABAergic inhibition in the
BLA that is further regulated by the dopamine system. Briefly, glutamatergic principal neurons project into the BLA from the cortex, thalamus, and lateral amygdala (Fig. 6.1:1). These projections synapse onto local principal neurons that, in turn, project to the CeA, among other brain regions. This local glutamate system is tightly regulated by the inhibitory GABAergic system. Although the GABAergic system of the BLA is comprised of multiple populations of interneurons (McDonald and Mascagni, 2001; McDonald et al., 2005; Muller et al., 2007), only two have been extensively characterized. Lateral paracapsular cells (LPCs) are found in distinct clusters along the external capsule (EC; lateral of the BLA; Fig. 6.1:2) (Marowsky et al., 2005; Silberman et al., 2007). These GABAergic interneurons function as feedforward inhibitors, meaning they hyperpolarize principal neurons before those neurons can respond to excitatory cortico-thalamic inputs. Interestingly, LPCs receive cortical inputs that regulate their function (Rosenkranz and Grace, 2001; Marowsky et al., 2005). Although these interneurons can synapse onto several principal neurons, they have been shown to synapse only onto distal dendrites (Marowsky et al., 2005; Muller et al., 2007). The second GABAergic population is comprised of local interneurons (Fig. 6.1:3). They are dispersed throughout the BLA. Local interneurons function as feedback inhibitors, and
are activated by local principal neurons. Furthermore, local interneurons have been shown to innervate one another, in turn creating networks that can self-propagate, chemically and electrically (through gap junctions), resulting in robust feedback inhibition (Woodruff et al., 2006; Woodruff and Sah, 2007). Unlike LPCs, local interneurons have been shown to form perisomatic synapses onto principal neurons. Despite this complexity, the GABAergic system is further regulation from the dopamine system. The BLA receives direct dopamine projections from the ventral tegmental area.
that alter GABAergic transmission, in turn significantly regulating BLA activity (Kroner et al., 2005; Marowsky et al., 2005). In fact, the DA system within the BLA has been referred to as the “gate” of the amygdala (Marowsky et al., 2005). In other words, the DA system can regulate how much inhibitory tone is provided to the BLA and from which population of interneurons it arises, therefore modulating BLA-mediated activity (i.e. fear and anxiety) (Weldon et al., 1991; Lamont and Kokkinidis, 1998; Timothy et al., 1999; Greba et al., 2001; Phillips et al., 2002a, b; Rogoz et al., 2004; de la Mora et al., 2005; Phillips and Hitchcott, 2009). Although all types of DA receptors are found within the BLA (Camacho-Ochoa et al., 1995; Gurevich and Joyce, 1999; Ciliax et al., 2000; Fuxe et al., 2003; Martina and Bergeron, 2008), only the D1R and, now, D3R have been predominantly characterized. In addition to the intricate neuroanatomy of the BLA, dopaminergic modulation of GABAergic transmission in alcohol-naïve animals appears to be age-dependent.

Dopamine, GABA, and Glutamate Systems

The dopamine system in the basolateral amygdala plays a major role in modulating GABAergic transmission. In chapter 4 we found that D3Rs can suppress both feed-forward (LPCs) and feed-back (local) inhibition in young rats (4-5 weeks). Similar reports have shown that D3Rs can decrease GABAergic activity in the hippocampus (Hammad and Wagner, 2006; Swant et al., 2008) and the nucleus accumbens (Chen et al., 2006) through a PKA-dependent increase in endocytosis of GABA_A receptors. Although we did not determine the specific cellular mechanism for the D3R-mediated suppression
of GABAergic transmission in these young animals, we did find that D3Rs were preferentially activated at low DA concentrations (30nM).

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<td>LPC</td>
<td>Local</td>
<td>LPC</td>
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<tr>
<td>D1R</td>
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<td>D3R</td>
<td>↓</td>
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Table 6.1 Summary of results of the effects of dopamine on GABAergic transmission.

This D3R-mediated effect represents a novel finding for GABA/DA circuitry in younger animals. In addition we found that DA-mediated suppression of GABAergic transmission in LPCs also required D1R activation. This is consistent with previous reports of a D1R-mediated hyperpolarization of LPCs which results in a decrease in excitability of LPC interneurons (Marowsky et al., 2005). Given that LPC activity regulates cortical inputs entering the BLA through the EC, our data suggest that at low concentrations, DA functions to gate the flow of cortical information into the BLA via
D3Rs and D1Rs. Similarly, we found that low concentrations of DA (30nM) also suppressed local, feed-back GABAergic transmission, again through the D3R and D1-like receptors in these young animals. Together these data suggest that at low concentrations, DA functions to reduce both feed-forward (LPC) and feed-back (local) inhibition within the BLA in 4-5 week old animals. Previous reports have found a D1R-mediated potentiation of local interneuron excitability (Kroner et al., 2005) that would lead to a decrease in BLA excitability. However, these findings were in the presence of a high DA concentration (10µM). In light of this research, we used a higher DA concentration (50µM), and also found a D1R-mediated facilitation of local GABAergic transmission in these young animals. Surprisingly, while D1Rs were blocked with the D1-like receptor antagonist SCH23390, 50µM DA suppressed GABAergic transmission at these same local synapses, suggesting that the predominant facilitatory effect of high DA is mediated by D1R. These findings suggest a concentration-dependent effect of DA within the BLA of 4-5 week old rats. It is worth noting that both D1R and D3R blockade was required to completely attenuate the inhibitory effect of 30nM DA (low concentration) at both local and LPC synapses. Yet, following D1R blockade, we were unable to attenuate the residual inhibitory effect of a high DA concentration on local GABAergic transmission. We were also unable to block the inhibitory effect of 50µM DA at LPC synapses. We hypothesize that this was related to the competitive nature of the D3R antagonist (Audinot et al., 1998), given that we only tested a 10µM concentration of U99194 to block a 50µM concentration of DA. Nevertheless, the paradox that both the facilitatory effect of 50µM DA on local GABA-IPSCs as well as the inhibitory effect of 30nM DA on local and LPC activity was sensitive to the D1R
antagonist suggests the presence of multiple D1-like receptors within the BLA that function to modulate local GABAergic transmission. Interestingly, it has been shown that D5Rs are localized within the BLA (Ciliax et al., 2000) and are known to have a high affinity for DA (Tupala and Tiihonen, 2004). Therefore, it is reasonable to suggest that D5Rs may be partly responsible for mediating the inhibitory effect of low DA concentrations on GABAergic transmission within the BLA. Nonetheless, we conclude that in young rats DA, at low concentrations, suppresses feed-forward LPC and local feed-back inhibition via D3Rs and D1-like receptors, potentially D5Rs. Our hypothesis is that this would lead to an overall decrease in inhibitory tone and ultimately result in an increase in BLA excitability and output. Yet, with an increase in DAergic transmission (for example, in response to stress (Inglis and Moghaddam, 1999; Yokoyama et al., 2005) or alcohol (Yoshimoto et al., 2000)), DA function switches to potentiate local feed-back GABAergic transmission via D1Rs, while continuing to suppress feed-forward LPC GABAergic transmission. This bi-directional effect of DA on BLA GABAergic transmission would ameliorate BLA excitability, perhaps as a means to shunt the anxiety circuit to prevent ‘hyperexcitability.’ Whatever the reason for the increase in DA levels in the BLA, it does not alter cortically-driven feed-forward inhibition. This suggests that cortical input into the BLA is resistant to alterations in the DA system. This set of studies demonstrates the important role of DA in the BLA of young rats in modulating GABAergic transmission. Still, preliminary data suggests that DA plays a different role in the BLA of adolescent/young adult animals.

The DA system modulated the GABAergic system differently in the BLA of animals that were only 2-3 weeks older (adolescent/young adults) than those from chapter
4 (juvenile). We found that a high concentration of DA (50µM) no longer had the same D1R-mediated facilitatory effect on local interneurons. Instead, 50µM DA suppressed local GABAergic transmission, similar to its effect on LPC activity in young animals. Furthermore, this inhibitory effect on GABAergic transmission was mimicked by the D3R agonist PD128907. This age-dependent switch was surprising given the important role of DA in increasing feed-back inhibition during times of elevated DA release (i.e. stress (Inglis and Moghaddam, 1999)). However, it has been shown that the development of the DA system within the BLA ends around 4 weeks of age in gerbils, with increases in DA fibers from P14-P30 (Brummelte and Teuchert-Noordt, 2006). Although we don’t know if this holds true in rats, the age range of our animals did fall in this window; juvenile animals from chapter 4 were between 4-5 weeks old, while adolescent/young adult animals from chapter 5 were between 7-8 weeks old. Although it is not known why the DA system in the BLA finishes developing at this age nor why DA has opposing effects before and after this point in time, we hypothesize that DA’s role in the BLA of juvenile animals may be to gate plasticity in order to establish rudimentary learned fear and anxiety. This hypothesis suggests that the BLA and the DA system play a significant role in early-life experience. This hypothesis would also support our earlier findings that higher concentrations of DA in young animals lead to inhibition of the BLA. In addition, it has been shown that in juvenile animals DA functions to dis-inhibit the lateral amygdala via bi-directional effects on GABAergic transmission in order to gate the induction of LTP (Bissiere et al., 2003). In contrast, in adolescence, DA’s role is to suppress GABAergic activity at both types of inhibitory synapses, when ‘innate’ anxiety-like behaviors should presumably be engrained. Although these findings represent an
important developmental stepping stone in DA modulation of the BLA GABA system, much remains to be examined. Despite these age-dependent changes in DA modulation of GABAergic transmission, the dopamine system can successfully modulate GABAergic function in the BLA to regulate glutamatergic excitation resulting in stable and ‘normal’ anxiety-like behavior.

Anxiety-like Behavior

Anxiety-like behavior in rodents can be evaluated using several behavioral assays. Our studies utilized a light/dark box apparatus to measure changes in anxiety-like behavior. This assay provides a broad measurement of fear and anxiety, as rodents prefer dark areas over lit areas (McCool et al., 2003; Lack et al., 2007; Lack et al., 2008). An increase in time spent in the light side following a treatment (i.e. drug microinjection, or exposure to alcohol) is suggestive of a decrease in anxiety-like behavior. Our studies also employed the elevated-plus maze, another behavioral assay that uses a rodents’ preference to be in dark confined spaces, and not open spaces (Pellow et al., 1985). This apparatus has two open arms and two closed arms where the animals can freely move around. Animals that spend more time in the open arms following a treatment (i.e. drug administration) are thought to express decreased anxiety-like behavior. Using these behavioral tools, we examined changes in innate anxiety-like behavior following manipulation of several neurotransmitter systems within the BLA.

Manipulation of the DA system within the BLA can alter anxiety-like behavior in alcohol-naïve rats. Previous reports have shown that D3R knockouts express significantly less anxiety-like behavior on an elevated plus maze (Steiner et al., 1997). Furthermore,
D3Rs within the BLA have also been shown to be involved in other BLA-mediated behaviors, such as taste-aversion (Phillips et al., 2002a, b), and more importantly, drug seeking behaviors (Di Ciano, 2008). We tested whether D3R blockade within the BLA would alter BLA-mediated anxiety-like behavior. Following microinjections of U99194, a competitive D3R antagonist with a 14-fold higher affinity for D3Rs than D2Rs (Audinot et al., 1998), into the BLA, we found a robust dose-dependent decrease in anxiety-like behavior in both the light/dark box and elevated plus maze. It is worth noting that these findings led us to investigate the physiology underlying this behavioral change. The anxiolytic properties of D3R blockade within the BLA were consistent with the electrophysiological findings that showed a D3R-dependent suppression of GABAergic transmission. Thus, blockade of D3Rs eliminates the suppression of GABAergic transmission within the BLA, ultimately resulting in reduced output from the BLA and a decrease in anxiety-like behavior. Based on these microinjection studies and electrophysiological findings, we confirmed a role of D3Rs in the BLA, and propose that D3Rs may be a putative target for treating anxiety.

As previously discussed, D1Rs also played a role in modulating GABAergic transmission although their specific role appears to be age-dependent. While we did not examine the role of D1Rs in modulating anxiety-like behavior, several studies have shown behavioral data consistent with our novel findings of an age-dependent switch. Specifically, D1R blockade within the BLA decreased anxiety-like behavior (de la Mora et al., 2005) as well as blocked fear expression (Lamont and Kokkinidis, 1998; Guarracci et al., 1999). Consistent with our preliminary findings in older animals, these studies
suggest that D1Rs function to suppress GABAergic transmission within the BLA, making them a potential target for treating anxiety.

While direct manipulation of the DA system within the BLA alters anxiety-like behavior, we have previously hypothesized that alterations in anxiolytic behavior are modulated through the GABA system. Our electrophysiological data indicates this occurs specifically through a suppression of GABAergic transmission. We know that direct manipulation of the GABAergic system influences anxiety-like behavior; in chapter 2 we microinjected muscimol, a GABA_A agonist, into the BLA and found that muscimol significantly decreased anxiety-like behavior in alcohol-naïve animals similar to D3R and D1R blockade. These studies demonstrate the intimate interaction between the dopamine and GABA systems within the BLA that ultimately regulate glutamatergic function resulting in the observed behaviors. Moreover, these studies suggest that chemically-induced alterations of neurotransmitters, whether via direct injection of receptor-specific drugs or more importantly exposure to chronic alcohol, can lead to profound changes in anxiety-like behavior.

**Chronic Alcohol State**

According to the DSM IV, alcohol dependence is described as a maladaptive pattern of alcohol use which includes the occurrence of tolerance and withdrawal as part of the criteria (American Psychological Association, 1994). In animals, chronic ethanol exposure can be used to produce alcohol-dependence (Roberts et al., 1996) verifying the validity of this animal model. Interestingly, chronic exposure to alcohol has been shown to significantly alter BLA physiology and BLA-mediated behaviors. We hypothesize that
as a result of the changes in BLA synaptic transmission in response to chronic ethanol, plastic changes may take place that ultimately lead to changes in anxiety-like behaviors observed during withdrawal.

*Dopamine System*

Although there is no evidence for a change in BLA circuitry, we did find that chronic intermittent ethanol (CIE) exposure altered dopaminergic modulation of GABAergic transmission. Specifically, following CIE, high concentrations of DA (50µM) potentiated local GABAergic transmission. Interestingly, this DA-mediated facilitation in adolescent chronic ethanol-exposed animals resembled the effect seen in juveniles. What’s more, with a pre-treatment of the D1-like antagonist, SCH23390, DA suppressed local GABA-IPSCs. These findings suggest that a D1R-mediated facilitation of local GABAergic transmission, similar to the one previously described in young animals (chapter 4), occurs during exposure to chronic ethanol. One possibility for this switch is D1R-modulation may be increased in response to chronic ethanol exposure. In fact, previous research has shown that D1R numbers increase in the lateral and intercalated nuclei of the amygdala following chronic ethanol exposure (Sari et al., 2006). While there were no reported changes in D1R expression in the BLA, we incorporate the lateral nuclei into the ‘BLA’ under our recording conditions, due to the difficulty in separating the two. Another possible reason for this re-occurrence of a D1R-mediated facilitation of local GABAergic transmission is D1Rs that facilitate local GABAergic transmission are preferentially activated with high DA concentrations, as described in chapter 4. Although there have been no reports of increased DA levels during chronic
ethanol, the presence of alcohol in the system has been shown to significantly elevate DA levels in many different brain regions ((Weiss et al., 1993); (for review see (Tupala and Tiihonen, 2004)). Our data suggest that DA levels within the BLA are increased during chronic ethanol exposure (also see review in (Grace, 2000)), in turn allowing these D1Rs to be more easily activated. We also found that during chronic ethanol exposure DA suppressed LPC GABAergic transmission to the same extent as in control cells. This DA-mediated effect was also mimicked by the D3R agonist. Interestingly, these data demonstrate that DA function at LPC synapses is resistant to the effects of chronic ethanol. More importantly, these data suggest that DA modulation of GABAergic transmission in older animals following CIE may be shifting towards to mirror that which is seen in younger animals, represented by a more plastic state (Bissiere et al., 2003) when the system may be functioning to establish new fear and anxiety-like behaviors.

GABA System

GABAergic transmission within the BLA is unaffected immediately following exposure to CIE. In chapter 3, we stated that release probability from feed-forward GABAergic interneurons remained unchanged in CIE animals. The lack of an effect on GABA release from LPCs might be explained by opposing changes with a net result of zero. In one direction there is an increase in cortical activity during chronic alcohol intake (Conte et al., 2008) that drives LPC activity (Rosenkranz and Grace, 2001; Marowsky et al., 2005). In fact, DA can facilitate mPFC activity, resulting in increased cortical input into the BLA (Rosenkranz and Grace, 2001). In addition, if DA levels are increased in the mPFC during chronic ethanol exposure (see review in (Grace, 2000)), elevated DA in the
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<td>mIPSCs (frequency &amp; amplitude)</td>
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<td>α1-expression &amp; contribution</td>
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<td>γ2- expression</td>
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<td>α4-expression &amp; contribution</td>
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<td>Acute Ethanol</td>
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<td>Acute Ethanol</td>
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Table 6.2 Summary of results for effects of chronic intermittent ethanol and withdrawal on GABAergic transmission. ↓ means decrease; ↑ means increase; -- means no change.
mPFC would lead to enhanced cortical input into the BLA. This enhanced input would further increase LPC activity. In the other direction, there is still significant D3R-mediated suppression, with a trend toward an enhanced suppression of LPCs during CIE. We hypothesize that these two opposing effects ultimately negate each other, resulting in a lack of change in LPC GABA release. GABA release from local interneurons also remains unchanged in chronic ethanol exposed animals. These data contrast reports of increased spontaneous activity in the central amygdala of animals exposed to chronic ethanol (Roberto et al., 2004a) and reports of reduced GABA function in the hippocampus following chronic ethanol exposure (Cagetti et al., 2003). One possibility for the contrasting findings with the central amygdala is that under our recording conditions the frequency of spontaneous events was so high in control (CON) slices, that any additional increase in frequency would most likely be undetectable. Therefore, any additional increase in the inhibitory efficacy at local synapses would not be evident with our measure of spontaneous activity. If we are in fact underestimating the frequency of spontaneous events during CIE, an increase in local inhibitory function during CIE would be consistent with an increase in CIE-induced D1R-mediated facilitation of local interneurons. This would ultimately lead to a stronger inhibition of glutamatergic principal neurons, in turn preventing a major increase in glutamatergic transmission. Although CIE had no detectable effect on presynaptic GABA release, chronic ethanol exposure did result in postsynaptic alterations. Specifically, postsynaptic GABA_A receptor composition was differentially altered by chronic ethanol exposure. In chapter 3, we demonstrated a decrease in α1-subunit function and expression. These findings are consistent with decreases seen in BLA α1 mRNA in rats (Falco et al., 2008) and non-
human primates (Floyd et al., 2004). Similar decreases in α1-subunit have been reported in the hippocampus using measures of mRNA (Morrow et al., 1994) and protein (Charlton et al., 1997) expression following chronic ethanol exposure. Likewise, α1-protein levels are reduced in the VTA following 14 weeks of chronic ethanol treatment (Charlton et al., 1997). Interestingly, this decrease in α1 protein in the BLA appears to be specific to LPC synapses, as we found a decrease in the effect of zolpidem, an α1-specific agonist, only when electrically activating LPCs. While α1 expression is evident in the BLA (Marowsky et al., 2004), it is unclear why CIE affects α1-function only at LPC synapses. One possible reason is that the relative intracellular distribution of α1-containing GABA_A receptors on glutamatergic principal neurons may be different. In fact, zolpidem potentiated GABA_A-mediated currents in CON slices only at LPC synapses, while practically having no effect on local GABA_A-currents. Although this decrease in α1-subunit during CIE is consistent across brain regions, the specific purpose for this decrease in the BLA needs further investigation.

We also found an increase in γ2-subunit protein expression in the BLA following CIE. Similar increases in γ2 protein levels have been reported in several other brain regions, including the hippocampus and cerebellum, following chronic ethanol exposure (Marutha Ravindran et al., 2007). The presence of the γ2-subunit is required for GABA_A clustering and synaptic localization in cultured cortical neurons (Essrich et al., 1998), as well as in the maturing rat brain (Schweizer et al., 2003). Furthermore, changes in mIPSC frequency have been associated with changes in γ2-protein levels (Essrich et al., 1998). γ2 expression is also essential for the postsynaptic accumulation of gephyrin, a multifunctional microtubule-associated protein, that is required for synaptic accumulation
of GABA<sub>A</sub> receptors containing the γ2-subunit (Essrich et al., 1998; Kneussel et al., 1999). We did not find changes in mIPSC frequency that would presumably arise from local synapses, following chronic ethanol exposure. Yet, it is possible that the increases in γ2-protein expression are specific to LPC synapses given that changes in α1-subunit were also specific to LPC synapses. Unfortunately, with our recording tools we cannot detect spontaneous activity from these synapses. Additional investigation is required. Furthermore, it would also be beneficial to examine gephyrin levels, given its intimate relation with the γ2-subunit.

In contrast to the previously discussed changes in GABA<sub>A</sub> subunit expression, we found that neither α2, α3, nor α4-subunit expression were altered by CIE. The findings from α2 and α3 contrast previous reports in cultured cortical synaptoneurosomes that have shown a decrease in α2- and α3-subunit protein levels following chronic exposure to ethanol (Mhatre et al., 1993) as well as a decrease in α2 in the cortex and cerebellum of animals exposed to chronic ethanol (Marutha Ravindran et al., 2007). In addition, we have previously found decreases in α2- and α3-subunit mRNA in the BLA of chronic ethanol exposed cynomolgus macaques (Floyd et al., 2004). Our contrasting findings with the α2- and α3-subunits may be related to the differences in brain regions (amygdala vs. hippocampus and cerebellum) and the species (rat vs. non-human primate). In addition, and more probable, is that changes in mRNA that have been previously reported may not be directly related to protein expression, as has been reported in other systems (Richichi et al., 2008). This disconnect between mRNA and protein expression could be attributed to numerous factors such as mRNA half-life and post-translational modifications (i.e. phosphorylation (Ravindran and Ticku, 2006), palmitoylation
(Rathenberg et al., 2004), etc.) that can ultimately influence the translation of mRNAs to protein or the trafficking of the protein. Similar to the lack of change in α2- and α3-subunits, α4-subunit expression and function were also unchanged during chronic ethanol exposure. As with the other alpha-subunits, these findings contrast evidence from cerebral cortex where α4 mRNA expression was upregulated during CIE (Devaud et al., 1995). This increase in α4 expression has been proposed to be a compensatory mechanism for the decrease of α1 levels in cortex. However, unlike consistent chronic ethanol induced decreases in α1 across multiple brain regions, the effects of chronic ethanol on α4 are not homogeneous. Several studies from our laboratory have shown that expression of α4 in the BLA is tolerant to chronic ethanol exposure. Using the benzodiazepine midazolam to indirectly measure α4 contribution to GABA_A-mediated currents, it was shown that GABA-IPSCs were unchanged in acutely dissociated neurons from rats treated with a liquid ethanol diet (McCool et al., 2003). Consistent with these findings from McCool et al. and the lack of change in α4-protein expression from chapter 3, α4-mRNA expression in the BLA of chronic ethanol exposed cynomolgus macaques was also unchanged (Floyd et al., 2004). One possible reason why this subunit is not affected by chronic ethanol is that the BLA has been shown to express low levels of the α4-subunit (Wisden et al., 1992; Benke et al., 1997). Taken together the data from our lab indicate that the α4-subunit within the BLA is resistant to chronic ethanol exposure across species, unlike the other α-subunits. Based on the CIE-induced postsynaptic GABAergic alterations, the beginning of a shift in the balance between excitation and inhibition, in favor of glutamatergic excitation, appears to take place in the BLA of CIE animals.
Glutamate System

Our studies indicated that presynaptic glutamatergic function is elevated during CIE exposure; specifically, that the frequency of glutamatergic spontaneous activity was increased. Given that alterations of release probability paralleled increases in sEPSC frequency, without any change in the frequency of mEPSCs, we concluded that the presynaptic changes are most likely attributed to an increase in glutamatergic synapse excitability. As the only GABAergic alteration during CIE was altered subunit expression (decreased α1 and increased γ2), this change in glutamatergic synapse excitability may be associated with the changes in GABA_A conformation. It is also reasonable to suggest that CIE-induced increases in excitability of brain regions upstream from the BLA (i.e. cortex (McMahon et al., 2000; Conte et al., 2008)) may ultimately influence glutamatergic transmission within the BLA. In contrast to the increased glutamatergic excitability of the BLA and cortex, glutamate release in the central amygdala is not altered by chronic ethanol exposure (Roberto et al., 2004b). While the differences between the BLA and CeA could be attributed to where the glutamate projections arise from, it is also possible that these differences are due to the differential effects of chronic ethanol exposure on their respective GABAergic systems. As mentioned above, there are no net changes in GABA release in the BLA following CIE. In contrast, GABA levels have been shown to be significantly elevated in the CeA following chronic ethanol treatment (Roberto et al., 2004a), potentially arising from the ICM (Collins and Pare, 1999; Royer et al., 1999; Delaney and Sah, 2001; Royer and Pare, 2002). This pronounced increase in GABAergic transmission in the central amygdala may potentially maintain the glutamate system in a suppressed state. Although the exact
mechanism for the increase in BLA principal neuron excitability is unknown, an increase in glutamate release could potentially lead to postsynaptic glutamatergic changes.

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<tr>
<th>Glutamatergic Transmission</th>
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<tr>
<td></td>
<td>CIE</td>
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<tr>
<td>NMDAR function</td>
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<td>sEPSC amplitude</td>
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<td>sEPSC frequency</td>
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<td>Presynaptic release (PP)</td>
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<td>mEPSC amplitude</td>
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<td>mEPSC frequency</td>
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Table 6.3 Summary of results for the effects of chronic intermittent ethanol and withdrawal on glutamatergic transmission.

During chronic ethanol treatment NMDAR function was shown to be potentiated in BLA cells. Given that NMDARs are inhibited by acute alcohol in the BLA (Lack et al., 2008), it is reasonable to suggest that chronic inhibition of NMDARs could result in an increase in receptor function or an increase in receptor number. Although we did not
measure NMDAR protein or mRNA levels from CIE-treated animals, an earlier study from our lab found increased mRNA expression of the BLA NR1 subunit in rats exposed to a chronic liquid ethanol diet (Floyd et al., 2003). This same study also showed elevated NMDAR densities in the BLA following chronic ethanol ingestion. Similar increases in NMDAR subunit mRNA and protein (NR1, NR2A, and NR2B) have been reported in the central amygdala following exposure to chronic ethanol (Roberto et al., 2005). In addition, increases in NMDA receptor densities (Grant et al., 1990; Gulya et al., 1991) as well as subunit mRNA and protein levels (Follesa and Ticku, 1995; Chandler et al., 1999; Qiang et al., 2007) have been shown in several other brain regions. Our findings of increased NMDAR function are consistent with increased NMDAR expression. Interestingly, it is well established that the process of fear conditioning induces long-term potentiation (LTP) within the BLA that requires NMDAR contribution (Gewirtz and Davis, 1997; Gale et al., 2004). In fact, during times of strong stimulation, consistent depolarization of a postsynaptic neuron provides a relief of the voltage-dependent block of NMDARs by Mg$^{2+}$. This provides a positive feedback for NMDAR Ca$^{2+}$ currents into the postsynaptic compartment that has been suggested to result in the induction of LTP (reviewed in (MacDonald et al., 2006)). It is possible that the CIE-induced increase in neuronal excitability provides enough of a robust stimulation within the BLA that results in the facilitation of NMDAR-function. We hypothesize that this would ultimately lead to the induction of LTP during exposure to chronic ethanol. In support of this hypothesis, we also reported changes in the DA system following chronic ethanol exposure that suggests an induction of plasticity in the BLA (discussed in the DA section above). The
induction of LTP would result in many other postsynaptic changes that may be relevant to BLA activity.

Postsynaptic AMPAR function was not altered by chronic ethanol exposure in our studies. This was evident given that the amplitude of mEPSCs was unaffected by CIE. Interestingly, only a few studies have shown changes in AMPAR function and expression following chronic ethanol exposure. These changes occurred under specific conditions, such as cultured cortical neurons in low glutamine-containing media (Chandler et al., 1999), and cultured cerebellar Purkinje neurons in specific postsynaptic compartments (dendritic vs. somatic) (Netzeband et al., 1999). It is reasonable to suggest that in our chronic ethanol paradigm more permanent changes, such as increased AMPAR function/surface expression, do not occur as only the induction of LTP is taking place. Consistent with our hypothesis that the induction of LTP occurs during CIE, it has been shown that AMPAR surface trafficking and insertion takes place only during the expression and/or maintenance of LTP (Bliss and Collingridge, 1993; Malinow and Malenka, 2002; Bredt and Nicoll, 2003; Derkach et al., 2007). This requires extensive further investigation and is only speculative. Nevertheless, the changes in glutamatergic, as well as GABAergic and dopaminergic, function following chronic exposure to ethanol demonstrate a disruption in the BLA that ultimately leads to changes in anxiety-like behavior.

Anxiety-like Behavior

We hypothesized that chronic ethanol induced alterations in DA modulation of GABAergic transmission leads to adaptations in GABAergic function. Subsequently, an
enhanced glutamate system resulted in a change in anxiety-like behavior. Yet, in chapter 2 we found that animals exposed to chronic ethanol showed no change in anxiety-like behavior, with a trend toward a decrease in anxiety-like behavior. In contrast, a previous study from our lab found that chronic ethanol ingestion produced anxiolysis in rats (McCool et al., 2003) with a concurrent increase in NMDA function (Floyd et al., 2003). It is apparent that changes in BLA activity contrast the BLA-mediated behavior regardless of the type of ethanol exposure. This discrepancy can be explained in several ways. First, the lack of change in feed-forward and feed-back inhibition that we found in chapter 3 may be sufficient to counter increases in BLA glutamatergic excitability. This process may be modulated by the potential enhancement of feed-back inhibition mediated by D1R-activation shown above. Another possibility is that, the central nucleus of the amygdala functions as a ‘filter’ for the BLA anxiety-circuit. In fact, similar to the BLA, the CeA is comprised of several inhibitory GABAergic interneuron populations (Pitkanen and Amaral, 1994) that together function to regulate the output of relevant information from upstream brain regions, including the BLA (Royer et al., 1999; Royer and Pare, 2002). Moreover, GABAergic function is significantly enhanced within the central amygdala following chronic ethanol exposure (Roberto et al., 2004a), suggesting that this ‘filter’ is enhanced after an exposure to chronic alcohol. This increase in inhibitory function within the CeA could be a compensatory response to the increase in excitability of the BLA. Although chronic ethanol itself does not appear to alter anxiety-like behavior, the presence of residual ethanol in this brain region may lead to the lack of behavioral changes.
Withdrawal State

While long-term exposure to alcohol can have a multitude of negative effects, the removal of alcohol from the system also has detrimental effects. The occurrence of alcohol withdrawal symptoms is one criterion for diagnosing alcohol dependence (Grant, 2004). The development of alcohol withdrawal symptoms has been thought to be a major cause of relapse in recovering alcoholics (Breese et al., 2005). This may be due to the changes that the brain undergoes upon cessation of alcohol. In particular, it has been hypothesized that the brain is in a ‘hyperexcitable’ state during alcohol withdrawal (Crews et al., 1996; Little, 1999) that leads to several typical withdrawal symptoms including increased anxiety. In addition, following withdrawal from a chronic ethanol exposure similar to ours, rats have been shown to show increases in alcohol self-administration that has been suggested to represent an animal model of ‘relapse’ (reviewed in (Gilpin et al., 2008)). Nevertheless, this hypothesis of withdrawal-induced ‘hyperexcitability’ arises from evidence of suppressed GABAergic inhibition (Cagetti et al., 2003; Olsen et al., 2005). Interestingly, it has been shown that withdrawal from chronic alcohol significantly alters dopaminergic transmission throughout the brain (Diana et al., 1992; Rossetti et al., 1992a; Rossetti et al., 1992c). These studies suggest that the dopamine system, by modulating GABAergic transmission, may play a role in mediating this withdrawal-induced ‘hyperexcitability’ of the brain.

Dopamine System

Dopaminergic modulation of GABAergic transmission within the BLA following exposure to chronic ethanol is altered at 24 hours of withdrawal. In particular, in chapter
we demonstrated that D1R-mediated facilitation of GABAergic transmission at local synapses is diminished in cells from WD animals. In fact, DA appeared to have little effect on local GABAergic transmission during WD. However, upon D1R-blockade, DA significantly suppressed local GABAergic transmission suggesting a D1-mediated facilitation during withdrawal balanced by an additional suppressing effect of DA. These findings were intriguing given that the suppressing effect of DA was still present at local synapses during withdrawal, but only detectable upon D1R-blockade. This suggests that CIE-induced activation of D1Rs that potentiate local GABAergic transmission begin to reverse during withdrawal. We hypothesize that this may be related to a decrease in DA transmission during alcohol withdrawal. Consistent with this hypothesis, several reports indicate that DA release is significantly decreased during withdrawal from chronic ethanol treatment (Rossetti et al., 1992c; Rossetti et al., 1992b; Diana et al., 1993; Gil-Martin et al., 1994). Findings from chapter 4 demonstrated that D1R-mediated facilitation of local GABAergic transmission only occurs with high concentrations of DA. High concentrations of DA presumably occur during chronic ethanol exposure following synaptic DA release (see review by (Grace, 2000). It is possible that these D1Rs are less readily activated due to withdrawal-induced decreases in DA release. However, there is evidence that D1Rs may still be active during withdrawal. In the VTA, decreased dopaminergic neuronal firing during withdrawal from chronic alcohol has been associated with increased sensitivity of striatal D1-like receptors to $^{3}$H-SCH23390 (Gil-Martin et al., 1994; Bailey et al., 2001). This fluctuation in DA modulation of GABAergic transmission between chronic ethanol and withdrawal appears to only target local synapses.
DA continued to suppress feed-forward LPC GABAergic inhibition in WD slices. Interestingly, preliminary findings from chapter 5 show a trend toward a stronger D3R-mediated suppression of GABAergic transmission. This trend was shown using a D3R agonist, at LPC synapses during WD compared to CON and CIE slices. These data may indicate that D3R expression is elevated during withdrawal. Another explanation for this change in D3R-mediated effect is that the efficacy of these receptors may be increased. Further experiments will be required to examine these mechanisms. The continued presence of D3R-mediated inhibition of GABAergic transmission within the BLA is consistent with several reports that suggest that D3Rs play a major role in ethanol seeking and relapse (Boyce and Risinger, 2000, 2002; Vengeliene et al., 2006; Heidbreder et al., 2007). Together, the effects of withdrawal on the DA system within the BLA are reflected in the GABA system.

GABA System

The ongoing hypothesis that the brain, in our case the BLA, is in a 'hyperexcitable' state during withdrawal arises from evidence of suppressed GABAergic inhibition. In chapter 3, we found evidence that GABAergic feed-forward inhibition from LPCs was suppressed. This effect of withdrawal on feed-forward inhibition is not related to changes in cortical inputs, given that cortically-driven inputs into the BLA (through the external capsule) are significantly increased during withdrawal (Lack et al., 2009). Instead, the decrease in GABA release during withdrawal may be associated with potential increases in D3R-mediated suppression of LPC GABAergic transmission. Decreases in GABA release have also been shown to occur at 2 and 40 days of
withdrawal from chronic ethanol in the hippocampus (Kang et al., 1996). In addition, a separate study showed a complete suppression of GABAergic transmission in 77% of cortical neurons following withdrawal from chronic alcohol (Ibbotson et al., 1997). Together, these studies all support the hypothesis that GABAergic inhibition is reduced during withdrawal, supporting the ‘hyperexcitability’ hypothesis. Interestingly, in the same study by Ibbotson et al. (1997), the other 23% of the cells did not appear to be affected by withdrawal from chronic ethanol. The differential effect of alcohol withdrawal on GABAergic transmission may also be occurring in the BLA, as we found that local feed-back inhibition within the BLA was unaffected during WD. Given a possible ceiling effect observed in our spontaneous GABAergic transmission recordings it is possible that there was an unseen increase in GABA release. Yet, a lack of change in local GABAergic transmission would be consistent with the D1R-mediated facilitation of local GABAergic transmission that is still partially present during WD. It is important to note that this D1R-mediated facilitation is enough to counter the DA-mediated inhibition that is presumably D3R-mediated. Therefore, this D1R-mediated facilitation may function to maintain local feed-back inhibition. These findings do contrast reports of decreased feed-back inhibition (presumably from local interneurons) in the BLA during withdrawal from a liquid ethanol diet (Isoardi et al., 2007). However, this reported decrease in feed-back inhibition occurred after 3 days of withdrawal, suggesting that the GABAergic system may be dynamic throughout withdrawal. Another possibility for the discrepancy is the delivery method, given that a liquid ethanol diet was used in the study (Isoardi et al., 2007). Regardless, further studies will be needed to measure changes in
GABAergic transmission at later withdrawal time points and to gather more accurate measure of GABA release from local synapses.

Postsynaptic changes in GABAAR composition that occurred following chronic ethanol exposure are maintained during withdrawal. $\alpha_1$-subunit expression remained reduced and $\gamma_2$-subunit expression continued to be elevated at 24 hours of withdrawal. These changes in $\alpha_1$ and $\gamma_2$ are consistent with changes in the hippocampus at 2 days of withdrawal following 60 days of chronic ethanol ingestion (Cagetti et al., 2003; Olsen et al., 2005). Although we did not extensively examine the role of these postsynaptic modifications, we did find that the sensitivity to zolpidem, an $\alpha_1$-agonist, also remained suppressed at LPC synapses during withdrawal. Furthermore, similar to CIE, neither $\alpha_2$, $\alpha_3$, nor $\alpha_4$ protein levels were altered by withdrawal. Interestingly, it has been suggested that the $\alpha_1$-subunit is required for only the sedative effect of benzodiazepines, and not the anxiolytic effects (Rudolph et al., 1999; McKernan et al., 2000). Despite the decrease in $\alpha_1$, the results on $\alpha_2$, $\alpha_3$, and $\alpha_4$ protein levels suggests that the sensitivity of benzodiazepines is unaffected. Consistent with this hypothesis, the effects of the benzodiazepine, midazolam, on GABA$_A$-mediated IPSCs were not altered by withdrawal. The relevance of the residual sensitivity to benzodiazepines will be discussed below.

These findings contrast reports from the hippocampus where $\alpha_4$ expression was increased (Cagetti et al., 2003). Specifically, there was a ‘switch’ between $\alpha_1$ and $\alpha_4$ in the hippocampus that occurred two days following chronic ethanol exposure. Consistent with this ‘switch’ there were also changes in the kinetics of GABAergic currents (smaller amplitudes and faster decay). Furthermore, it has now been shown that the increases in hippocampal $\alpha_4$ function during withdrawal arose from a switch between $\alpha_4$-containing
extrasynaptic GABA_A receptors and α4-containing synaptic GABA_A receptors (Liang et al., 2006). Although we did not specifically examine extrasynaptic GABA_A R activity, we found no evidence for changes in α4 protein levels or changes in the kinetics of GABAergic currents, all consistent with the lack of a “switch” in the BLA. Although removal of alcohol from the system leads to pre- and post-synaptic adaptations in the GABAergic system of the BLA during withdrawal from chronic alcohol exposure, it is therefore important to understand what the effects of re-introducing alcohol into the system would be.

In chapter 3, we demonstrated that the sensitivity of GABAergic transmission to acute alcohol is not altered by withdrawal. These data contrast with findings in the hippocampus that show an increase in the effect of acute ethanol on GABAergic transmission in slices from withdrawal animals compared to alcohol naïve controls (Kang et al., 1998). It is important to note that neither the hippocampus nor BLA show tolerance to the effects of acute ethanol. Furthermore, the lack of tolerance to acute ethanol in the BLA is an extremely important finding for two reasons. First, it suggests that a reason for the lack of change in feed-forward inhibition from LPCs in CIE animals compared to CON is the presence of alcohol in this brain region. Although there was no ethanol present in the buffer solutions used during the experiments, the biochemical processes that would occur during “acute in vitro withdrawal” (during experiments) must occur at body temperature (Lack et al., 2007). This suggests that the system is ‘frozen’ in the state that it was in during euthanasia, when alcohol was present. This holds true given that the removal of ethanol resulted in a decrease in GABA release from feed-forward LPC synapses. More importantly, these data demonstrate a possible explanation for why
relapse can relieve withdrawal-induced anxiety. This hypothesis will be discussed further in a following section. The changes in GABAergic function following withdrawal from chronic ethanol exposure may contribute to the adaptations that occur in the glutamate system during withdrawal.

Glutamate System

In chapter 2, we found that during alcohol withdrawal pre-synaptic glutamatergic adaptations are more pronounced. Principal neuron excitability continues to be elevated during withdrawal given the increase in release probability, as well as the increase in excitability of cortical inputs (Lack et al., 2009). However, there are also presynaptic terminal-specific changes on upstream principle neurons at 24 hours of withdrawal. Specifically, we found that action potential-independent (mEPSC) event frequency was increased during withdrawal. We have actually measured the changes in presynaptic transmission up to 72 hours of withdrawal and have found that sEPSCs continue to be elevated at 72 hours of withdrawal. These data suggest that the longevity of these adaptations past 24 hours of withdrawal can indicate more permanent changes (i.e. LTP-like processes (Sastry and Bhagavatula, 1996)). Nevertheless, a switch from excitability to terminal-specific increases in glutamate release can be attributed to several processes. The D3R-dependent decreases in feed-forward GABAergic inhibition during withdrawal may be responsible for these significant adaptations. In addition, the possibility of decreased local feed-back inhibition may contribute to these changes (Isoardi et al., 2007). Another potential explanation is increases in Ca$^{2+}$ channel currents during alcohol withdrawal (N’Gouemo and Morad, 2003) (potentially via increased NMDAR function)
that could ultimately alter neurotransmitter release. These withdrawal-induced terminal-specific adaptations further support the hypothesis that the BLA is in a ‘hyperexcitable’ state.

In chapter 2 we also found that increased NMDAR function continues to be elevated at 24 hours of withdrawal. This increased NMDAR function at 24 hours of withdrawal is consistent with elevated NMDA function in mouse hippocampus during ethanol withdrawal (Whittington et al., 1995; Hendricson et al., 2007). Although we only examined NMDAR function at 24 hours of withdrawal, our data may indicate transient changes in NMDA contribution. This may be as NMDA-subunit mRNAs (NR1 and NR2B) are significantly decreased compared to control levels at 1 week of withdrawal from chronic ethanol not 2 weeks in the central amygdala (Roberto et al., 2005). In contrast, Roberto et al. (2005) showed that NMDA subunit protein expression remained unchanged during chronic ethanol, in addition to 1 and 2 weeks of withdrawal. Importantly, these findings clearly show that changes in gene expression do not always correlate to changes in protein expression. Regardless, changes in NMDAR contribution may function to initiate the neurochemical processes that result in ‘hyperexcitability’ of the amygdala, i.e. increased AMPAR function. An increase in NMDAR function or expression would ultimately increase intracellular Ca\(^{2+}\), which is evidence for the increase in glutamate release that occurs during withdrawal. This persistent increase in NMDAR function in the BLA coincides with an increase in AMPAR function during withdrawal.

We also showed in chapter 2 that AMPAR function is facilitated during withdrawal from chronic ethanol. This was evident by increased mEPSC amplitude and a
decrease in the decay kinetics for AMPA-mediated mEPSCs (Malenka and Nicoll, 1999). As the mEPSC frequency was also increased during WD, it is difficult to dissociate whether the changes in amplitude and frequency are related, as previously discussed (frequency-dependent summation of events at the post-synapse may appear as increased amplitude). Yet, since release probability was also elevated, we can suggest that the changes in amplitude and frequency are independent of each other. These data support an increase in postsynaptic AMPAR function. As we did not measure protein or mRNA levels of AMPAR-subunits we cannot rule out changes in receptor or gene expression. Nonetheless, increased AMPAR function may be associated with increased surface trafficking of specific AMPAR-subtypes. For example, faster AMPA-mediated EPSC decay, as those reported in chapter 2 during withdrawal from alcohol, have been shown to involve increased delivery of GluR1 containing channels to the synapse (Clem and Barth, 2006). Interestingly, Clem et al. found that this phenomenon occurred in response to sensory stimulation of the barrel cortex, a brain region that is highly plastic in rodents (reviewed in (Fox, 2002). The increase in AMPAR function in the BLA as described in chapter 2 suggests that processes involved in plasticity may have taken place regardless of the mechanism. This is because LTP, an in vivo form of plasticity, is initiated via NMDARs, particularly in the BLA, and is maintained with an increase in AMPAR contribution (McKernan and Shinnick-Gallagher, 1997; Shi et al., 1999). Furthermore, the ATPA-induced LTP via kainate receptors has been shown to be significantly suppressed during CIE and WD, suggesting that other LTP-like processes may be occluding this KA-R-mediated LTP (Lack et al., 2009). The withdrawal-induced increase in AMPARs, as well as the change in KA-R-mediated LTP further support the hypothesis
of ‘hyperexcitability’ of the brain, in particular in the BLA. Although the increases in AMPAR function significantly contribute to the increases in BLA excitability during withdrawal, all of the changes in the three neurotransmitter systems ultimately help to shape the BLA-mediated anxiety-like behavior observed in withdrawal.

Anxiety-like Behavior

Anxiety-like behavior was significantly increased during withdrawal from chronic ethanol exposure. Specifically, animals spent more time in the dark side of a light/dark box than control animals. In addition, they took more time to re-enter the light side once they entered the dark side. Increases in anxiety-like behavior have been shown to result from alterations in anxiety-related neural circuitry, specifically the amygdala, due to withdrawal from chronic ethanol (Koob et al., 1998; Roberto et al., 2008). Supporting this, we found that changes in DA modulation of GABAergic transmission occurred during exposure to chronic ethanol and subsequent withdrawal that ultimately led to significant increases in glutamatergic transmission. This increase in glutamatergic function resulted in increased output from the BLA (Lack et al., 2009) that was associated with increased anxiety-like behavior. Interestingly, we found that the anxiogenic response to 24 hours of withdrawal was attenuated by microinjections of DNQX, an AMPA/KA-R antagonist, into the BLA. This finding supported our hypothesis that manipulations of neurotransmitter systems within the BLA (in this case, the glutamate system) can attenuate withdrawal-induced anxiety-like behaviors. Furthermore, based on the functional findings from chapters 3 (decreased feed-forward inhibition), 4 (D3R-sensitive anxiety-like behavior) and 5 (persistent D3R-mediated
suppression of GABAergic transmission), we suggest that microinjections of a GABA_\text{A} agonist or a D3R antagonist into the BLA of withdrawal animals would also attenuate the anxiogenic effect of withdrawal. Although they were only examined in alcohol-naïve animals, we did find that microinjections of muscimol, a GABA_\text{A} agonist, and U99194 a D3R antagonist, significantly decreased anxiety-like behaviors. Several groups have also demonstrated that D3R antagonists can also alter various aspects of drug reward. For example, microinjections of U99194, a selective D3R antagonist, into the amygdala, but not the nucleus accumbens, significantly attenuated responding for cocaine under a 2^{\text{nd}}-order schedule of reinforcement (Di Ciano, 2008). More relevant to this dissertation and further evidence suggesting a therapeutic use of D3R antagonists in preventing alcohol relapse, it has been shown that i.p. injections of SB-277011A, a selective D3R antagonist, significantly reduced reinstatement of alcohol seeking (Vengeliene et al., 2006; Heidbreder et al., 2007). In addition, Heidbreder et al (2007) found that SB-277011A also reduced several aspects of operant self-administration of alcohol, including alcohol intake (g/kg), the number of reinforcers, and the number of active lever presses. Our studies also suggest that activation of D1Rs could also reduce withdrawal-induced anxiety-like behavior, given that under states of high DA concentration D1R activation can facilitate GABAergic feed-back inhibition. As DA levels are decreased during withdrawal (Rossetti et al., 1992c; Rossetti et al., 1992b; Diana et al., 1993; Weiss et al., 1993; Gil-Martin et al., 1994), a D1R agonist would hypothetically increase local GABAergic transmission to counter the increased glutamate function. Although the effects of a D1R agonist on anxiety-like behavior have never been investigated, it has been shown that microinjections of a D1-like receptor antagonist, SCH23390, into the
BLA decreased anxiety-like behavior in drug-naïve animals (de la Mora et al., 2005). Although this is in opposition of what we hypothesize, we previously discussed the potential contribution of D5Rs that could be associated with the study by de la Mora (2005). It is important to remember that CIE induces a switch in D1R contributions by increasing the facilitatory effect of D1Rs at local GABAergic synapses that is subtly present during 24 hours of withdrawal. Nevertheless, these drugs (GABA<sub>A</sub> agonist, D3R antagonist, and D1R agonist) would need to be tested in withdrawn animals to further validate our functional findings during withdrawal.

One of the major findings of these experiments was that the effect of acute ethanol and midazolam on GABAergic transmission was not altered by either chronic ethanol exposure or withdrawal. These findings suggest that the presence or removal of alcohol on GABAergic transmission in the BLA may be responsible for the prevention or induction of anxiety-like behavior, respectively. Unfortunately, as acute alcohol has been shown to enhance GABAergic transmission in the BLA during withdrawal (and ameliorate anxiety), recovering alcoholics revert back to alcohol use (bouts of acute alcohol) which ultimately leads to the vicious cycle of relapse (Breese et al., 2005). However, as we found that midazolam sensitivity was unaltered by withdrawal, our study also suggests a possible reason why benzodiazepines are extremely useful in treating withdrawal symptoms (anxiety), in newly abstaining or recovering alcoholics (Holbrook et al., 1999). These data contrast findings from alcohol-dependent non-human primates that showed a decrease in benzodiazepine sensitivity of BLA GABA<sub>A</sub> receptors at the end of their chronic alcohol exposure (Anderson et al., 2007). Interestingly, γ2 levels were also decreased in these monkeys, while we found an increase in γ2 levels. This suggests
that γ2 expression and benzodiazepine sensitivity may be related. In addition, another study using multiple ethanol withdrawals in mice showed that the use of a benzodiazepine during each acute withdrawal can ameliorate the initial withdrawal symptoms (seizure susceptibility) during a longer withdrawal. Unfortunately, withdrawal symptoms were shown to be exacerbated following a protracted withdrawal due to a combination of ethanol and benzodiazepine withdrawal (Becker and Veatch, 2002). Nevertheless, as acute ethanol and midazolam positively modulate GABAergic transmission during alcohol withdrawal, the use of a GABA_A agonist may be extremely beneficial for alleviating (at least initially) withdrawal-induced anxiety-like behavior.

Taken together, this set of studies demonstrates significant roles for the dopaminergic, GABAergic, and glutamatergic systems within the BLA and their interactions in shaping BLA-mediated anxiety-like behavior. More importantly, these studies revealed that chronic ethanol exposure and withdrawal induced alterations in these neurotransmitter systems that ultimately influence BLA output, resulting in anxiety-like behavior.

**Significance and Future Directions**

Previous research has placed an emphasis on the extended amygdala, in particular the central amygdala, as the primary origin for the negative effects of chronic ethanol and withdrawal (Koob et al., 1998; Koob, 2004, 2008). Although the CeA does play a role in observed increases in withdrawal-induced anxiety-like behavior (Rassnick et al., 1993; Menzaghi et al., 1994; Lack et al., 2005), the present studies demonstrate significant roles for the BLA in driving the negative effects of alcohol withdrawal. By examining the
changes and interactions in the neurotransmitter systems within the BLA we now have a better understanding of how the BLA can drive and mediate the observed changes in anxiety-like behavior following withdrawal from chronic alcohol. We have also presented novel findings regarding D3R contributions to BLA-mediated anxiety-like behavior in young drug-naïve animals. In addition, we have characterized dopaminergic modulation of two populations of GABAergic interneurons within the BLA under CIE and withdrawal conditions. Changes in these populations may relate to increased glutamatergic function seen during chronic ethanol exposure and withdrawal. Despite the current set of studies, many questions remain regarding the specific role each of these neurotransmitter systems.

Given the age-dependent alterations in DA modulation of GABAergic transmission found in chapter 5, it would be extremely beneficial to further investigate these changes. Establishing the age at which D1R-mediated facilitation of local GABAergic transmission is diminished would suggest an explanation for differences in the age of onset for alcohol abuse (i.e. early-onset alcoholism (type 2 alcoholics) vs. late-onset (type 1 alcoholics) (Cloninger, 1995; Tupala and Tiitonen, 2008). In addition to the age-dependent changes, it would be important to complete a characterization and analysis of the effects of chronic ethanol exposure and withdrawal on DA modulation of GABAergic transmission. Our studies only examined some primary effects of this interaction. Conducting a pharmacological and cellular mechanistic investigation would help to address questions about changes that occur in both the GABA and glutamate systems.
More experiments are needed to complement the findings from chapter 3. First, determining a way to measure specific presynaptic changes from LPCs (i.e. sIPSCs and mIPSCs) would enable us to examine the effects of chronic ethanol and withdrawal as well as the effects of dopamine on GABAergic transmission at distal synapses. Furthermore, it would allow us to investigate mechanisms that underlie decreases in release probability from LPC synapses in CIE and WD slices. One potential target for further investigation lies within the dopamine system given that D3Rs suppressed GABAergic transmission from LPCs. Identification of other modulatory neurotransmitter systems potentially involved in these neuroadaptations of LPCs is also warranted. Specifically, the noradrenalin system has been shown to specifically modulate GABAergic transmission only at LPCs and not local interneurons, and is also involved in mediating the effects of acute ethanol on GABAergic transmission within the BLA (Silberman et al., 2007; Silberman et al., 2009). One possible technique that could be useful in measuring presynaptic changes at distal LPC synapses is including strontium (Sr$^{2+}$) in the external solution (aCSF). As Sr$^{2+}$ is a larger ion than Ca$^{2+}$, replacing Ca$^{2+}$ with Sr$^{2+}$ drastically slows the electrically-induced release of neurotransmitters from presynaptic boutons. By manipulating GABA release, artificial asynchronous (Sr$^{2+}$-induced) action-potential dependent spontaneous activity following electrical stimulation of the targeted synapses can be used to measure presynaptic changes. This technique has been shown to be useful for glutamatergic transmission (Jones et al., 2007), in regards to the effects of chronic ethanol and withdrawal on NMDARs (Hendricson et al., 2007). We have successfully used this technique in our own slice preparations for glutamate related experiments. Unfortunately, further technical fine-tuning is required for recording
GABAergic responses. Nevertheless, we hope to employ this technique to address questions regarding presynaptic release from LPCs. Another issue in measuring GABA release within the BLA is the extremely high frequency of local spontaneous events from control (CON) slices. As previously discussed in this document, any treatment-induced change in frequency of local spontaneous activity was largely undetectable given that we may have reached a ceiling effect in CON slices. Although previous attempts have been unsuccessful it should be possible to use an internal solution with a high Cl\(^-\) concentration. This would shift the reversal potential of Cl\(^-\) allowing us to record inward Cl\(^-\) currents at more physiologically relevant membrane potentials (~ -60mV). This would decrease the driving force for Cl\(^-\) and would theoretically decrease the frequency of spontaneous events in CON slices allowing us to better detect changes induced by CIE and WD. Further characterization of the changes that occur in GABAergic transmission following CIE and WD may help illustrate how this complex circuitry adapts during exposure to chronic ethanol and subsequent withdrawal. In addition, these future studies may suggest possible mechanisms for changes that occur in the glutamate system.

We found a robust increase in glutamatergic transmission following exposure to chronic ethanol and withdrawal and these changes likely arise from different factors. As the BLA receives glutamatergic information from various brain regions, it is necessary to examine each of these inputs into the BLA and how they might adapt to CIE and WD. We have collected preliminary data that suggests a dual-stimulation protocol can be employed to simultaneously and independently measure electrically-induced glutamatergic transmission from cortical and thalamic pathways into the BLA, similar to the one previously described to measure GABAergic transmission. In fact, recent data
collected from Daniel Christian, a current graduate student in our lab, indicates that glutamatergic release probability from cortical inputs is not affected by either CIE or WD, while glutamate release from thalamic inputs may be enhanced (unpublished). These findings from cortical inputs are consistent with an earlier study from our lab that showed that CIE- and WD-induced increases in excitability in the BLA are independent of presynaptic terminal changes from the cortical input pathway (Lack et al., 2009). Although more experiments are required to further characterize these cortical and thalamic pathways, these preliminary findings suggest that the observed increase in glutamatergic activity during CIE and WD potentially arise from changes in the lateral amygdala. In addition to these future directions, we propose a major set of future experiments to more closely examine time-points at which our observed changes occur. As repeatedly discussed and mentioned throughout this discussion, the length of exposure to alcohol is a potentially significant confound that may contribute to many of the discussed discrepancies. As the GABAergic system was not significantly altered by CIE, we propose to characterize the glutamate system (i.e. measuring sEPSCs/mEPSCs and input-output curves) with fewer exposure days (<10) in addition to a longer exposure (>10 days). In addition to exposure length, we are also interested in elucidating the duration of withdrawal-induced increases in glutamatergic function within the BLA. Understanding the extent of glutamatergic changes would help clarify the physiology underlying the persistence of withdrawal symptoms, in particular anxiety.

Finally, investigating putative pharmacological therapeutics is of extreme interest. Specifically, manipulating the dopamine system via D3Rs antagonists or D1R agonists and measuring those effects on withdrawal-induced anxiety-like behavior. With these
studies we would have further evidence to support our hypothesis that chronic ethanol exposure alters the BLA dopamine system, leading to a shift in the interactions between GABAergic and glutamatergic transmission, ultimately resulting in increased anxiety-like behaviors.
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