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Synaptic Targets: Chronic Alcohol Actions

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Abstract

Alcohol acts on numerous cellular and molecular targets to regulate neuronal communication within the brain. Chronic alcohol exposure and acute withdrawal generate prominent neuroadaptations at synapses, including compensatory effects on the expression, localization and function of synaptic proteins, channels and receptors.

The present article reviews the literature describing the synaptic effects of chronic alcohol exposure and their relevance for synaptic transmission in the central nervous system. This review is not meant to be comprehensive, but rather to highlight the effects that have been observed most consistently and that are thought to contribute to the development of alcohol dependence and the negative aspects of withdrawal. Specifically, we will focus on the major excitatory and inhibitory neurotransmitters in the brain, glutamate and GABA, respectively, and how their neuroadaptations after chronic alcohol exposure contributes to alcohol reinforcement, dependence and withdrawal.

Keywords

alcohol/ethanol; GABA; glutamate; synaptic transmission; presynaptic; postsynaptic; protein phosphorylation; intoxication; tolerance; dependence

1.0 Chronic Ethanol Effects on Synaptic Transmission

Alcohol use disorders (AUDs) cause large medical, economic and social burdens worldwide. It is widely accepted that alcohol/ethanol (EtOH) acts in the central nervous system (CNS) to alter neuronal function. The synapse is the central point of communication between neurons and it is among the most sensitive sites of ethanol's actions. In the CNS, a number of cellular and molecular targets involved in synaptic transmission are profoundly altered by both acute and chronic EtOH exposure. Generally, low to intermediate concentrations of EtOH (5–50 mM, corresponding to a few drinks to complete intoxication) act on a variety of

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synaptic targets, including, but not limited to, ion channels, neurotransmitter receptors and intracellular signalling proteins.

Animal models of chronic EtOH exposure provide important information relevant to the human neurobiology and pathophysiology of long-term alcohol abuse. Chronic EtOH exposure produces both tolerance and dependence in humans, and critically, similar effects are observed in rodent and non-human primate models. Alcohol tolerance, which is acquired after repeated exposures, is characterized by decreased behavioral responses to EtOH, such that more EtOH is required to achieve the same intoxicating effects observed after an initial exposure. Alcohol dependence is a chronic relapsing disorder characterized by alcohol preoccupation, loss of control over intake and a negative emotional state (Koob and Volkow, 2010). Dependence is generally described by the symptomology elicited during and following EtOH withdrawal (Heilig et al., 2010; Koob, 2003; Koob and Volkow, 2010, 2016; Tsai and Coyle, 1998), including anxiety, dysphoria, increased seizure susceptibility, hyperalgesia and sleep disturbances (Enoch, 2008; Grobin et al., 1998; Kumar et al., 2009; Ron and Barak, 2016). Central to understanding and treating the pathophysiology of alcohol dependence is characterizing the brain region-specific neuroadaptions in glutamatergic and GABAergic synaptic transmission that occur with chronic alcohol exposure.

1.1 Glutamate and EtOH Effects

Acute EtOH generally inhibits glutamatergic neurotransmission, while chronic EtOH exposure and acute withdrawal tend to enhance it. It is important to note here that many of the studies that have investigated the effects of *in vivo* chronic ethanol exposure on synaptic function have been conducted during an acute *in vitro* withdrawal period (i.e. the absence of ethanol for ~2–8 hours) for technical reasons. Since the synapse is highly sensitive to the presence of EtOH, we will distinguish as best as possible experiments conducted under this acute *in vitro* withdrawal paradigm from work that assesses the effects of *in vitro* chronic EtOH exposure (usually in primary neuronal cultures), *in vivo* chronic EtOH exposure (where experiments are performed in the presence of EtOH) and *in vivo* withdrawal of chronic EtOH-exposed animals.

The effects of both acute EtOH and chronic EtOH/acute *in vitro* withdrawal on glutamatergic signaling are primarily centered on postsynaptic glutamate receptors (Bliss et al., 2014; Lovinger and Roberto, 2013; Roberto and Gilpin, 2014; Szumliski and Woodward, 2014). Glutamate receptors include three major classes of ionotropic receptors (iGluRs), 1) α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA receptors or AMPARs), 2) *N*-methyl-D-aspartate receptors (NMDA receptors or NMDARs) and 3) kainate receptors (KARs), as well as various sub-classes of metabotropic glutamate receptors (mGluRs) that are G protein-coupled receptors (GPCRs) (Lovinger and Roberto, 2013).

1.1.1 EtOH Effects on Ionotropic Glutamate Receptors

Ionotropic Glutamate Receptors (iGluRs) are ligand-gated ion channels (LGICs), which are well characterized targets for EtOH actions (Forstera et al., 2016; Lovinger, 1997; Szumliski and Woodward, 2014; Vengeliene et al., 2008). LGICs are heteromeric proteins that bind

extracellular neurotransmitters or intracellular messengers and transduce that binding energy into the opening of an intrinsic ion pore (Collingridge et al., 2009). These receptors are present on all CNS neurons, where they mediate fast synaptic transmission and the activation of intracellular signalling. iGluRs are specifically activated by extracellular glutamate binding and transport cations into the cell, with each receptor class displaying different selectivity for sodium, potassium and calcium ions.

NMDA Receptors—Acute ethanol has generally inhibitory actions on iGluRs (although see (Lu and Yeh, 1999)). Most consistently, EtOH inhibits NMDAR function (Criswell et al., 2003; Dildy and Leslie, 1989; Hoffman et al., 1989; Lima-Landman and Albuquerque, 1989; Lovinger et al., 1989, 1990), as well as the synaptic responses mediated by NMDARs (Lovinger et al., 1990; Morrisett and Swartzwelder, 1993; Ren et al., 2013; Roberto et al., 2004b; Wang et al., 2007; Xu et al., 2015; Zhao et al., 2015). Functional NMDARs contain an obligatory NR1 subunit in combination with at least one NR2 or NR3 subunit. Although, EtOH inhibits all NMDAR subtypes, differences in their EtOH sensitivity are observed according to their subunit compositions. Generally, EtOH more potently inhibits receptors with NR1/2A or NR1/2B subunit compositions than NR1/2C-containing receptors (Chu et al., 1995; Jin et al., 2008; Jin and Woodward, 2006; Kuner et al., 1993; Lovinger, 1995; Lovinger and Roberto, 2013; Masood et al., 1994).

Chronic ethanol exposure tends to increase both the function of NMDARs and NMDAR-mediated glutamatergic synaptic transmission (see Figure 1A) (Cebere et al., 1999; Grover et al., 1998; Gulya et al., 1991; Lack et al., 2007; Smothers et al., 1997). These changes in NMDAR activation are consistently larger than the EtOH-induced activation of other ionotropic glutamate receptors (i.e. AMPA and kainite receptors) (Chandler et al., 1999; Chandler et al., 1997; Gulya et al., 1991; Smothers et al., 1997).

Generally, EtOH-treated cultured primary neurons and tissue/cultured neurons from EtOH-exposed animals were used to study EtOH's effects on glutamate receptor activation and calcium influx. In these studies, chronic EtOH exposure (days to weeks) enhanced NMDAR agonist-induced increases in intracellular calcium (Chandler et al., 1997; Grover et al., 1998; Gulya et al., 1991; Iorio et al., 1992; Smothers et al., 1997) and upregulated the flow of ion current through the NMDAR pore (Floyd et al., 2003; Grover et al., 1998). Moreover, increased currents specific to NR2B-containing receptors have been observed in the basolateral amygdala (BLA) (Floyd et al., 2003), bed nucleus of the stria terminalis (Kash et al., 2009) and central amygdala (CeA) (Roberto et al., 2006; Roberto et al., 2004b) of chronic EtOH-exposed rodents during acute *in vitro* withdrawal.

Notably, the effects of acute EtOH on NMDAR function remained intact, or were even enhanced, after chronic exposure in most brain regions (Floyd et al., 2003; Roberto et al., 2006; Roberto et al., 2004b). Tolerance to acute EtOH-induced inhibition has been reported in hippocampal slices (Grover et al., 1994; Miyakawa et al., 1997) and in medial septum neurons (Grover et al., 1998), but not, for example, in the BLA (Floyd et al., 2003) or CeA (Roberto et al., 2004b). Since increased NMDAR-mediated calcium influx is associated with increased susceptibility to the excitotoxic effects of NMDA (Chandler et al., 1993; Iorio et al., 1993), the combination of chronic EtOH/acute withdrawal-induced increases in NMDAR

function and the conclusion of acute EtOH-induced NMDAR inhibition during the early stages of withdrawal are likely to generate a hyperexcitable state. Generally, this excitotoxicity during EtOH withdrawal contributes to alcohol-related neuronal loss in the brain (Bliss et al., 2014). Overall, these studies show that NMDAR function is still depressed by acute EtOH in animals exposed to chronic EtOH, and imply significantly enhanced NMDAR function during the acute stages of withdrawal to produce hyperexcitability/excitotoxicity.

The mechanisms underlying chronic EtOH's enhancement of NMDAR function are still not fully understood. Similar to the acute effects of EtOH, studies on receptor subunit expression, function and location indicate that NR2B-containing receptors are most strongly affected by chronic EtOH exposure and acute *in vitro* withdrawal (Carpenter-Hyland et al., 2004; Floyd et al., 2003; Kash et al., 2009; Roberto et al., 2004b). Some studies, conducted both *in vivo* and *in vitro*, report increases in NR2B mRNA expression following chronic EtOH exposure (Follesa and Ticku, 1995; Hu et al., 1996; Kash et al., 2009; Roberto et al., 2006; Snell et al., 1996), but these results are not always consistent (Cebere et al., 1999; Floyd et al., 2003; Lack et al., 2005). Notably, postmortem human brain gene expression studies have observed a similar increase in NMDA Type Subunit 2B (*GRIN2B*) mRNA levels in both the prefrontal cortex and hippocampus of human alcoholics (Farris and Mayfield, 2014; Zhou et al., 2011) (for more details see article by Warden and Mayfield in this Special Issue). Increases in NR2B protein expression, and sometimes NR2A protein expression, have also been observed in rodents after both *in vitro* and *in vivo* chronic EtOH exposure (Kash et al., 2009; Kash et al., 2008; Obara et al., 2009; Snell et al., 1996). Furthermore, increased expression of mRNA and protein for other NR subunits and specific NR1 splice variants have been reported following chronic EtOH exposure (Roberto et al., 2006; Trevisan et al., 1994; Winkler et al., 1999) (but see (Morrow et al., 1994)), but a clear correlation with increased receptor function has not been observed.

For instance, a chronic EtOH exposure paradigm that produced alcohol-dependent rats significantly increased NR1, NR2A and NR2B mRNA transcription, protein expression and immunohistochemical staining in the CeA (Roberto et al., 2006). The protein levels of all subunits returned to control values at 1 week of withdrawal, but the NR1 and NR2B mRNA expression reversed, leading to a significant decrease at 1 week of withdrawal and recovery to control levels after 2 weeks (Roberto et al., 2006). These data suggest that prolonged EtOH exposure and withdrawal induced NMDAR neuroadaptations within CeA glutamatergic synapses by selectively altering the expression of its subunits and thus, their contribution to the final NMDAR subunit composition. Parallel functional studies in CeA neurons of EtOH-naïve rats showed that acute EtOH (5–66 mM) significantly decreases evoked NMDAR- and non-NMDAR-mediated excitatory postsynaptic currents (EPSCs), while chronic EtOH/acute *in vitro* withdrawal produced an enhancement of the acute EtOH-induced depression of NMDA-EPSCs (but not non-NMDAR-mediated EPSCs) (Roberto et al., 2004b). Similarly, local application of exogenous NMDA and acute EtOH elicited a greater inhibition of NMDAR currents in CeA slices taken from alcohol-dependent rats compared to those from naïve animals, suggesting that chronic EtOH sensitizes NMDARs to the acute effects of the drug. In addition, ifenprodil, a specific NR2A and NR2B subunit-specific antagonist, occludes EtOH's effects and is more effective in blocking CeA NMDA-

EPSCs in alcohol-dependent rats compared to naïve rats (Roberto et al., 2004b). Notably, acute EtOH significantly decreased the paired-pulse ratios of compound, non-NMDA and NMDA-EPSCs in CeA slices of alcohol-dependent rats, indicating that after chronic EtOH exposure there is an acute EtOH-induced increase in glutamate release (Roberto et al., 2004b). This finding was supported by *in vivo* experiments showing that infusion of EtOH via reverse microdialysis significantly increases glutamate release in CeA dialysate only in rats that underwent chronic EtOH exposure (Roberto et al., 2004b). Moreover, baseline CeA glutamate content was significantly higher in alcohol-dependent animals relative to naïve controls. These combined studies indicate that alcohol dependence and withdrawal lead to neuroadaptations of glutamatergic transmission at both pre- and postsynaptic sites in the CeA, and that CeA glutamatergic synapses may play an important role in the development of alcohol dependence.

Notably, chronic intermittent ethanol (CIE) induced a significant increase in NMDAR function in dopamine D1 receptor-containing medium spiny neurons (MSNs) during acute *in vitro* withdrawal, as measured by the NMDA/AMPA ratio and input-output curves of isolated NMDAR currents and a decrease in D1- MSNs in the mouse nucleus accumbens (NAcc) shell (Renteria et al., 2016). This reversal of NMDAR function may account for the CIE-induced alterations in neuronal plasticity. In fact, in ethanol naïve mice, low frequency stimulation induced synaptic depression (LTD) in D1+ MSNs, but induced synaptic potentiation (LTP) in these cells after chronic ethanol exposure (Renteria et al., 2016). These cell-type specific alterations in excitatory signaling in the NAcc shell may constitute an important neuroadaptation for the expression of increased ethanol consumption induced by intermittent ethanol vapor exposure.

In another set of elegant studies, the NMDA/AMPA current ratio of medial prefrontal cortex (mPFC) layer V pyramidal neurons was increased with both acute *in vitro* withdrawal from CIE and one week of *in vivo* withdrawal (Kroener et al., 2012). In the CIE mice, these changes were associated with increased NMDA-EPSCs and increased NMDA NR1 and NRB subunit expression. After one week of withdrawal, increased NR1 and NR2B expression was no longer observed, despite the persistent increase in synaptic NMDA currents. Notably, no change in expression of the AMPA GluR1 subunit was observed in either animal group. Finally, there were mPFC-related cognitive flexibility deficits in these CIE mice when tested up to one week into withdrawal using an attentional set-shifting task (Kroener et al., 2012). These results are consistent with those in human alcoholics, who show protracted deficits in executive function (cite), and overall, this work suggests that these behavioral deficits may be associated with the persistent alterations in mPFC glutamatergic signaling observed here.

Consistent with the Kroener study, CIE exposure (for 15 days) increased the baseline amplitude of evoked NMDA currents in mPFC layer V pyramidal neurons of rats withdrawn for 1 week or 4 weeks (Trantham-Davidson et al., 2014). However, this increase in evoked NMDA current after 1 week of withdrawal did not appear to be associated with increased NMDAR subunit expression (Trantham-Davidson et al., 2014). The reason for this discrepancy is not clear, but may relate to circuit specific changes that are not detected when analyzing tissue samples containing diverse neuronal types and synaptic sites of excitatory

afferent projections from numerous brain regions (Trantham-Davidson et al., 2014). Notably, exposing rats to alcohol during early-mid adolescence (PD28–42) did not alter the expression of glutamatergic proteins in the adult mPFC. However, there was a pronounced reduction in dopamine D1 receptor modulation of both intrinsic firing and evoked NMDA currents in pyramidal cells, whereas D2 receptor function was unaltered (Trantham-Davidson et al., 2014; Trantham-Davidson et al., 2016).

Finally, glutamatergic transmission was enhanced in layer 2/3 pyramidal neurons from the infralimbic division of the mPFC in 48-hr withdrawn CIE mice compared to control mice (Pleil et al., 2015). While this study did not separate out NMDAR- and AMPAR-mediated currents, neurons from the IfL of CIE mice had larger sEPSC amplitudes, indicating altered postsynaptic receptor expression/function. Also, this enhancement of IfL glutamatergic transmission was accompanied by a reduction in sEPSC amplitudes in the medial subdivision of the central amygdala (CeA) of CIE mice.

One of the mechanisms driving increased NMDAR function following chronic ethanol exposure may involve its subcellular distribution and/or its phosphorylation state. For example, chronic EtOH exposure specifically increases the trafficking of NR2B-containing NMDARs to dendritic spines, enhancing glutamatergic transmission in cultured hippocampal neurons (Carpenter-Hyland et al., 2004). Additionally, several studies have investigated the effects of EtOH-induced kinase activity on NMDARs, with varying results. EtOH increased Fyn-mediated tyrosine phosphorylation of NR2B, resulting in an acute tolerance of NMDA currents to EtOH's effects (Miyakawa et al., 1997) (for review see (Ron and Barak, 2016)), while brief EtOH exposure (seconds to min) rapidly increased NR2B-containing receptor function via tyrosine phosphorylation by a Fyn-like kinase (Wang et al., 2007; Yaka et al., 2003). Despite these results suggesting a role for Fyn-mediated NR2B tyrosine phosphorylation in EtOH effects on glutamatergic signaling, Woodward (2004) reported that in a co-expression system, Fyn kinase did not directly influence the acute EtOH sensitivity of NR1/NR2A receptor function (Woodward, 2004). Additionally, Wu et al. (2010) reported that a relatively short-term (2 weeks) chronic EtOH liquid-diet consumption altered hippocampal NMDAR activity, which was associated with decreased levels of phospho-Y-1472 NR2B and increased levels of STEP33 and phospho-p38 mitogen-activated protein kinase (pp38 MAPK), but not NMDAR subunit expression (Wu et al., 2010). Behavioral studies with these rats following an EtOH challenge also revealed a tolerance to EtOH's sedative effects, suggesting that the NMDAR system's functional and biochemical changes are associated with behavioral tolerance to its acute sedative effects after chronic exposure. Therefore, the question remains whether tyrosine phosphorylation or de-phosphorylation can directly modulate the acute and chronic effects of EtOH on NMDARs.

AMPA Receptors—Acute ethanol also inhibits the function of AMPARs (Akinshola et al., 2001; Akinshola et al., 2003; Dildy-Mayfield and Harris, 1992; Moykkynen et al., 2003; Nieber et al., 1998; Wirkner et al., 2000) although with lower potency than NMDARs (Frye and Fincher, 2000; Lovinger, 1995; Lovinger et al., 1989). EtOH slightly alters AMPAR-mediated synaptic responses [the amplitude or time course of EPSCs] at most synapses due to increased receptor desensitization (Ariwodola et al., 2003; Lovinger et al., 1990;

Moykkynen et al., 2003; Moykkynen et al., 2009), but see (Mameli et al., 2005; Nie et al., 1993; Roberto et al., 2004b; Zhu et al., 2007).

The effects of chronic ethanol on AMPARs are more variable. Increased AMPAR subunit mRNA expression in the hippocampus (Bruckner et al., 1997) and protein expression in cortical cultures (Chandler et al., 1999) have been reported following chronic EtOH exposure. Notably, a postmortem human brain gene expression studies identified the AMPA Type Subunit 1 (*GRIN1*) as a hub gene in the human alcoholic prefrontal cortex (Farris and Mayfield, 2014) (for more details see article by Warden and Mayfield in this Special Issue). Alcohol also up-regulated neuronal activity dependent pentraxin (Narp) levels, concomitant with increased AMPAR subunit levels in the mouse NAcc (Ary et al., 2012). Additionally, Marty and Spigelman (2012) reported that the amplitude and conductance of AMPAR-mediated miniature EPSCs were enhanced in the NAcc of CIE-treated rats during acute *in vitro* withdrawal, due to an increase in a small fraction of functional postsynaptic GluA2-lacking AMPARs (Marty and Spigelman, 2012). Similarly, CIE/acute *in vitro* withdrawal induced a significant increase in baseline AMPAR-mediated signaling in D1+ MSNs, but not D1- MSNs, in the rat NAcc (Renteria et al., 2016). Other studies have demonstrated similar increases in AMPAR function in cerebellar Purkinje neurons (Netzeband et al., 1999), and AMPAR-mediated synaptic responses in the BLA during *in vivo* withdrawal (Lack et al., 2007). A more recent study from the McCool group (Christian et al., 2012) specifically dissected CIE- and withdrawal-induced changes in glutamatergic signaling using electrophysiological and biochemical approaches, with a particular focus on cortical glutamatergic inputs to BLA principal neurons. Both animal treatments induced postsynaptic alterations in the AMPAR that were associated with its surface expression, and AMPAR subunit phosphorylation that was associated with changes in total protein levels and/or the phosphorylation status of several key, plasticity-associated protein kinases, such as calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC). Together, these data show that CIE- and withdrawal-induced changes in BLA glutamatergic signaling both functionally and biochemically mimic plasticity-related states that likely contribute to the long-term increases in anxiety-like behavior that result from chronic EtOH exposure (Christian et al., 2012). The same team of investigators (Christian et al., 2013) also reported that withdrawal from chronic EtOH exposure also induced presynaptic alterations at thalamic-BLA synapses. The functional changes observed include altered paired-pulse ratios, decreased failure rates of unitary events and increased concentrations of synaptic glutamate release. These alterations were also associated with increased expression of vesicle-associated proteins. Overall, these data demonstrate that chronic EtOH modulation of glutamate neurotransmission in the rat basolateral amygdala is afferent-specific (Christian et al., 2013).

Notably, high-dose EtOH (BAL 0.32 g/dl) exposure during the equivalent to the last trimester of human pregnancy (postnatal days P3–5) can persistently increase the frequency of spontaneous and miniature EPSCs in the BLA of P36–50 rats, correlating with an increase in anxiety-like behaviors (Baculis et al., 2015). A similar paradigm of early EtOH exposure did not significantly affect the frequency, amplitude, rise-time and half-width of sEPSCs in hippocampal CA3 pyramidal neurons 2 weeks later (Baculis and Valenzuela, 2015). Previous studies also reported that AMPAR expression and function in the

hippocampus are not altered following chronic EtOH exposure (e.g. (Smothers et al., 1997)). Chronic EtOH appears to produce variable effects on glutamatergic transmission, though this may stem from differences in brain regions and type of tissue/cell preparation, the paradigm of EtOH exposure, and whether experiments were performed immediately prior to the end of drug exposure or during acute *in vitro* or *in vivo* withdrawal.

Kainate Receptors—Although, acute EtOH also inhibits KAR-mediated responses at quite low (10 mM) concentrations (Costa et al., 2000; Lack et al., 2008; Valenzuela et al., 1998; Weiner et al., 1999) it is unclear whether EtOH's actions on KAR function are direct or indirect (Dildy-Mayfield and Harris, 1992). More consistently, chronic EtOH exposure/acute *in vitro* withdrawal increased KAR subunit protein expression and receptor function in cultured hippocampal neurons (Carta et al., 2002), as well as KAR-mediated synaptic transmission in the basolateral amygdala (Lack et al., 2009). However, KAR expression was unchanged in cultured cortical neurons treated with chronic ethanol (Chandler et al., 1999).

Overall, acute EtOH's effects on iGluRs are generally thought to dampen neuronal excitability across the brain by reducing excitatory synaptic drive and inhibiting the synaptic plasticity that requires iGluR activation. Chronic EtOH exposure tends to have the opposite effect of enhancing iGluR function, though there generally remains a lack of tolerance to the acute effects of the drug. This combination of chronic EtOH-induced increases in iGluR function and the conclusion of acute EtOH-induced iGluR inhibition are likely to generate the hyperexcitable state that characterizes early withdrawal.

1.1.2 EtOH Effects on Metabotropic Glutamate Receptors

The majority of the brain's glutamate receptors are G protein-coupled receptors (GPCRs) and are termed metabotropic glutamate receptors (mGluRs). mGluRs bind glutamate, which alters their protein conformation to activate intracellular signaling proteins, known as G proteins. GPCRs act on three main G protein subclasses: $G_{i/o}$, G_s and G_q (Wickman and Clapham, 1995). $G_{i/o}$ proteins tend to inhibit neuronal function via the downstream effects of its α and β/γ protein subunits. Specifically, $G_{i/o}$ α subunits inhibit adenylyl cyclase (AC) activity to prevent the production of the second messenger 3',5'-cyclic adenosine monophosphate (cAMP), while its β/γ subunits activate G protein-activated inward rectifier potassium channels (GIRKs) to inhibit neuronal activity and inhibit voltage-gated calcium channels to prevent neurotransmitter release (Dolphin, 2003a, b; Elmslie, 2003; Wickman and Clapham, 1995; Wu and Saggau, 1997). In contrast, G_s α subunits stimulate AC/cAMP formation to promote synaptic transmission and inhibit some potassium channels (Wickman and Clapham, 1995). Finally, the G_q α subunits induce protein and lipid signaling pathways to activate ion channels that induce neuronal excitability, inhibit potassium channels and increase neurotransmitter release (Wickman and Clapham, 1995). Overall, G_s and G_q activation generally have net excitatory effects on neuronal activity and synaptic transmission. Therefore, mGluRs can directly alter cellular physiology and can also induce long-lasting effects on neuronal function.

The direct effects of acute alcohol on GPCRs and G proteins tend to be small and the physiological consequences unclear. Early studies demonstrated that acute EtOH stimulated

cAMP formation (Luthin and Tabakoff, 1984; Rabin and Molinoff, 1981), likely via actions on AC (Bjork et al., 2008). More recent work confirms that acute EtOH exposure increases neurotransmitter release via AC activity (Cruz et al., 2012; Kelm et al., 2008; Roberto et al., 2010; Ron and Barak, 2016; Talani and Lovinger, 2015). Of particular interest here, acute EtOH inhibits the activation of some G_q-type GPCRs, including mGluRs (Kelm et al., 2011; Ron and Barak, 2016). More consistently, chronic alcohol exposure enhances mGluR expression/function in several brain regions. Specifically, chronic ethanol activates mGluR-associated intracellular signaling pathways, such as mGluR5 in the NAcc (Cozzoli et al., 2012; Cozzoli et al., 2009). However, while chronic EtOH increases both mGluR1 and mGluR5 protein expression in the NAcc and amygdala (Cozzoli et al., 2014; Obara et al., 2009; Szumlinski et al., 2008; Szumlinski and Woodward, 2014), the enhancement of NAcc mGluR5 downstream signaling is not always associated with its increased expression (Szumlinski et al., 2008). Chronic EtOH exposure also decreased mGluR-induced dendritic calcium signals in cultured cerebellar Purkinje neurons (Netzeband et al., 2002).

1.1.3 Ethanol Effects on Glutamate Release

Chronic EtOH treatment increased synaptic glutamate release in several amygdalar nuclei (Lack et al., 2007; Roberto et al., 2004b), while also decreasing glutamate reuptake (Melendez et al., 2005a; Melendez et al., 2005b) (see Figure 1A). A similar decrease in glutamate reuptake leads to increased basal extracellular glutamate in the NAcc core after alcohol consumption (Pati et al., 2016), though there are likely to be multiple other factors that contribute to these observed changes following chronic EtOH exposure and withdrawal.

Chronic EtOH exposure and withdrawal also increase extracellular glutamate levels in the central amygdala, hippocampus and striatum, as measured with *in vivo* microdialysis (Dahchour and De Witte, 1999, 2003; Roberto et al., 2004b; Rossetti and Carboni, 1995). However, in the lateral subdivision of the CeA, Pleil et al. (2015) observed a lower sEPSC frequency in 48 hr withdrawn CIE mice compared to control mice, indicating reduced glutamate release in this region (Pleil et al., 2015). Since these electrophysiology recordings assess local synaptic currents from a restricted population of neurons (lateral CeA), while *in vivo* microdialysis measures regional neurochemistry, it is not so surprising that these results differ. Furthermore, it is important to keep in mind that both synaptic and non-synaptic sources of glutamate contribute to the extracellular pool measured with microdialysis methods (Kalivas, 2009; Rao et al., 2015).

Finally, gene expression studies in the frontal cortex of human alcoholics revealed an up-regulation in several genes associated with glutamatergic synaptic transmission, including the NMDA Type Subunit 1 (*GRIN1*), dynamin (*DNMI*), syntaxin (*STX1A*), synapsin 1 (*SYN1*), synaptophysin (*SYP*), and the vesicular glutamate transporter 1 (*VGLUT1*, *SLC17A7*) (Ponomarev et al., 2012), suggesting that chronic ethanol exposure produces a coordinated increase in glutamatergic release (for more details see article by Warden and Mayfield in this Special Issue).

1.2 GABA and EtOH Effects

The γ -aminobutyric acid (GABA) system plays a well-established role in the behavioral and cellular effects of acute and chronic alcohol consumption (Koob, 2009). There are 3 main types of GABA receptors; GABA_AR and GABA_CR are LGICs, while the GABA_BR is a GPCR (Aguayo et al., 2002; Forstera et al., 2016; Harris, 1999; Lovinger, 1997). Early rodent studies showed that systemic administration of GABA_AR agonists increased voluntary EtOH consumption, whereas GABA_AR antagonists and benzodiazepine inverse agonists decreased drinking (Boyle et al., 1993; Rassnick et al., 1993). Additionally, intra-amygdala infusion of GABA_A antagonists decreased EtOH drinking by non-dependent rats (Hyttia and Koob, 1995), while a similar study using a GABA_AR agonist suppressed drinking by alcohol-dependent rats without affecting intake by the non-dependent controls (Roberts et al., 1996). GABA_BR agonists also reduced the alcohol intake, anxiety-like behaviors and seizure susceptibility of alcohol-dependent rodents (Colombo et al., 2000; File et al., 1991; Knapp et al., 2007; Walker and Koob, 2007).

The GABA-mimetic effects of EtOH are well described and further support the relevance of the GABAergic system, and so in this section we will focus mostly on the effects of ethanol on GABA_AR transmission. In particular, GABA_ARs can be potentiated by low to intermediate concentrations of ethanol (5–50mM, corresponding to a few drinks up to complete intoxication). In recent years there has been an increase in the understanding of ethanol effects on GABA_A and GABA_B receptors, however, several critical questions remain. What are EtOH's mechanisms of action on GABA_A and GABA_B receptors? Are these mechanisms direct or indirect? Which GABA_A subunits compositions are most influenced by EtOH, and in what way?

1.2.1 EtOH Effects on GABA_A Receptors

GABA_ARs are pentameric “cys-loop” LGICs that are activated by extracellular GABA binding and transport chloride ions. This class of channels is characterized by an obligatory cysteine double bond in the N-terminal binding domain. Other “cys-loop” LGICs include the strychnine-sensitive glycine receptor (GlyR), nicotinic acetylcholine receptor (nAChR) and serotonin 3 receptor (5-HT₃). Acute EtOH tends to enhance cys-loop LGIC function (Aguayo et al., 2002; Forstera et al., 2016; Harris, 1999; Lovinger, 1997) by increasing their probability of channel opening and/or increasing agonist affinity (Tonner and Miller, 1995; Welsh et al., 2009). This EtOH effect has been observed in both synaptic and extrasynaptic receptors (Eggers and Berger, 2004; Sebe et al., 2003; Ye et al., 2001; Ziskind-Conhaim et al., 2003). For example, acute EtOH increases the amplitude and/or duration of GABA_AR and GlyR-mediated inhibitory postsynaptic currents (IPSCs) (Sebe et al., 2003; Ziskind-Conhaim et al., 2003). It is important to note, that acute EtOH can also inhibit nAChRs and GABA_ARs (Aguayo et al., 2002; Cardoso et al., 1999; Davis and de Fiebre, 2006; Marszalec et al., 1994; Roberto et al., 2003).

Generally acute EtOH facilitates GABA_AR function. There are 19 subunit proteins (α 1–6, β 1–3, γ 1–3, δ , ϵ , θ , π and ρ 1–3) that can combine in specific patterns to form different types of GABA_ARs (Aguayo et al., 2002; Forstera et al., 2016; Harris, 1999; Lovinger, 1997). Most GABA_ARs comprise 2 α and 2 β subunits, together with a single γ 2 or δ

subunit. The differential subunit composition of GABA_ARs determines its ligand binding affinity and gating properties. For instance, recombinant $\alpha 4\beta 2\gamma 2$ GABA_ARs are less sensitive to GABA and benzodiazepines compared to $\alpha 1\beta 2\gamma 2$ receptors (Whittemore et al., 1996). GABA_AR subunit composition also affects the receptor localization, such that receptors containing the γ subunit are expressed within the synapse where they mediate phasic inhibition, while receptors containing $\alpha 4$, $\alpha 5$, $\alpha 6$ and δ subunits are largely extra- or non-synaptic (Cherubini and Conti, 2001; McKernan and Whiting, 1996). In contrast, extrasynaptic high affinity GABA_ARs that contain the δ receptor subunit produce a tonic GABA_AR-mediated current in many CNS neurons (Hancher et al., 2005). Many studies have shown that EtOH potentiates the function of synaptic $\alpha/\beta/\gamma$ -containing receptors and extrasynaptic GABA_AR containing either $\alpha 4$ or $\alpha 6$ paired with β and δ subunits (Harris et al., 2008; Herman et al., 2013; Lobo and Harris, 2008; McCool et al., 2003; Mihic, 1996; Olsen et al., 2007). These findings remain inconsistent, and have not been uniformly replicated across all studies on EtOH/GABA_ARs in heterologous systems (reviewed in (Aguayo et al., 2002; Forstera et al., 2016; Lovinger and Homanics, 2007; Lovinger and Roberto, 2013)). Acute EtOH potentiation of extrasynaptic tonic current has been observed in recordings from the cerebellum, hippocampus, accumbens, amygdala and thalamus (Botta et al., 2007; Chandra et al., 2006; Forstera et al., 2016; Glykys et al., 2007; Hancher et al., 2005; Herman et al., 2013; Jia et al., 2008; Liang et al., 2014; Pirker et al., 2000; Valenzuela and Jotty, 2015; Wei et al., 2004).

Chronic ethanol exposure induces many neuroadaptations within GABAergic synapses in a brain region-specific manner. The effects of chronic EtOH are critical for the development of ethanol dependence (Eckardt et al., 1998; Grobin et al., 1998) and accumulating evidence suggests a role for the GABA_AR system in chronic EtOH-induced tolerance for the anxiolytic, sedative, ataxic and positive reinforcing effects of acute EtOH (Kumar et al., 2004; Kumar et al., 2009; Kumar et al., 2002). Specifically, these behaviors involve marked adaptations in GABAergic signaling, including GABA_A receptor subunit expression (Grobin et al., 1998) and pharmacology (see Figure 1B) (Kang et al., 1998b).

Chronic EtOH differentially altered the expression of specific GABA_AR subunits at both the transcriptional and translational levels in several brain regions. While it can be difficult to compare the work of multiple groups even within a single region due to differences in the EtOH exposure paradigm (*in vitro* vs. *in vivo*), here we describe the major findings in three brain regions that are known to play central roles in the development of alcohol dependence: 1) the cerebral cortex, 2) the hippocampus and 3) the amygdala.

Cerebral Cortex—In the cerebral cortex, chronic EtOH decreased mRNA and protein expression of the $\alpha 1$, $\alpha 2$ and $\alpha 3$ subunits (Devaud et al., 1997; Devaud et al., 1995) and increased expression of the $\alpha 4$, $\beta 1$, $\beta 2$, $\beta 3$, $\gamma 1$ and $\gamma 2$ subunits (Devaud et al., 1997; Devaud et al., 1995). Notably, human RNAseq studies have also identified several genes encoding for GABA_AR subunits that were associated with lifetime alcohol consumption, including $\alpha 3$ (*GABRA3*) in the prefrontal cortex (Farris and Mayfield, 2014) and $\alpha 2$ (*GABRA2*), $\gamma 1$ (*GABRG1*) and $\gamma 2$ (*GABRG2*) in the hippocampus (Enoch et al., 2012; Zhou et al., 2011) (for more details see article by Warden and Mayfield in this Special Issue). These changes in the subunit expression could affect the assembly of GABA_ARs and thus their ligand binding

affinities and function. For instance, cerebral cortical synaptoneurosome exhibit decreased sensitivity to GABA (Morrow et al., 1988) and cortical membrane vesicles (microsacs) to benzodiazepines (Buck and Harris, 1990) after chronic EtOH treatment. Similarly, acute EtOH no longer facilitated GABA- or muscimol-stimulated Cl⁻ uptake in the cortex (Morrow et al., 1988) and cerebellum (Allan and Harris, 1987) after chronic EtOH exposure. More recently, the Morrow laboratory has investigated two distinct populations of synaptic and extrasynaptic $\alpha 4$ -containing GABA_ARs in cultured rat cortical neurons (Carlson et al., 2016a; Carlson et al., 2016b). They found that chronic EtOH treatment alters the abundance of both types of subunits, while six weeks of chronic EtOH diet, but not 2 weeks, increased mouse thalamic GABA_AR $\alpha 4$ subunit levels (Werner et al., 2016). Chronic EtOH also decreased $\alpha 1$ mRNA and increased $\alpha 6$ mRNA in the cerebellum, and altered δ protein levels in the rat cerebellum and hippocampus (Mhatre and Ticku, 1994; Morrow et al., 1992). However, a down-regulation of native δ subunit-containing GABA_AR assemblies in the cerebellum has also been observed after rats received chronic EtOH administration (Marutha Ravindran et al., 2007), while no changes in δ subunit protein levels were reported in the cerebral cortex (Marutha Ravindran et al., 2007). Finally, CIE exposure did not affect the amplitude of baseline evoked IPSCs in Layer V pyramidal neurons of mPFC at 1 and 4 weeks of withdrawal (Trantham-Davidson et al., 2014).

Hippocampus—Alterations in GABA_AR expression induced by chronic EtOH exposure can also vary based on the time period of exposure (as alluded to above), and these temporal effects are demonstrated by work that assessed changes in hippocampus subunit expression at multiple time points during a single study. Accordingly, chronic EtOH consumption for 40 days, but not 14 days, produced a significant increase in the level of GABA_AR $\alpha 4$ subunit protein expression in the hippocampus (Matthews et al., 1998). However, neither exposure altered $\alpha 1$, $\alpha 2$, $\alpha 3$, $\beta(2/3)$, or $\gamma 2$ protein levels (Charlton et al., 1997; Matthews et al., 1998). Hippocampal $\alpha 1$ subunit immunoreactivity and mRNA content were also significantly reduced and $\alpha 5$ mRNA content was increased after 12 weeks of EtOH exposure, but not after 4 weeks of exposure. In contrast, chronic EtOH consumption for both 14 days (Devaud et al., 1997) and 40 days (Devaud et al., 1997; Matthews et al., 1998) increased cerebral cortical GABA_AR $\alpha 4$ subunit expression, but decreased $\alpha 1$ subunit expression (Devaud et al., 1997; Matthews et al., 1998). Overall, these findings indicate that chronic EtOH consumption differentially alters GABA_AR expression in both a temporal- and brain region-specific manner (Grobin et al., 1998).

Other studies from the Spiegelman and Olsen laboratories used a longer CIE treatment (120 day paradigm that ended with 2 days of *in vivo* withdrawal) in rats to correlate observed alternations in GABA_AR subunit expression with changes in the channel's function. These animals had increased levels of $\alpha 4$, $\gamma 2S$ and $\gamma 1$ in the hippocampus, a decrease in $\alpha 1$ and δ expression and no significant change in $\alpha 5$ (Cagetti et al., 2003; Mahmoudi et al., 1997). This increased hippocampal $\alpha 4$ subunit protein expression was paralleled by alterations in the pharmacological responses of GABA_ARs to benzodiazepine agonists and inverse agonists (Cagetti et al., 2003). CA1 pyramidal neurons from these CIE rats also exhibited decreases in the GABA_AR miniature IPSCs (mIPSCs) amplitude and decay time, which can be interpreted as altered receptor expression/composition, and reflects the observed changes

in the expression of $\alpha 1$ and $\alpha 4$ subunits (described above). The mIPSC frequency was also slightly decreased, suggesting that chronic EtOH exposure may also be associated with a presynaptic decrease in GABA release at these synapses. These adaptive changes in GABA_AR expression are thought to lead to a pronounced hypofunction of GABAergic neurotransmission and possibly the development of tolerance to the acute effects of EtOH at these synapses. Additionally some of these GABA system neuroadaptations persist during abstinence, including the decrease in hippocampal GABA_AR activity detailed above, which lasts for at least 40 days into withdrawal (Cagetti et al., 2003; Kang et al., 1996; Liang et al., 2004; Liang et al., 2009).

Additionally, Cagetti et al. identified several pharmacological alterations in the properties of GABAergic synapses that were consistent with their observed changes in subunit expression (Cagetti et al., 2003). Overall, CIE primarily decreased CA1 pyramidal neuron mIPSC sensitivity to positive modulation (by compounds such as diazepam and the steroid anesthetic alphaxalone); however, mIPSC potentiation by bretazenil, a α -preferring benzodiazepine ligand, was maintained, and mIPSC potentiation by Ro15-4513 was increased (Cagetti et al., 2003; Liang et al., 2009). Critically, the loss of the effect of diazepam and alphaxalone on mIPSCs in the CIE rats compared to the control rats (Cagetti et al., 2003) reflects the loss of $\alpha 1$ and γ -subunits, respectively. In contrast, drugs with some selectivity for $\alpha 4$ -subunits [e.g.; RO 15-4513 and a benzodiazepine inverse agonist (DMCM)] showed an increased modulation of mIPSCs, potentially due to the observed increase in $\alpha 4$ subunit expression (Kang et al., 1998a; Kang et al., 1996; Kang et al., 1998b). Notably, electrically-evoked IPSCs were still sensitive to alphaxalone (Kang et al., 1998b), suggesting differences in the populations of GABA_ARs that underlie evoked and miniature IPSCs in terms of their sensitivity to chronic EtOH exposure. In addition, the acute effect of EtOH on evoked IPSCs was significantly increased in hippocampal slices from chronic ethanol exposed rats (Kang et al., 1998a; Kang et al., 1998b).

Liang et al., (Liang et al., 2004) have also compared the effects of chronic EtOH exposure on synaptic and extrasynaptic GABA_AR function in hippocampal CA1 neurons and observed similar effects of chronic EtOH exposure on synaptic mIPSCs and the extrasynaptic tonic conductance. Specifically, both mIPSCs and the tonic current exhibit a high tolerance to doses of diazepam and zolpidem that are selective for $\alpha 1$ -containing GABA_ARs (Cagetti et al., 2003). Previous reports identified that chronic EtOH exposure decreased the benzodiazepine-sensitive $\alpha 1$ -subunits and increased the benzodiazepine-insensitive $\alpha 4$ -subunits at synaptic receptors (Grobin et al., 2000). Accordingly, THIP (a high affinity and efficacy agonist of the $\alpha 4$ -containing GABA_AR) activated the tonic GABA current and depressed mIPSCs in slices from control rats; the THIP effect on tonic conductance was lost after chronic EtOH exposure and it now strongly increased mIPSCs (Liang et al., 2004). As observed for glutamatergic signaling, high-dose EtOH (BAL 0.32 g/dl) exposure during pregnancy did not significantly affect the frequency, amplitude, rise-time and half-width of sIPSCs in hippocampal CA3 pyramidal neurons (Baculis and Valenzuela, 2015).

In the last decade, non-human primates (*Cynomolgus* macaques) have emerged as a powerful model for studying the neurobiology of long-term EtOH consumption (Vivian et

al., 2001). The first evidence of GABAergic synapse neuroadaptation in the monkey hippocampus was obtained using a paradigm of ethanol-self administration where cynomolgus macaques were trained using operant 4% EtOH solution self-administration and then given 22 hr daily access to the ethanol solution (Vivian et al., 2001). For the electrophysiology studies, tissue slices were prepared immediately following the last day of 18 months of daily EtOH drinking, and a significant increase in paired-pulse facilitation (PPF) of GABA_A IPSCs in hippocampal dentate granule cells was observed (Ariwodola et al., 2003; Weiner, 2005). These findings indicate a decrease in hippocampal GABA release after chronic ethanol exposure, similar to that observed in CIE rats (Cagetti et al., 2003).

Amygdala—In the amygdala, $\alpha 1$ and $\alpha 4$ subunit expression was significantly decreased after two weeks of chronic EtOH consumption (Papadeas et al., 2001), while (1–12) weeks of EtOH exposure led to decreased $\alpha 4$ subunit expression in the NAc, but no change in $\alpha 1$ subunit expression (Charlton et al., 1997; Ortiz et al., 1995). Additionally, muscimol-stimulated Cl⁻ uptake was enhanced in the extended amygdala, but not the NAc of EtOH-dependent rats (after acute *in vitro* withdrawal) (Papadeas et al., 2001). Western blot analysis of surface subunit levels also revealed selective decreases in $\alpha 1$ and δ and increases in $\alpha 4$, $\alpha 5$, and $\gamma 2$ GABA_AR subunits in rat NAc after CIE treatment and withdrawal (Liang et al., 2014). Additionally, in this study there was decreased in the ethanol and Ro15-4513 potentiation of extrasynaptic GABA_ARs and decreased diazepam sensitivity of both tonic and phasic GABA_AR signaling, suggesting a reduction in somatic GABAergic synapses onto NAcc MSNs in CIE rats (Liang et al., 2014). Collectively, these results suggest that chronic EtOH exposure alters GABA_AR expression in the amygdala and NAcc and that decreased expression of $\alpha 4$ subunits is associated with increases in receptor function in the amygdala, but not the NAcc (Papadeas et al., 2001).

In the CeA of alcohol-dependent rats (with acute *in vitro* withdrawal), the baseline frequency of mIPSCs and the baseline evoked IPSCs was significantly higher, while the baseline PPF ratios of IPSCs was significantly lower, suggesting that basal amygdala GABA release is augmented by chronic EtOH exposure (Roberto et al., 2004a). In addition, acute EtOH superfusion significantly enhanced evoked IPSCs and mIPSC frequencies to an extent equivalent to that in slices from non-dependent rats, suggesting lack of tolerance for these acute EtOH effects (Roberto et al., 2004a). Further, *in vivo* EtOH administration directly into the CeA via a microdialysis probe dose-dependently increased CeA GABA release in both alcohol-dependent and non-dependent rats. Interestingly, in dependent rats there was about a 4-fold increase in baseline GABA microdialysate content compared to the non-dependent rats, further suggesting increased GABAergic ‘tone’ during alcohol dependence (Roberto et al., 2004a). These studies point to a dysregulation by acute and chronic EtOH of presynaptic GABAergic function in the CeA. Recent studies in mice also show that CIE produced profound and long-lasting changes in local inhibitory circuits in the CeA (Herman and Roberto, 2016). In particular, a loss of tonic inhibition in a population of neurons occurred in parallel with an increase in phasic and tonic signaling mediated by δ -containing GABA_AR. These effects include a complete loss of tonic inhibition that persisted 5–7 days into withdrawal in a subtype of CeA neurons (Herman and Roberto, 2016).

Electrophysiology experiments conducted using the same cynomolgus macaque model of long-term EtOH consumption as described earlier, showed a decrease in the effect of flunitrazepam on the currents gated by exogenous GABA application in acutely dissociated amygdala neurons from ethanol-exposed animals compared to control animals (Anderson et al., 2007; Floyd et al., 2004). However, the modest inhibition of GABA-gated currents induced by acute EtOH was not affected by the chronic EtOH consumption. In addition, chronic EtOH significantly reduced amygdala mRNA expression levels for GABA_AR β 1 and γ 2 subunits. Overall, these findings demonstrate that chronic EtOH self-administration reduces the benzodiazepine sensitivity of amygdala GABA_ARs and this reduced sensitivity may reflect decreased expression of the γ subunit. Therefore, correlations between chronic EtOH-induced GABA_AR subunit expression and channel function have been observed in multiple brain regions, though, it remains unclear whether this link is causal and whether/how it contributes to the development of dependence.

Molecular Mechanisms—The mechanisms underlying alcohol-induced adaptation of GABA_ARs, both in terms of their expression and composition, can be varied and are not well understood. GABA_AR membrane expression involves a highly regulated process of synthesis, assembly, trafficking, endocytosis, and recycling or degradation. Therefore, chronic EtOH could act on a variety of cellular processes to change the overall number and/or composition of GABA_ARs, including endocytosis and recycling/degradation to selectively remove membrane receptors and/or trafficking of newly synthesized receptors to the cell surface. Several groups have shown that the altered GABA_AR function could stem from altered subunit assembly and does not necessarily require changes in the total number of receptors expressed on the membrane (Devaud et al., 1995; Kumar et al., 2009; Morrow et al., 1992). These chronic EtOH-induced changes in GABA_AR composition (and consequent change in receptor pharmacological properties) are hypothesized to contribute to the development of alcohol dependence (Kumar et al., 2004).

Notably, chronic EtOH administration can affect GABA_AR internalization via clathrin and the adaptor complex (AP). For example, chronic EtOH exposure selectively increases the internalization of α 1-containing GABA_ARs into clathrin coated vesicles of the cerebral cortex, with no change in the internalization of α 4 GABA_ARs (Kumar et al., 2003). Decreases in α 1 GABA_AR subunit peptides, as well as concomitant increases in α 4, have been also observed in the synaptic fraction following chronic EtOH exposure, while the clathrin- α 1-GABA_AR complex is increased in the intracellular fraction (Kumar et al., 2004). Additionally, the presence of β 2 and/or γ 2GABA_AR subunits allowed for receptor recognition by AP-2, resulting in clathrin-dependent endocytosis (Herring et al., 2003; Kittler et al., 2008).

Although, many protein kinases, including PKC, PKA and fyn, regulate GABA_AR trafficking, the role of these protein kinases is not completely understood following chronic EtOH exposure. In the cerebral cortex, chronic EtOH increases the expression of α 4-, β 2-, and β 3- GABA_AR subunits and decreases α 1, α 2, and α 3, and the differential PKC-dependent phosphorylation of these GABA_AR subunits is thought to be involved in the selective endocytosis of specific GABA_ARs (Brandon et al., 2002; Mohler et al., 1996). Moreover, in a separate study in the cerebral cortex, chronic EtOH consumption decreases

the association of PKC γ with α 1-containing GABA $_A$ Rs and increases the association of PKC γ with α 4 GABA $_A$ Rs, which is accompanied by a decreased α 1 subunit expression and increased α 4 expression at the cell surface (Kumar et al., 2002). Considering that phosphorylation of GABA $_A$ R subunits reduces their binding to AP-2 (which is necessary for clathrin-dependent internalization, as described above) (Kittler et al., 2008), the increased association of PKC γ with α 4 GABA $_A$ Rs may prevent their internalization (Kumar et al., 2004). Conversely, a reduction in PKC-dependent GABA $_A$ R phosphorylation disinhibits receptor binding to AP-2 to enable GABA $_A$ R endocytosis (Terunuma et al., 2008).

Interestingly, EtOH alters PKA expression and translocation (Diamond and Gordon, 1994; Newton and Messing, 2006) and chronic PKA activation in cerebellar granule cells increases α 1 subunit membrane expression (Ives et al., 2002), suggesting that PKA might also regulate GABA $_A$ R membrane localization after chronic EtOH exposure. Recently, the Morrow laboratory reported that either PKA activation or PKC inhibition prevented ethanol-induced increases in α 4 subunit expression and decreases in the decay of GABA mIPSCs, whereas PKA inhibition had no effect (Bohnsack et al., 2016; Carlson et al., 2016a). This work suggests that PKA and PKC have opposing effects on synaptic α 4-containing GABA $_A$ Rs, with PKA activation negatively modulating, and PKC activation positively modulating, their abundance and function (Bohnsack et al., 2016; Carlson et al., 2016a). Furthermore, they reported that PKA activation decreases synaptic α 4 expression via increased β 3 S408/409 phosphorylation, while PKC activation acts via γ 2 S327 phosphorylation to increase α 4. Additionally, PKA activation increases extrasynaptic α 4 and δ subunit expression (Bohnsack et al., 2016). Similar mechanisms and regulatory phosphorylation patterns (PKC γ and PKC δ) have been also observed in thalamic GABA $_A$ R α 4 subunit expression following acute and chronic EtOH administration where they are likely dependent on neuroactive steroids (Werner et al., 2016).

Additionally, post-translational modifications, such as the phosphorylation of GABA $_A$ Rs, have been demonstrated to modulate receptor function and play a role in ethanol dependence. CAM kinase II (CAMKII) or tyrosine kinase activity phosphorylates GABA $_A$ Rs to enhance their function (Churn et al., 2002; Valenzuela et al., 1995), and in the rat hippocampus, chronic EtOH exposure decreases tyrosine kinase phosphorylation of α 1 subunits, increases its phosphorylation of β 2 subunits and has no effect on γ 2 subunits (Marutha Ravindran et al., 2007). Overall, it is hypothesized that chronic EtOH exposure may produce persistent changes in second messenger systems, such as kinases, to alter GABA $_A$ Rs composition and expression, and ultimately function. This possibility is complicated by the *in vivo* heterogeneity of GABA $_A$ Rs, and it is important to note that there has been no direct demonstration of EtOH-induced phosphorylation of GABA $_A$ Rs altering receptor function. Thus, the exact mechanisms employed by chronic EtOH to regulate GABA $_A$ receptor function remain unknown at this point.

1.2.2 EtOH Effects on GABA $_B$ Receptors

GABA $_B$ receptors tend to localize to the presynaptic terminal, where they can act as auto-receptors to limit GABA release, as well as postsynaptic sites, and are thought to play a critical role in the effects of ethanol (Ariwodola and Weiner, 2004). Accordingly, GABA $_B$ R

antagonists enhance the effects of acute EtOH on GABA_AR-mediated transmission in the hippocampus (Ariwodola and Weiner, 2004; Wan et al., 1996; Wu and Saggau, 1994) and NAc (Nie et al., 2000). These effects appear to be brain region-specific, as the presence of GABA_BRs accounted for the difference in sensitivity to EtOH's influences on GABA transmission in specific subfields of the hippocampus (Weiner et al., 1997), and GABA_BRs did not influence GABA release from neurons in the CeA (Roberto et al., 2003). Thus, the presence or absence of presynaptic GABA_BRs may be an important determinant for the regional specificity of ethanol to affect GABA transmission (Ariwodola and Weiner, 2004).

Several studies have investigated the effects of chronic EtOH exposure on the role of the GABA_BRs in regulating GABA release, but the results appear to be mixed and perhaps brain region-specific. For example, Peris et al., (Peris et al., 1997) showed that chronic EtOH treatment (along with acute *in vitro* withdrawal) increased hippocampal 3H-GABA release, while decreasing long-term potentiation (LTP). Baclofen (a GABA_BR agonist) decreased this stimulated presynaptic release, while 2-OH-saclofen (a GABA_BR antagonist) enhanced it, suggesting presynaptic GABA_B autoreceptor activity. This chronic EtOH-induced change in presynaptic regulation of GABA release may potentially represent an underlying mechanism for the concomitant reduction in hippocampal LTP observed after chronic EtOH exposure (Peris et al., 1997).

In a second hippocampal study, the role of postsynaptic GABA_BRs in EtOH's effects was assessed. Baclofen produced a concentration-dependent hyperpolarization in CA1 pyramidal neurons, and this sensitivity was unchanged by chronic ethanol exposure/acute *in vitro* withdrawal, suggesting that these GABA_BRs were essentially insensitive to EtOH (Frye and Fincher, 1996).

In contrast, in the ventral tegmental area (VTA) systemic ETOH (and acute *in vitro* withdrawal) employed presynaptic GABA_BRs to produce a long-lasting potentiation of GABAergic synapses (Melis et al., 2002). These ethanol-exposed VTA neurons also had higher mIPSC frequencies (but not amplitudes) compared to controls, supporting an increased probability of action potential-independent GABA release after EtOH exposure (Melis et al., 2002).

Finally, in the CeA, we have previously shown that chronic ethanol exposure/acute *in vitro* withdrawal produces neuroadaptations in GABA_BR function (Roberto et al., 2008). Overall, chronic EtOH reduced the sensitivity of GABA IPSCs to CGP 55845A (a GABA_BR antagonist) and baclofen, suggesting an overall downregulation of GABA_BR function. Specifically, CGP 55845A significantly increased the amplitude of evoked IPSCs and decreased PPF ratios in the CeA of naïve rats, indicating that presynaptic GABA_BRs basally constrain GABA release. In alcohol-dependent rats, however, CGP 55845A has no effect on evoked IPSCs and PPF ratios. Conversely, baclofen markedly depressed evoked IPSCs in the CeA of naïve rats and chronic ethanol exposure attenuated this effect. Collectively, these data suggest that the downregulation of the GABA_BR system associated with EtOH-dependence may partially explain the increased GABAergic tone reported in dependent rats (Roberto et al., 2008).

1.2.3 EtOH Effects on GABA Release

While most of the results described above relate to ethanol's effects on postsynaptic GABA_ARs to alter GABAergic transmission, EtOH can also regulate GABA release through its actions on GABA_ARs (Criswell and Breese, 2005; Siggins et al., 2005; Weiner and Valenzuela, 2006). Specifically, acute EtOH increases GABA release in several brain regions, including the cerebellum, hippocampus, VTA, hypoglossal nucleus, BLA and CeA (Ariwodola and Weiner, 2004; Kelm et al., 2007, 2011; Ming et al., 2006; Roberto et al., 2003; Sebe et al., 2003; Theile et al., 2008; Zhu and Lovinger, 2006; Ziskind-Conhaim et al., 2003). It is important to note that in these studies, EtOH-induced increases in spontaneous and/or miniature GABAergic IPSCs frequencies reflect enhanced GABA release, while changes in m/sIPSC amplitudes and/or kinetics are considered an index of postsynaptic GABA_AR sensitivity (i.e. localization or function) (De Koninck and Mody, 1994; Otis et al., 1994). Accordingly, the studies described above consistently reported that acute EtOH increased s/mIPSC frequencies when applied at concentrations that are associated with intoxication (Ariwodola and Weiner, 2004; Kelm et al., 2007; Roberto et al., 2003; Theile et al., 2008; Zhu and Lovinger, 2006), but this is not true of all regions (e.g. the thalamus) (Jia et al., 2008). Furthermore, these studies observed that EtOH's actions to promote GABA release are rapid in onset and rapidly reversible following EtOH washout from the tissue.

Only a few studies reported that chronic EtOH exposure alters GABAergic transmission via changes in presynaptic GABA release, and the results appear to be highly dependent on the paradigm of exposure (*in vitro* vs. *in vivo*). For instance, *in vivo* chronic EtOH exposure paired with acute *in vitro* withdrawal decreased LTP via increased electrically-stimulated (but not basal) GABA release in the CA1 region of hippocampal slices (Tremwel et al., 1994). This effect was likely produced by alterations in the muscarinic receptor regulation of GABA release, and not changed GABA uptake or GABA_AR function (Hu et al., 1999), as well as by altered GABA_B autoreceptor function (as described above) (Peris et al., 1997). Chronic EtOH consumption also induces tolerance to the impairing effects of acute EtOH treatment on induction of LTP in rat hippocampal CA1 slices (Fujii et al., 2008). In addition, voluntary EtOH drinking is associated with a significant increase in GABAergic synapse paired-pulse plasticity (consistent with a reduction in GABA release probability) in cynomolgus macaque dentate gyrus neurons (Weiner, 2004).

In the CeA, we have shown (Roberto et al., 2010; Roberto et al., 2004a) that chronic EtOH exposure can affect GABA release, perhaps via an action on GABAergic terminals. Using *in vitro* electrophysiology and *in vivo* microdialysis, baseline GABA release was higher in CeA from EtOH-dependent rats compared to non-dependent rats (Roberto et al., 2010; Roberto et al., 2004a). However, Pleil et al. (2015) observed no change in GABA release in the CeA of 48 hr withdrawn CIE mice compared to control mice; though there was decreased GABA release in the infralimbic mPFC cortex of these EtOH-exposed mice (Pleil et al., 2015). Collectively, these studies strengthen the possibility that chronic EtOH, as well as acute EtOH, may alter the function of the GABAergic synapses by acting at presynaptic terminals.

Overall, we conclude that chronic ethanol exposure can produce critical neuroadaptations at GABAergic synapses to alter local inhibition. These adaptations appear to be both brain

region- and temporally-specific, and more studies are required to precisely determine the necessary EtOH exposure paradigms that elicit these synaptic changes, as well as their underlying molecular mechanisms and behavioral consequences with respect to withdrawal and dependence.

1.3 Conclusions

Therefore, chronic alcohol exposure produces significant neuroadaptation at GABAergic and glutamatergic synapses, particularly with regards to the expression, localization and function of synaptic proteins, channels and receptors, and these effects can be both cell type- and brain region-specific. The development of alcohol tolerance and dependence are of particular clinical relevance, and it is likely that these adaptive changes in GABA and glutamate receptor function play a critical role in these processes. Indeed, it is well known that chronic EtOH treatment can produce tolerance and physical dependence (Chandler et al., 1998) and that withdrawal following long-term EtOH consumption is associated with increased neuronal excitability (Kliethermes, 2005; Weiner and Valenzuela, 2006). These alterations have been hypothesized to represent, in part, a compensatory adaptation to the *in vitro* acute facilitatory effects of EtOH on GABAergic synapses (Siggins et al., 2005; Weiner and Valenzuela, 2006), as well as its inhibitory effects on glutamatergic synapses (Szumliski and Woodward, 2014).

For a general review of brain-region specific EtOH actions on the GABA system see (Criswell and Breese, 2005; Siggins et al., 2005; Weiner and Valenzuela, 2006). From our review, it is clear that the majority of early studies characterizing chronic effects of EtOH on GABAergic transmission focused mainly on postsynaptic properties and the subunit composition of the GABA_ARs themselves (see Figure 1B). Some of the differences in the results reported across laboratories may reflect the differences in the chronic EtOH exposure duration and protocol, brain region examined, and methods of assessing receptor function. Most of these studies were in general agreement that chronic EtOH exposure and withdrawal did not result in dramatic decreases in the number of GABA_ARs in most brain regions. However, many of these studies reported marked alterations in the expression of specific GABA_AR subunits and hypothesized that those changes in the subunit composition of the GABA_ARs may account for the physiological and pharmacological alterations in GABAergic signaling associated with chronic EtOH administration (Grobin et al., 1998).

Another area in which action of EtOH on GABA function has been implicated is *in vivo* withdrawal from chronic EtOH treatment. Withdrawal results in an increased sensitivity to the induction of seizures (Allan and Harris, 1987; Frye et al., 1983), and several studies have found that benzodiazepines and other drugs with GABA-mimetic actions can reduce withdrawal-related hyperexcitability (Breese et al., 2006; McCown and Breese, 1990; McCown et al., 1985; Roberto et al., 2008; Ticku and Burch, 1980).

Likewise, in terms of chronic EtOH's effects on glutamatergic signaling, the main effects appear to involve changes in the postsynaptic expression (see Figure 1A), composition and function of iGluRs (see (Bliss et al., 2014; Lovinger and Roberto, 2013; Rao et al., 2015; Szumliski and Woodward, 2014)) for more general reviews of EtOH's effects on the

glutamate system). Most significant were chronic EtOH-induced increases in the function of NR2B-containing NMDARs and NMDAR-mediated glutamatergic synaptic transmission (Carpenter-Hyland et al., 2004; Floyd et al., 2003; Kash et al., 2009; Roberto et al., 2004b). These findings were correlated with altered NMDAR subunit expression (and in particular NR2B levels) after both *in vitro* and *in vivo* chronic EtOH exposure (Follesa and Ticku, 1995; Hu et al., 1996; Kash et al., 2009; Roberto et al., 2006; Snell et al., 1996), but the results were not always consistent (Ceberé et al., 1999; Floyd et al., 2003; Lack et al., 2005). More generally, EtOH's actions on iGluRs dampen neuronal excitability by reducing excitatory synaptic drive and inhibiting the synaptic plasticity that requires iGluR activation. Chronic EtOH has the opposite effect and enhances iGluR function, though there generally remains a lack of tolerance to the acute effects of the drug. This combination of chronic EtOH-induced increases in iGluR function and the conclusion of acute EtOH-induced iGluR inhibition are likely to generate the hyperexcitable state that characterizes early withdrawal.

Collectively, these results offer strong support for the hypothesis that at least some of the actions of chronic EtOH are mediated by its effects on neural functions associated with GABA and glutamate synaptic transmission and these effects play an important role in the maintenance of addictive drinking behavior.

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Chronic alcohol exposure produces neuroadaptation at GABA and glutamate synapses.

The main effects are altered GABA_AR/NMDAR expression, composition and/or function.

It is hypothesized that these adaptations play a role in addictive drinking behavior.

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Figure 1.

Chronic ethanol effects on glutamatergic and GABAergic transmission. A: Schematic illustration of a glutamatergic synapse after chronic ethanol exposure. Presynaptically, glutamate release is enhanced. Postsynaptically, NMDAR function is increased, likely due to increased receptor expression. AMPA and kainate receptor function can also be enhanced by chronic ethanol, though these effects tend to be less consistent. B: A GABAergic synapse after chronic ethanol exposure. Presynaptically, GABA release is altered in a brain region-specific manner, potentially due to changes in GABA_BR function. Postsynaptically, GABA_AR composition is altered by chronic ethanol, such that there is an increase in synaptic α4-containing receptor expression and a concomitant decrease in synaptic α1-containing receptor number, though the functional outcome of these changes is less clear.