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Glutamate Plasticity in the Drunken Amygdala: the Making of an Anxious Synapse

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Introduction

The enduring behavioral changes wrought by chronic alcohol abuse and alcoholism are among the most challenging obstacles for the development of effective therapies. These behaviors are currently believed to represent a shift from the initially rewarding effects of ethanol to an avoidance of the negative consequences during alcohol withdrawal following long-term abuse (Koob, 2003). One pervasive manifestation of chronic drug abuse in general and alcohol dependence in particular is the robust and persistent anxiety experienced during withdrawal and throughout abstinence. Long-abstinent alcoholics frequently cite anxiety as a significant contributing factor to relapse (Brown et al., 1990; Noone et al., 1999; Schneider et al., 2001; Thevos et al., 1991). Independent measures of anxiety in recently abstinent alcoholics suggest this negatively reinforcing emotion persists weeks-to-months after the acute withdrawal (Cohn et al., 2003) and can predict relapse rates in some populations (Roberts et al., 1999). Cause-effect relationships between alcohol dependence, relapse, and anxiety are none-the-less difficult to study in humans (Kushner et al., 2000; Merikangas et al., 1998; Rodgers et al., 2000). However, animal models clearly suggest that withdrawal-associated increases in anxiety-like behavior persist well after the acute withdrawal syndrome (Santucci et al., 2008) and contribute to subsequent increases in ethanol drinking (Valdez et al., 2002). While antecedent anxiety disorders contribute to ethanol drinking and abuse and are likely to make important contributions to the transition from use to dependence, we will focus specifically upon dependence-related anxiety and the excitatory synaptic mechanisms that potentially help regulate this persistent increase in anxiety following chronic ethanol exposure.

Enduring changes in behavior, whether initiated by environmental cues or by chronic drug and alcohol exposure, are most certainly dictated by long-term changes in neurotransmitter signaling and neuronal excitability. Recent evidence in animal models of cocaine (Borgland et al., 2004; Goussakov et al., 2006; Ungless et al., 2001) and opiate (Glass et al., 2005) exposure support the hypothesis that abused drugs can engage many of the cellular mechanisms that are believed to govern long-term changes in synaptic plasticity, particularly

within reward- and anxiety-related neural circuits. This neurobiological ‘allostasis’ likely reflects the subversion of synaptic and intrinsic mechanisms that normally serve such functions as learning and memory. This review will attempt to consolidate recent findings that suggest chronic ethanol exposure, like cocaine and opiates, can specifically up-regulate glutamatergic synaptic function in ways that parallel activity-related changes in synaptic efficacy.

What then are the neuro-anatomical substrates that govern anxiety-like behavior in general? There have been many excellent reviews discussing the neuroanatomical contributions to learned fear behaviors as well as innate anxiety-like responses to more naturally aversive environments (Charney and Deutch, 1996; Davidson, 2002; Davis, 2006; Mathew et al., 2008; Phelps and LeDoux, 2005). Critical to both learned fear-like and innate anxiety-like behaviors is the amygdala. As part of the limbic system, the amygdala serves as a central “hub” for information dealing with emotional behaviors and therefore sends/receives information from a number of brain regions. Highly processed sensory and cognitive information flows into the amygdala from the cortex and thalamus as well as the hippocampus (LeDoux, 1996). The amygdala subsequently projects to the lateral hypothalamus to regulate the HPA axis responses to learned fear (Gray et al., 1989) and to the parabrachial nucleus and dorsal motor nucleus of the vagus which modulate the autonomic and physical components of the conditioned fear (Davis, 1992). In addition, the amygdala modulates innate anxiety-related behaviors via projections to forebrain regions such as the prefrontal cortex, nucleus accumbens and bed nucleus of the stria terminalis which have been associated with risk-assessment (Jinks and McGregor, 1997; Simpson et al., 2001), reward (Prado-Alcala and Wise, 1984), and unconditioned fear (Walker and Davis, 1997), respectively. Hence the central contributions by the amygdala to both conditioned fear-responses and unconditioned anxiety-like behavior make it a likely candidate when looking for neural adaptations related to chronic ethanol exposure/withdrawal-associated increases in anxiety.

The amygdala itself contains several distinct nuclei which are all associated with specific efferent and afferent projections that serve to modulate conditioned and unconditioned behaviors. The lateral and basolateral nuclei (BLA) are the primary “input” regions of the amygdala. We refer to these regions together as ‘BLA’ to emphasize their cytoarchitectural similarities and their extensively overlapping efferent/afferent arrangements. These nuclei represent the initial ‘stop’ for cognitive and sensory information flowing into the anxiety circuit. The BLA integrates information from sensory and executive cortices, hippocampus, and sensory thalamus and sends glutamatergic efferents to the central nucleus of the amygdala (CeA), the bed nucleus of the stria terminalis, and the nucleus accumbens (De Olmos et al., 1985). Ninety to ninety-five percent of BLA neurons are large, pyramidal-shaped glutamatergic projection neurons and 5–10% small, non-pyramidal GABAergic interneurons (McDonald, 1982). The information processing in the BLA is dictated by local inhibitory GABAergic inputs and excitatory glutamatergic synaptic inputs arising both locally and from distant brain regions. The delicate balance between excitation and inhibition in the BLA ultimately influences the activity of the principal glutamatergic projection neurons.

Neuroadaptations within the BLA due to chronic ethanol and withdrawal would significantly impact the flow of information both within the amygdala as well as along amygdala efferent pathways and ultimately influence the expression of anxiety/fear-related behaviors. For example classic behavioral pharmacology experiments have shown that the BLA plays a central role in the acquisition of learned-fear behaviors (Blair et al., 2001; Fanselow and LeDoux, 1999; Maren, 2005) and can also modulate the expression of innate anxiety-like behaviors (McCool and Chappell, 2007; Sajdyk and Shekhar, 1997). Both behavior

measures are dependent upon BLA neurotransmitter systems and can be modulated by direct manipulation of glutamatergic (Fanselow and Kim, 1994; Sajdyk and Shekhar, 1997), GABAergic (Muller et al., 1997; Sanders and Shekhar, 1995), catecholaminergic (Gonzalez et al., 1996; Guarraci et al., 1999), and neuropeptide (Gutman et al., 2008; Sajdyk et al., 1999) systems. Consistent with this, we have also recently shown that microinjection of the AMPA receptor antagonist DNQX into the BLA can alleviate withdrawal-associated increases in anxiety-like behavior as they are represented in the light/dark transition test (Lack et al., 2007). Previous work has also found increased c-fos expression in the BLA following multiple withdrawals from an ethanol liquid diet (Borlikova et al., 2006). While these findings do not directly implicate glutamatergic dysregulation during ethanol withdrawal, the data do suggest a central role of the lateral/basolateral amygdala in the regulation of withdrawal-associated anxiety. We therefore began our investigations with examining glutamatergic alterations following chronic ethanol and withdrawal. Our rationale centered upon the important role this system plays in both learned and unconditioned amygdala-dependent behaviors.

Use-Dependent Synaptic Plasticity

Synaptic plasticity has been generally defined as a modification in synaptic efficacy resulting from some salient behavioral experience or overt experimental manipulation. This plasticity is currently believed to be the neurobiological mechanism that underlies long-term behavioral changes that manifest as learning and memory (Sigurdsson et al., 2007) or, in the context of the amygdala, Pavlovian fear conditioning (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997). The physiological manifestation of synaptic plasticity has a direction (increase/decrease or potentiation/depression of activity) and a time course (lasting minutes to days or short-term/long-term plasticity) that ultimately defines its character. While short-term changes in synaptic efficacy are clearly important for signal integration and processing, longer-lasting changes in synaptic function are arguably more relevant for enduring changes in behavior.

The hippocampus has provided the most detailed insights into the neurobiological mechanisms governing long-term synaptic plasticity at glutamatergic synapses. Hippocampal long-term potentiation (LTP) has been suggested as a possible mechanism underlying learning and memory formation. At the Schaffer collateral CA3-CA1 glutamatergic synapse, activity-dependent N-Methyl-D-Aspartate (NMDA) receptor activation initiates plastic changes in glutamatergic signaling via calcium influx and initiation of downstream second messenger systems. NMDA receptor-dependent activation of calcium-calmodulin dependent protein kinase II (CamKII) has been identified as a critical step in this process (Barria and Malinow, 2005) with additional important roles played by other second messenger systems (cAMP) and protein kinases (PKC and src-family kinases). Initiation of NMDA-independent forms of LTP at Schaffer collateral synapses likewise involves increases in intracellular calcium via voltage-gated calcium channels and subsequent activation of calcium-dependent kinases and signaling pathways (Jilla Sabeti, 2007; Stefani et al., 1998). These Schaffer collateral/CA1 initiation mechanisms, particularly calcium influx and calcium-dependent signaling processes, parallel similar initiation mechanisms at other hippocampal glutamatergic inputs (Colino and Malenka, 1993) and at glutamate synapses in many other brain regions (Berretta et al., 2008; Feldman et al., 1999) including the amygdala (see below). These similarities underscore the fundamental importance of calcium-dependent signaling pathways during the initiation of synaptic plasticity.

However, the mechanisms responsible for the expression of LTP at hippocampal Shaffer collateral-CA1 synapses has been the subject of some debate. It is now generally

accepted that LTP expression is the result of increased contributions by α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) receptors at the affected synapses. Calcium-dependent protein kinases (CamKII, PKC) or other calcium-activated signaling pathways, presumably activated by the influx of calcium through NMDA receptors, appear to phosphorylate AMPA receptors (Barria et al., 1997; Hayashi et al., 2000; Lee et al., 2009). This can directly increase receptor function (Derkach et al., 1999) but has also been shown to increase receptor trafficking from intracellular pools to dendritic membranes (Kerchner and Nicoll, 2008). As with initiation, AMPAR-dependent expression of synaptic plasticity is characteristic of LTP in other brain regions (Takegawa and Yuzaki, 2005; Yu et al., 2008).

In contrast to the postsynaptic initiation/expression at Schaffer collateral-CA1 synapses, the hippocampus also provides an example of presynaptic initiation and expression of synaptic plasticity. At the mossy fiber-CA3 glutamatergic synapses, use-dependent LTP is initiated by activation of calcium-permeable kainate-type glutamate receptors (Schmitz et al., 2003). This is believed to initiate calcium-dependent signaling in the presynaptic terminal and enhance neurotransmitter release by increasing quantal content (Xiang et al., 1994) and/or the number of release sites (Tong et al., 1996). Presynaptic initiation/expression of use-dependent synaptic plasticity is also found in other brain regions like the lateral (Tsvetkov et al., 2002) and central amygdala (Samson and Pare, 2005) and cerebellum (Bender et al., 2009). Thus synaptic plasticity can involve either pre- or post-synaptic neurobiological mechanisms depending upon the specific synapse involved.

For fear conditioning, the acquisition phase of the behavioral training was ultimately shown to involve NMDA-dependent (Fanselow and Kim, 1994), long-lasting plastic changes in glutamatergic synaptic transmission in the lateral/basolateral amygdala (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997). These LTP-like changes in lateral/basolateral amygdala neurophysiology were among the first to provide a direct link between synaptic plasticity in a specific brain region and long-term changes in behavior. LTP at thalamic- and cortical-lateral/basolateral amygdala synapses can utilize either NMDA-dependent activity (Drephal et al., 2006) or voltage-gated calcium channel signaling (Bauer et al., 2002; Chapman and Bellavance, 1992) depending upon the precise stimulation parameters and recording conditions. Both 'initiation' pathways result in the activation of CamKII (Chapman et al., 2003) and subsequent down-stream effectors (Huang and Kandel, 1998). These molecular pathways are very similar to those found in the hippocampus. These calcium-dependent signals are believed to increase AMPA receptor trafficking to the plasma membrane (Yu et al., 2008) which ultimately regulates the postsynaptic expression of plasticity in these studies. In contrast to these post-synaptic pathways, recent evidence also suggests that LTP at cortico-amygdala glutamatergic synapses can be attributed to presynaptic increases in neurotransmitter release (Humeau et al., 2003; Tsvetkov et al., 2002) and cAMP-dependent increases in presynaptic calcium channel function (Fourcaudot et al., 2009; Fourcaudot et al., 2008). Thus, use-dependent plasticity in the lateral/basolateral amygdala shares many of the same characteristics as hippocampal plasticity including the input-specific mechanisms (both pre- and post-synaptic) related to LTP initiation and expression.

Amygdala Use-dependent Synaptic Plasticity and Ethanol

Direct measures of the acute effects of ethanol on amygdala-dependent behaviors are complicated by the amnesic and sedative properties of ethanol. Despite this, doses of acute ethanol that suppress the acquisition of fear-conditioned behaviors in rats can be separated from higher doses that suppress motor function (Sonner et al., 1998), thus segregating a potential confound for measures of both fear-potentiated startle and anxiety-like behavior. Similar findings have been reported in mice (Gould, 2003) and importantly, in humans

(Moberg and Curtin, 2009). Given the critical role of the lateral/basolateral amygdala in the acquisition of fear-conditioned and anxiety-related behaviors, these findings suggest that the well-established learning/memory-impairing effects of acute ethanol likewise disrupt amygdala-dependent fear-learning.

The modulation of amygdala-dependent behaviors has also been studied in the context of chronic exposure and withdrawal. A history of binge drinking in humans can impair auditory fear conditioning (Stephens et al., 2005), a behavioral learning process that is dependent upon an intact amygdala (Bechara et al., 1995). This is paralleled by findings that withdrawal from chronic ethanol exposure can disrupt the acquisition of auditory fear-conditioning in rats (Stephens et al., 2001) and can impair contextual fear conditioning in mice (Celerier et al., 2000). Since the acquisition of contextual fear conditioning depends upon NMDA-dependent processes in the basolateral amygdala (Fanselow and Kim, 1994), these findings together imply that chronic ethanol and withdrawal must influence amygdala neural plasticity and subsequently impair the acquisition of fear-conditioning. In fact, repeated withdrawal from a chronic ethanol liquid diet attenuates calcium-dependent postsynaptic LTP induction at rat cortical-lateral amygdala and Schaffer collateral-CA1 glutamatergic synapses (Roberto et al., 2002; Stephens et al., 2005). This might be interpreted as a simple ‘inhibition’ of plasticity initiation or expression in this brain region following chronic ethanol and withdrawal. However, withdrawal from chronic ethanol increases anxiety-like behavior; and glutamate signaling in the lateral/basolateral amygdala plays a critical role in this withdrawal-associated anxiety (Lack et al., 2007). Both seem inconsistent with any direct suppression of glutamate signaling. These inconsistencies set the stage for our recent attempts to directly measure the glutamate signaling pathways responsible for the initiation and expression of amygdala LTP following chronic ethanol exposure and withdrawal.

Lateral/Basolateral Amygdala, Chronic Ethanol, and Glutamatergic Mechanisms Associated with Plasticity Initiation

NMDA Receptors and Chronic Alcohol/Withdrawal

To understand the inhibitory effect of chronic ethanol/withdrawal on LTP initiation/ expression in the amygdala, we began by exploring functional alterations of NMDA-type glutamate receptors. NMDA receptors are heteromeric receptors whose subunits include the NR1 as well as NR2A-D subunits; the NR1 subunit is necessary for the formation of a functional channel while NR2 subunits confer native pharmacological and biophysical properties to the receptor complex. All NMDA NR1-NR2 subunit combinations are inhibited by acute ethanol, however, ethanol appears to be most potent at NMDA receptors containing combinations of NR1 paired with either NR2A or 2B (Masood et al., 1994). Parallels to these heterologous system studies are evident in whole-cell NMDA-gated currents expressed by cultured neurons (Lovinger, 1995; Lovinger et al., 1989; Popp et al., 1999) and by acutely isolated neurons (Criswell et al., 2003; Grover et al., 1998). In addition to the acute ethanol inhibition of NMDA receptors, chronic ethanol exposure up-regulates native NMDA receptor function in many different brain regions (Grover et al., 1998; Gulya et al., 1991). This functional increase is sometimes associated with increased subunit mRNA and protein expression (Follesa and Ticku, 1995; Snell et al., 1996) but may also be related to increased trafficking of NMDA receptors from intracellular pools to the cell surface (Carpenter-Hyland et al., 2004). Given the central role of NMDA receptors in the initiation of synaptic plasticity, it seemed reasonable to hypothesize that the ethanol-related effects on amygdala LTP might involve alterations in NMDA receptor function.

We recently examined the impact of chronic intermittent ethanol vapor inhalation on rat NMDA receptor synaptic function in principal neurons found in the lateral/basolateral amygdala. This paradigm exposes rats to ethanol vapor 12 hours/day for 10 consecutive days; and blood ethanol levels at the end of the exposure are typically >200mg/dL. Glutamatergic synaptic currents were measured using whole-cell patch-clamp methods and electrical stimulation of 'local' afferents. We found that electrically-evoked NMDA-mediated synaptic currents were increased in BLA slices prepared from intoxicated animals sacrificed immediately following the last ethanol exposure (CIE) as well as animals sacrificed after 24 hours of withdrawal (WD) (Lack et al., 2007) relative to control (air-exposed) animals. Both CIE and WD caused a left-and up-ward shift in a NMDAR-mediated stimulus-response relationship indicating greater synaptic activation of these receptors at a given electrical stimulus. These findings suggest that either NMDAR-mediated synaptic currents were more sensitive to electrically-evoked glutamate release or that NMDAR function/expression at the synapse was increased by chronic ethanol and withdrawal. The latter interpretation was supported by another set of experiments examining the relative contribution of NMDA- and AMPA-mediated synaptic currents. In this study, the amplitude of a NMDAR-mediated synaptic current was normalized to the amplitude of an AMPA receptor-mediated synaptic current. This NMDA:AMPA ratio was increased in BLA neurons recorded from both CIE and WD slices. Since AMPA receptor function is increased in animals following both chronic ethanol and withdrawal (see below), increases in the NMDA:AMPA ratio would be due to a relatively greater contribution by NMDA receptors. These findings are consistent with the hypothesis that chronic ethanol and withdrawal enhance synaptic NMDA receptor expression/function in the BLA. Additional studies on acutely isolated BLA neurons also found an increase in NMDAR-mediated current density following chronic exposure to an ethanol-containing liquid diet (Floyd et al., 2003). This increased function was associated with decreased calcium-dependent desensitization and increased sensitivity to the NR2B-selective allosteric inhibitor ifenprodil, but did not change acute ethanol sensitivity. Levels of BLA NR1 subunit mRNA were altered in these animals, but expression level and cellular distribution of NR2A and NR2B mRNAs remained unchanged. We also found that the chronic ethanol exposure did not alter NR1, NR2A, or NR2B peptide levels (unpublished observations). Together the synaptic and whole-cell measures suggest a substantial up-regulation in the function of BLA NMDA receptors following chronic ethanol exposure. Although the molecular mechanism responsible for this effect remains to be clearly established, the absence of any substantive modulation of NMDAR subunit expression suggest that non-genomic mechanisms, such as increased trafficking of NMDA receptors to the cell surface (Carpenter-Hyland et al., 2004), are likely to be engaged by chronic ethanol exposure in the BLA.

Chronic ethanol also alters NMDA receptors in the neighboring central amygdaloid nucleus. While many of these changes are similar to those we have identified in the BLA, some are distinct. For example chronic ethanol inhalation increased the ifenprodil sensitivity of NMDAR-mediated synaptic responses in central amygdala neurons (Roberto et al., 2004b) similar to our results in acutely isolated neurons (Floyd et al., 2003). However, unlike BLA NMDA receptors, the synaptic function of central amygdala NMDA receptors is unchanged by chronic ethanol exposure (Roberto et al., 2006; Roberto et al., 2004b) while the acute ethanol sensitivity of NMDA-mediated synaptic currents is increased (Roberto et al., 2006; Roberto et al., 2004b). Even the molecular alterations of BLA and central amygdala NMDA receptors in response to chronic ethanol/withdrawal share some characteristics but differ in others. Similar to the BLA, chronic ethanol inhalation increases NR1 subunit mRNA levels in the central amygdala but also increases NR2B mRNA levels and NR2A/2B subunit peptide expression (Roberto et al., 2006). These effects appear transient as NR1- and NR2-subunit mRNA expression is significantly decreased compared to control levels following 1 week of withdrawal from chronic alcohol and return to control levels 2 weeks after the last

ethanol exposure. Similarly, protein levels for these subunits also return to control levels, but much sooner—at 1 week of withdrawal (Roberto et al., 2006). It is important to note that methodological differences may influence the unique chronic ethanol/withdrawal-dependent alterations in central and lateral/basolateral amygdala NMDA receptors. For example, a more modest chronic exposure to an ethanol-containing liquid diet caused only minimal effects to NR2A subunit mRNA and peptide expression and did not alter NR1 or NR2B mRNA or protein expression (Lack et al., 2005). This suggests that exposure duration or intensity might dramatically influence NMDA receptor alterations. Since NMDA receptors initiate plastic changes in synaptic activity in the amygdala, we subsequently examined the influence of chronic ethanol/withdrawal on neurobiological mechanisms involved with the expression of synaptic plasticity.

Kainate receptor-dependent Glutamatergic Plasticity in the Amygdala

Kainate receptors are tetrameric cation channels made up of five possible subunits with GluR₅₋₇ needed for functional channels, as well as KA₁₋₂ (reviewed in (Braga et al., 2004)). Similar to AMPA receptors, kainate receptors exhibit rapid activation and desensitization kinetics (reviewed in (Pinheiro and Mulle, 2006)). The receptors can be subject to post-transcriptional RNA editing at a Q/R site in the membrane re-entrant domain that directly modulates calcium permeability of the receptor (Burnashev et al., 1995). Kainate receptor subunits are found throughout the nervous system including the cortex, striatum, hippocampus, cerebellum, retina, spinal cord, dorsal root ganglia and amygdala (reviewed in (Huettnner, 2003)). These receptors share many molecular and functional characteristics with AMPA receptors yet form their own unique receptor assemblies (Puchalski et al., 1994). The historical precedent grouping AMPA and kainate glutamate receptors together was based largely on the absence of any pharmacological tools to functionally segregate native channels. However, it is worth noting that, unlike AMPA receptors (see below), kainate receptors expressed in both heterologous and native systems are inhibited by relatively low concentrations of acute ethanol (Costa et al., 2000; Dildy-Mayfield and Harris, 1992; Valenzuela et al., 1998; Weiner et al., 1999). This suggests a potentially unique contribution of kainate receptors to glutamatergic alterations in response to chronic ethanol and withdrawal.

Over the last decade a number of receptor-selective compounds have been developed that differentiate between AMPA and kainate receptors (reviewed in (Jane et al., 2009)). The compound GYKI 53655 is a non-competitive antagonist of the AMPA receptors and has allowed direct measurement of native kainate receptor-mediated currents in the absence of a contaminating AMPA-mediated component (Paternain et al., 1995). Conversely, UBP 296, a selective and competitive kainate receptor antagonist, allowed investigators to confirm that isolated GYKI53655-insensitive currents were in fact kainate receptor-mediated (Andrasfalvy and Magee, 2004). This pharmacology has recently allowed the characterization of neurobiological processes mediated specifically by kainate receptors. In the lateral/basolateral amygdala for example, evidence from a number of studies indicate that GluR5-containing kainate receptors mediate excitatory synaptic responses that can be directly measured in principal neurons (Li et al., 2001; Li and Rogawski, 1998; Rogawski et al., 2001). Kainate receptor-mediated responses can represent up to 30% of the overall glutamatergic synaptic response measured in a principal BLA neuron (Li and Rogawski, 1998). Trains of electrical stimuli can increase the magnitude of this synaptic kainate current up to 190% suggesting that kainate receptors may be most active under conditions of “strong synaptic drive” (Li and Rogawski, 1998). These same studies showed that kainate receptor-mediated postsynaptic currents can be evoked from cortical inputs arriving via the external capsule but appear to be absent from other glutamatergic inputs. We recently showed that kainate receptor mediated BLA synaptic responses are inhibited by both the competitive

antagonist UBP296 and by acute ethanol (Lack et al., 2008). In fact acute ethanol was more potent at inhibiting kainate receptor-mediated synaptic currents than at NMDA-mediated synaptic responses in the same neurons.

Kainate receptor-dependent synaptic plasticity in the amygdala appears to have a much different biological profile from that expressed in the hippocampus (see above). BLA Kainate receptors mediate a form of heterosynaptic plasticity (Li et al., 2001) where receptor activation at one synapse can cause plastic changes in other adjacent glutamatergic synapses on the same neuron. This decidedly 'postsynaptic' plasticity can be initiated by both low frequency electrical stimulation (1Hz, 15min) and the kainate receptor-selective agonist (RS)-2-amino-3(3-hydroxy-5-tert-butylisoxazol-4-yl)propanoic acid (ATPA). Using field excitatory post-synaptic potentials we were recently able to replicate the findings that application of kainate receptor agonist ATPA leads to long-term increases in synaptic strength in the BLA (Lack et al., 2008). Acute ethanol blocked the increased synaptic strength seen with ATPA administration. Confirmation that this effect was kainate receptor-mediated was demonstrated when we were able to inhibit ATPA-LTP with the specific kainate receptor antagonist, UBP 296. Since activation of BLA kainate receptors increases anxiety-like behaviors (Lack et al., 2008), this research suggests that the inhibitory effects of acute ethanol on kainate receptors may contribute to the anxiolytic behaviors elicited by ethanol intoxication.

The acute ethanol sensitivity of BLA kainate receptors and their ability to initiate plastic changes in glutamatergic synaptic transmission suggests parallels with NMDA receptors in this brain region. However the effects of chronic ethanol or withdrawal on kainate receptors have not been extensively studied. Chronic ethanol/withdrawal does not affect protein expression for kainate receptor subunits in cultured cortical neurons (Chandler et al., 1999) but enhances both subunit protein and kainate receptor function in cultured hippocampal neurons (Carta et al., 2002). Supporting this latter study, chronic cocaine exposure increases kainate receptor mRNA and protein in both rats and monkeys (Ghasemzadeh et al., 1996; Hemby et al., 2005). Given these findings and the prominent role for kainate receptors in postsynaptic glutamatergic responses in the BLA, we recently examined the effects of chronic ethanol and withdrawal on kainate receptor neurophysiology in this brain region. Using the same ethanol exposure paradigm employed for NMDA receptors, we found that chronic ethanol exposure enhanced BLA kainate receptor-mediated synaptic responses relative to control neurons (Lack et al., 2009). Unlike NMDA receptors, this effect was entirely reversed after twenty-four hours of withdrawal from the exposure. In this same study, we subsequently showed that chronic ethanol and withdrawal significantly reduced ATPA-induced synaptic plasticity. These data provide remarkable parallels with our findings with NMDA receptors. Namely chronic ethanol and/or withdrawal enhance the function of both NMDA and kainate receptors—receptors that both initiate forms of synaptic plasticity in the lateral/basolateral amygdala—but paradoxically reduces glutamatergic synaptic plasticity in this same brain region.

Chronic Alcohol, Withdrawal, and the Expression of Synaptic Plasticity

Amygdala AMPA Receptors

AMPA-type ionotropic glutamate receptors mediate the vast majority of excitatory neurotransmission in the nervous systems. These receptors are composed of four closely related subunits, GluR1-4, that form tetrameric assemblies to produce functional channels (Rosenmund et al., 1998). And GluR1-4 subunits are all expressed within the rat BLA (Farb et al., 1995; McDonald, 1994). The specific subunit composition of the native channel dictates ion permeability. While all AMPARs are non-selectively permeable to cations like sodium and potassium, subsets of these channels, particularly those lacking the GluR2

subunit, are also permeable to calcium (Bochet et al., 1994). GluR2-less channels are in fact found in BLA interneurons (McDonald, 1996) where they mediate synaptic Ca^{2+} signaling and initiate plastic changes in GABAergic function (Szinyei et al., 2007). In BLA projection neurons, AMPA receptors are responsible for the expression of use-dependent plasticity at glutamatergic synapses (Humeau et al., 2007; Yu et al., 2008). Thus BLA AMPA receptors mediate a complex array of neurophysiological functions.

AMPA-type glutamate receptors seem to be relatively insensitive to modulation by acute ethanol. For example, 80mM ethanol did not alter synaptic AMPA-mediated currents measured in hippocampal dentate granule neurons in either rats or monkeys despite robust ethanol-inhibition of NMDA-mediated synaptic currents in these same neurons (Ariwodola et al., 2003). AMPA-mediated synaptic responses from locus coeruleus (Nieber et al., 1998) and spinal motoneurons (Ziskind-Conhaim et al., 2003) are likewise insensitive to the direct, postsynaptic effects of acute ethanol. This rather modest sensitivity of AMPA receptors to intoxicating concentrations of acute ethanol has also been noted in whole-cell currents from cultured cortical neurons (Lovinger, 1993), acutely isolated medial septum/diagonal band neurons (Frye and Fincher, 2000), and AMPA-gated whole-cell currents recorded from nucleus accumbens neurons in brain slices (Nie et al., 1994). This latter finding contrasts with earlier work in the nucleus accumbens showing robust ethanol sensitivity of AMPA-mediated excitatory postsynaptic potentials (Nie et al., 1993). This effect was reversed with the opioid antagonist naloxone suggesting an indirect ethanol modulation of the EPSP in this instance. However, it should be noted that 60mM acute ethanol modestly inhibits (20%) AMPA-mediated synaptic responses in the central amygdala (Roberto et al., 2004b). Similarly, acute ethanol robustly inhibits AMPA-gated whole-cell currents recorded from neonatal (P4) but not juvenile (P26) CA3 hippocampal neurons (Mameli et al., 2005). These findings suggest that intoxicating concentrations of acute ethanol produce relatively modest effects on AMPA receptor function in most cases but that the magnitude of this modulation may be developmentally regulated and brain region-dependent.

There has been very limited interest in the effects of chronic ethanol on AMPA-type glutamate receptors. Chronic exposure does seem up-regulate AMPA receptor subunit mRNA levels in hippocampus (Bruckner et al., 1997), AMPAR subunit protein expression in primary cortical cultures (Chandler et al., 1999), AMPA receptor-dependent calcium signaling in cerebellar Purkinje neurons (Netzeband et al., 1999), and AMPA receptor binding in cortical membranes (Haugbol et al., 2005). However, AMPA receptor synaptic function following chronic ethanol exposure has not been extensively studied.

Our laboratory recently found that AMPA receptor synaptic function was significantly up-regulated in the BLA during withdrawal from chronic ethanol inhalation. We showed specifically that the amplitude of AMPA-mediated, TTX-insensitive spontaneous glutamatergic events (mEPSCs) recorded from BLA projection neurons was potentiated in slices from 24 hour withdrawal animals, but not from slices prepared from CIE animals (Lack et al., 2007). This increased synaptic function was also associated with an increase in the decay kinetics of AMPA-mediated mEPSCs. These findings are consistent with altered BLA postsynaptic AMPA receptor synaptic function. Although the mechanism for the increase mEPSC amplitude is unclear at present, preliminary data suggests that neither chronic ethanol nor withdrawal increases AMPA receptor subunit peptide levels in the BLA (unpublished observations). Despite this, withdrawal from chronic benzodiazepine treatment increases AMPA-mediated whole-cell current density (Song et al., 2007), increases mEPSC amplitude (Van Sickel et al., 2004), and reduces hippocampal LTP expression (Shen et al., 2009). These findings are very similar to the effects of chronic ethanol/withdrawal in the BLA. Importantly, chronic benzodiazepine treatment increases the delivery of GluR1-containing AMPA receptors to hippocampal glutamatergic synapses (Das et al., 2008). This

is similar to the increased delivery of GluR1 subunits to the synaptic compartment following synaptic plasticity in the barrel cortex which is associated with faster mEPSC decay (Clem and Barth, 2006). Together these data suggest that the altered postsynaptic properties of BLA AMPA-mediated mEPSCs following withdrawal from chronic ethanol might involve increased trafficking of AMPA receptors to the cell surface.

BLA Extracellular Recordings

The implication of increased AMPA-receptor signaling is that BLA neurons will be more responsive to glutamatergic inputs during chronic ethanol/withdrawal. In support of this, we recently found that chronic ethanol/withdrawal increased extracellular excitatory postsynaptic potentials (field EPSPs) measured in BLA brain slices by electrical stimulation of cortical inputs (Lack et al., 2009). This increase was evident across a range of electrical stimulation intensities and occurred in the absence of any significant presynaptic effect of chronic ethanol or withdrawal at these particular glutamatergic synapses (see below). These data suggest that chronic ethanol and withdrawal increase the responses of BLA neurons to electrical stimulation via increased excitability of individual neurons and/or increased AMPA receptor-mediated synaptic responses. Since BLA fEPSPs are entirely sensitive to the AMPA receptor antagonist CNQX (unpublished observations), it is difficult to differentiate between these possible mechanisms. However chronic ethanol increases the extracellular population EPSP recorded in CA1 hippocampus (Whitington and Little, 1990) suggesting this may be a common outcome. Regardless, increased neuronal responsiveness during chronic ethanol/withdrawal would certainly interfere with traditional postsynaptic measures of LTP expression.

Presynaptic Glutamatergic Function

In addition to the altered postsynaptic characteristics seen during withdrawal from chronic ethanol, we also found that the glutamatergic synapses themselves were altered by the exposure. CIE and WD increased the frequency of mEPSCs recorded from BLA projection neurons (Lack et al., 2007). This is difficult to interpret in the face of the increased mEPSC amplitude found in that same study. However, there were two pieces of evidence that supported the hypothesis that chronic ethanol/withdrawal altered presynaptic glutamatergic function in this brain region. First, we found increases in the probability of glutamate release when measured by pairs of locally applied electrical stimuli. Second, we found an increased frequency of spontaneous EPSCs in CIE slices relative to control, but not when tetrodotoxin was present in the extracellular bath. These findings are consistent with the idea that glutamatergic release is significantly elevated in the BLA during both CIE and withdrawal, albeit via distinct, TTX-sensitive and -resistant mechanisms. Interestingly, chronic ethanol treatment also enhances presynaptic glutamate release (Zhu et al., 2007) and increases extracellular glutamate levels (Roberto et al., 2004b) in the neighboring central amygdala.

The precise mechanisms governing these presynaptic alterations are not clear. However chronic ethanol increases the number of glutamatergic terminals in the shell of the nucleus accumbens (Zhou et al., 2006). Alternatively acute ethanol can depress glutamate release via inhibition of voltage-gated calcium channels in some brain regions (Mameli et al., 2005; Zhu et al., 2007). It is possible then that adaptations increasing presynaptic voltage-gated calcium channels might underlie enhanced glutamate release during chronic ethanol/withdrawal. It should be noted that presynaptic effects of chronic ethanol are not consistently observed, even in the BLA. Our laboratory has shown that presynaptic release probability at cortical glutamatergic inputs seems insensitive to chronic ethanol/withdrawal (Lack et al., 2009). Given that 'local' stimulation within the BLA itself reveals clear increases in glutamatergic release probability (Lack et al., 2007), these data suggest that presynaptic effects of chronic ethanol/withdrawal may be input-specific. Regardless, the

increased glutamatergic presynaptic function at 'local' synapses, similar to enhanced postsynaptic function of AMPA receptors, parallels those mechanisms responsible for the expression of use-dependent synaptic plasticity at hippocampal mossy fiber terminals.

Ethanol-dependent Plasticity of Additional Amygdala Neurotransmitter Systems

This review has focused primarily upon the plasticity of glutamatergic ionotropic receptors following chronic ethanol and withdrawal. However, we must emphasize that there are many additional signaling pathways which can contribute to increased neuronal output from the BLA. Along with the pre- and post-synaptic glutamatergic alterations described here, these additional pathways could also help regulate the enhanced anxiety-related behaviors evident during withdrawal. For example, increased glutamate release from some synaptic inputs might potentially enhance signaling by G protein-coupled metabotropic glutamate receptors. In fact, chronic ethanol/withdrawal enhances mGluR-dependent phosphoinositide hydrolysis in hippocampus (Valles et al., 1995) and intracellular calcium levels in cultured Purkinje neurons (Netzeband et al., 2002). Since Group I mGluR activation in the BLA enhances the acquisition of fear-conditioned behaviors (Rudy and Matus-Amat, 2009), increased mGluR signaling during withdrawal might provide an additional glutamatergic influence to tip the balance towards enhanced BLA output and increased anxiety-like behavior.

In addition to excitatory neurotransmission, BLA output is regulated by a balance between excitatory and inhibitory synaptic and intrinsic activity. Like many brain regions, fast synaptic inhibition in this brain region is governed by GABAergic mechanisms. Chronic ethanol exposure alters BLA GABA_A receptor expression and function (Anderson et al., 2007; Floyd et al., 2004; McCool et al., 2003; Papadeas et al., 2001). Recent work also suggests that GABAergic synaptic responses evoked by electrical stimulation of the lateral external capsule are substantially reduced by withdrawal from chronic ethanol and diazepam treatments as well as repeated restraint-stress (Isoardi et al., 2007). While the effects of chronic ethanol/withdrawal on BLA GABAergic synaptic transmission remain to be fully characterized, chronic ethanol enhances both GABA presynaptic release and extracellular GABA levels in the neighboring central amygdala (Roberto et al., 2004a). This CeA up-regulation is contrasted with diminished GABAergic synaptic/extrasynaptic function in the hippocampus (Cagetti et al., 2003; Liang et al., 2007; Liang et al., 2006) and reduced expression of GABA_A subunit mRNAs/proteins in several different brain regions (reviewed in (Kumar et al., 2009)). Thus, GABAergic neuroadaptations to chronic ethanol/withdrawal in the BLA are likely to be complex and potentially specific to this brain region.

In addition to the glutamate and GABA systems, BLA catecholamine and neuropeptide neurotransmitters also play an important regulatory role during chronic ethanol and withdrawal. For example, D1 receptor activation enhances GABAergic synaptic activity arising from local feed-back neurons (Kroner et al., 2005) while providing simultaneous suppression of GABAergic activity arising feed-forward inhibitory synapses (Marowsky et al., 2005). Similarly, BLA mu-opioid receptors suppress GABAergic but not glutamatergic synaptic function (Finnegan et al., 2006). These data suggest that increased binding density of BLA D1-like receptors (Sari et al., 2006) and mu-opioid receptors (Djouma and Lawrence, 2002) following chronic ethanol drinking may dramatically alter GABAergic synaptic signaling in this brain region. However this remains to be more directly established.

Finally, chronic ethanol/withdrawal may modulate the intrinsic membrane properties of lateral/basolateral amygdala neurons. Oddly, single unit recordings of lateral amygdala neurons in awake animals have consistently shown *reduced* firing rates during ethanol

withdrawal (Feng and Faingold, 2008; Feng et al., 2007). This seems inconsistent with the emerging picture of an 'excitable' BLA following chronic ethanol. However, interpretation of *in vivo* BLA data is complicated by the robust tonic activity and high intrinsic firing rates of GABAergic interneurons relative to glutamatergic principal neurons (Rainnie et al., 1993; Washburn and Moises, 1992). These characteristics suggest that GABAergic interneurons might be over-represented in the population of cells sampled during *in vivo* unit recordings. Similarly glutamatergic projection neurons increase firing in response to emotionally-salient (Chang et al., 2005; Hobin et al., 2003; Pelletier et al., 2005) but not emotionally-neutral environmental stimuli (Cromwell et al., 2005). Since most *in vivo* BLA studies to date employ habituated behaviors during the unit recording, they would also tend to under-represent contributions by glutamatergic projection neuron firing during ethanol withdrawal. Regardless, the precise effect of chronic ethanol exposure on the excitability of projection neurons and local-circuit feed-forward and feed-back interneurons remains to be characterized. Ethanol withdrawal reduces the firing threshold and increases spontaneous firing rates of periaqueductal grey neurons (Yang et al., 2002). Potential cellular mechanisms regulating increased excitability might include increased voltage-gated calcium channel function (Whittington and Little, 1993) or reduced function of calcium-dependent potassium channels or non-selective hyperpolarization activated cation (I_h) channels (Hopf et al., 2007) that serve to re-polarize or delay recovery from the after-hyperpolarization following an action potential, respectively. While future studies will focus on the effects of chronic ethanol and withdrawal on intrinsic membrane properties and conductances, interactions between BLA neuron excitability and the synaptic contributions outlined in previous sections would ultimately govern the increased BLA-dependent anxiety-like behavior expressed during ethanol withdrawal.

Conclusions and Future Directions

Our work and the work of others have shown that chronic ethanol exposure and withdrawal can enhance function of glutamatergic receptors responsible for both the initiation and expression of BLA synaptic plasticity. The increased NMDA and kainate receptor function both speak to the diversity of LTP initiation-related signaling processes co-opted by a model of chronic alcohol exposure. Similarly, the enhanced AMPA receptor function, neuronal responsiveness, and presynaptic glutamatergic function that is evident following chronic ethanol/withdrawal suggest that mechanisms responsible for the expression of synaptic plasticity are likewise engaged. It is worth noting that these expression-related mechanisms are generally more resistant to the acute effects of ethanol suggesting that these alterations result from other cellular processes rather than a direct compensatory change to ethanol-related altered function. Our findings suggest that, rather than a simple inhibition of the mechanisms governing the use-dependent initiation or expression of glutamatergic plasticity in the lateral/basolateral amygdala, chronic ethanol and withdrawal appear to occlude experimental activation of these processes by engaging these very same mechanisms.

A model representing ethanol/withdrawal-related changes in glutamatergic function is shown in Figure 1. In the chronic ethanol/intoxicated condition, we found increased postsynaptic function of both NMDA and kainate receptors (Lack et al., 2009; Lack et al., 2007). It is not clear yet if this increase can be found across all the various glutamatergic inputs arriving at the principal neurons from which we made these measures or if these postsynaptic alterations are selectively expressed at specific synaptic inputs. We also found that chronic ethanol may increase the frequency/amplitude of these AMPA-mediated events via the enhanced function of tetrodotoxin-sensitive mechanisms. Specifically, tetrodotoxin, a potent blocker of voltage-gated sodium channels, appeared to inhibit the increased amplitude/frequency of spontaneous EPSCs recorded from CIE neurons. This suggests that action potential-dependent processes help mediate some effects on glutamatergic

neurotransmission immediately following chronic ethanol exposure. This represented in the model by increased numbers of voltage-gated channels (Brodie and Sampson, 1990; McMahon et al., 2000; Watson and Little, 1999) but might also be manifested as decreased expression of ethanol-sensitive potassium channels (Mulholland et al., 2009; Pietrzykowski et al., 2004; Sun et al., 2008) that serve to re-polarize or hyperpolarize the presynaptic compartment and thus reduce or control presynaptic neurotransmitter release. We have not included this later possibility in the model for simplicity, but both mechanisms are plausible in that they would ultimately produce a tetrodotoxin-sensitive increase in spontaneous EPSC amplitude/frequency in the chronic ethanol exposed animals.

In the withdrawal rats, postsynaptic alterations differ considerably between specific receptor families. The increased function of NMDA receptors measured immediately following chronic exposure is maintained at least 24hr after the exposure while kainate receptor function rapidly returns to control levels during this same period. In neither instance have we found any substantial changes in subunit protein levels (unpublished data). This suggests that any other mechanisms, like receptor trafficking or localization, must also be modified by chronic exposure/withdrawal in a receptor-dependent fashion. We also found that, unlike glutamatergic synapses measured during chronic ethanol exposure, presynaptic effects of withdrawal shift to a form that is resistant to tetrodotoxin and therefore independent of action potentials. Although the precise mechanism for this is unclear, increased presynaptic function can be associated with a change in release probability at individual synapses via an increase in the number of release sites (Bender et al., 2009; Tong et al., 1996), increased quantal size of individual vesicles (Bellingham et al., 1998; Wilson et al., 2005), or some combination of these mechanisms. Distinguishing between these possible mechanisms is an important future direction. However, increased release probability during withdrawal was evident at 'local' glutamate synapses (Lack et al., 2007) but not cortical synapses arriving via the external capsule (Lack et al., 2009). Thus, like chronic ethanol, the presynaptic alterations evident during withdrawal may be input-specific.

Future studies are likely to focus on the precise molecular mechanisms which govern both pre- and post-synaptic functional alterations resulting from chronic ethanol exposure and withdrawal. Additional aspects to be explored relate to the duration of exposure needed to achieve a particular adaptation, the durability of the glutamatergic alterations we and others have described to date, and potential contributions by other neurotransmitter systems. Regardless, our evidence suggests that chronic ethanol exposure and withdrawal 'chemically condition' the lateral/basolateral amygdala using glutamatergic mechanisms that closely parallel those engaged by Pavlovian fear-conditioning. The enduring behavioral changes resulting from environmental cue-dependent synaptic plasticity in this brain region likewise parallel the enhanced anxiety-like behaviors that are characteristic of ethanol dependence. It seems reasonable to propose that the cellular mechanisms governing the extinction of learned fear may also provide attractive therapeutic targets for ethanol withdrawal-associated anxiety.

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Figure 1.

Model describing glutamatergic alterations measured in lateral/basolateral amygdala principal neurons following chronic ethanol exposure and withdrawal. Pre-synaptic (purple) and post-synaptic (green) specializations represent a 'generic' glutamatergic synapse. Synaptic responses mediated by postsynaptic AMPA (red)-, kainate (blue)-, and NMDA (yellow)-type glutamate receptors can be measured in ethanol naïve (left) neurons (Lack et al., 2008; Lack et al., 2009; Lack et al., 2007). Chronic ethanol exposure (middle) enhances NMDA and kainate receptor synaptic function (here represented as increased numbers of receptors found at the postsynaptic specialization) and increases the probability of glutamate release measured by paired stimuli and the frequency spontaneous AMPA mediated synaptic events. Since this effect is sensitive to tetrodotoxin, we presume presynaptic alterations involve voltage- or calcium-dependent processes. During withdrawal (right), kainate receptor-mediated synaptic responses return to control levels while AMPA receptor-mediated responses are markedly increased, possibly due to increased numbers of receptors at the synapse. Like chronic ethanol, withdrawal also increases presynaptic function but these changes become resistant to tetrodotoxin—here represented as increased numbers of vesicles but could also involve increases in the probability of release or quantal content at single synapses or increases in the total number of release sites. See text for details.