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Synaptic Effects Induced by Alcohol

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Abstract

Ethanol (EtOH) has effects on numerous cellular molecular targets, and alterations in synaptic function are prominent among these effects. Acute exposure to EtOH activates or inhibits the function of proteins involved in synaptic transmission, while chronic exposure often produces opposing and/or compensatory/homeostatic effects on the expression, localization and function of these proteins. Interactions between different neurotransmitters (e.g. neuropeptide effects on release of small molecule transmitters) can also influence both acute and chronic EtOH actions. Studies in intact animals indicate that the proteins affected by EtOH also play roles in the neural actions of the drug, including acute intoxication, tolerance, dependence and the seeking and drinking of EtOH. The present chapter is an update of our previous Lovinger and Roberto (2013) chapter and reviews the literature describing these acute and chronic synaptic effects of EtOH with a focus on adult animals, and their relevance for synaptic transmission, plasticity and behavior.

Keywords

GABA; glutamate; monoamine; neuropeptide; neurotransmitter receptor; presynaptic; postsynaptic; protein phosphorylation; synaptic plasticity; intoxication; tolerance; dependence

1. Acute EtOH Actions

Ethanol (EtOH) produces intoxication through actions on the central nervous system (CNS) at concentrations ranging from low mM to ~100 mM (at least in non-tolerant humans and experimental animals) (Cui & Koob, 2017). Several proteins involved in synaptic transmission are altered by EtOH effects within this concentration range. The target proteins include, but are not limited to, ion channels, neurotransmitter receptors and intracellular signalling proteins (Abrahao et al., 2017; Cui et al., 2015). The first section of this article will review the literature describing the most prominent acute EtOH effects on synaptic transmission in the CNS. This review is not meant to be comprehensive, but rather to cover those effects that have been observed most consistently, and that are thought to contribute to intoxication.

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1.1 Ligand-gated Ion Channels and Postsynaptic EtOH Effects

Ion channels are among the best characterized targets for acute EtOH actions (Lovinger 1997, Vengeliene et al. 2008). Ligand-gated ion channels (LGICs) are heteromeric proteins that bind extracellular neurotransmitters or intracellular messengers and transduce that binding energy into opening of an intrinsic ion pore (Collingridge et al. 2009). Among those channels activated by extracellular neurotransmitters there are three classes.

1.1.1. Cys-loop LGICs—The "cys-loop" LGICs are pentameric proteins characterized by an obligatory cysteine double bond in the n-terminal binding domain. Each subunit protein contains an extracellular ligand-binding domain, four membrane spanning domains, and one large intracellular loop domain that also serves as a "portal" for ion permeability. This receptor class includes proteins with cation-permeable pores, the nicotinic acetylcholine (nAChR) and serotonin$_3$ (5-HT$_3$) receptors, as well as those with anion-permeable pores, the γ-aminobutyric acid$_A$ (GABA$_A$) and strychnine-sensitive glycine (GlyR) receptors. This class of receptors is distributed throughout the peripheral and central nervous systems.

Generally, acute EtOH exposure enhances the function of cys-loop LGICs (Aguayo et al. 2002, Harris 1999, Hendrickson et al., 2013; Lovinger 1997, Perkins et al. 2010; Rahman et al., 2016, Söderpalm et al, 2017), but instances of inhibition of the nAChRs and GABA$_A$Rs have been reported (Aguayo et al. 2002, Cardoso et al. 1999, Davis and De Fiebre 2006, Marszalec et al. 1994, Noori et al., 2018; Rahman et al., 2016; Roberto et al. 2003). The most common EtOH action is to potentiate channel opening in the presence of a low concentration of agonist by increasing probability of channel opening (Zhou et al. 1998), and/or increasing agonist affinity (Tonner and Miller 1995, Welsh et al. 2009). Direct EtOH binding to receptors is thought to underlie the potentiating action (Howard et al., 2014; Sauguet et al., 2013). This potentiating effect can influence both synaptic and extrasynaptic receptors (Sebe et al. 2003, Ye et al. 2001, Eggers and Berger 2004, Ziskind-Conhaim et al. 2003; Herman et al., 2016a; Herman and Roberto, 2016) (Figure 1). For example, EtOH has been shown to increase the amplitude and/or duration of GABA$_A$ and GlyR-mediated inhibitory postsynaptic currents (IPSCs) (Sebe et al. 2003, Ziskind-Conhaim et al. 2003).

Ethanol potentiation of GABA$_A$ receptor function has been extensively studied. There are 19 subunit proteins that contribute to the formation of GABA$_A$ receptors (International Union of Basic and Clinical Pharmacology, IUPHAR, database http://www.iuphar-db.org/index.jsp). Many of these subunit combinations have been examined for function and pharmacology in heterologous expression systems. To briefly summarize a large body of data, there is evidence that EtOH potentiates the function of α/β/γ-subunit containing receptors, as well as those containing α4 or α6 along with β and δ subunits (Olsen et al. 2007, Lobo and Harris 2008, Mihic et al. 1995, McCool et al. 2003). However, none of these findings has been uniformly replicated in all laboratories that have examined EtOH effects in heterologous systems (reviewed in Lovinger and Homanics 2007, Aguayo et al. 2002). Using cultured and isolated neurons, several investigators have observed potentiation of GABA$_A$R function (Celentano et al. 1988, Reynolds and Prasad 1991, Aguayo 1990, Nishio and Narahashi 1990, Sapp and Yeh 1998), but this sort of effect has not been observed

in every neuronal type examined (e.g. McCool et al. 2003, White et al. 1990, Yamashita et al. 2006). A tonic $GABA_A$-mediated current is observed in many CNS neurons, and is thought to reflect the function of extrasynaptic, high affinity GABA receptors containing the δ receptor subunit (Hanchar et al. 2005). Potentiation of this tonic current has been observed in recordings from cerebellum, hippocampus and thalamus using the brain slice preparation (Hanchar et al. 2005, Wei et al. 2004, Glykys et al. 2007, Jia et al. 2008, although see Botta et al. 2007). It should be noted that potentiation of GABAergic tonic current in cerebellar granule neurons does not require δ receptor subunits and involves EtOH-induced increases in interneuron firing (Diaz and Valenzuela, 2016; Wadleigh and Valenzuela, 2012). A recent study indicates a role for acetate-induced increases in GABA production in this EtOH action (Jin et al., 2021) Indeed, it has been suggested that EtOH inhibits GABAAR function in cerebellar granule neurons via a protein kinase C-dependent mechanism (Kaplan et al., 2013).

EtOH potentiation of $GABA_A$ receptor function appears to depend on protein phosphorylation. Messing and co-workers have shown that activity of the epsilon subunit of protein kinase C (PKC) is necessary for EtOH potentiation of γ2-subunit containing $GABA_A$ receptors expressed heterologously in a mammalian cell line (Qi et al. 2007). This PKC action appears to involve phosphorylation of a specific serine residue on the γ2 subunit. This finding may explain data from previous studies indicating the involvement of PKC in EtOH potentiation of GABAergic transmission (Weiner et al. 1994). However, in this earlier study it was not clear if the EtOH effects on transmission involved pre- or postsynaptic mechanisms. A parallel line of investigation indicates that PKCδ is necessary for EtOH potentiation of tonic current involving δ-subunit containing $GABA_A$Rs (Choi et al. 2008). It is not yet clear if acute EtOH exposure activates PKC phosphorylation of the $GABA_A$R or if phosphorylation on key amino acid residues is permissive for EtOH potentiation of receptor function, and this will be an interesting topic for future research.

Ethanol potentiation of glycine-activated chloride channels appears to be dependent on receptor subunit composition. Potentiation is consistently greater at receptors containing the α1 subunit (Davies et al. 2003, Mascia et al. 1996, Mihic et al. 1997), when expressed in xenopus laevis oocytes and in neurons that express this subunit (Förstera et al., 2017; Valenzuela et al. 1998b, although see McCool et al. 2003, Yevenes et al. 2008). Receptors containing the α2 subunit also exhibit EtOH potentiation (McCool et al. 2003; Gallegos et al., 2021), but may be less sensitive than those containing the α1 subunit (Mascia et al. 1996). Ethanol interactions with both membrane-spanning and intracellular domains within the receptor have been implicated in potentiation (Burgos et al., 2015; Mascia et al., 1996). Inclusion of the β subunit along with α2 eliminates potentiation (McCool et al. 2003). Potentiation has also been observed in neurons from brain and spinal cord, particularly in regions where the α1 subunit is expressed (Aguayo et al. 1996, Ye et al. 2001). Potentiation of the function of $GABA_A$ and glycine receptors is thought to increase inhibition of neurons. Indeed, in the prefrontal cortex, potentiation of glycine effects on GlyRs is implicated in EtOH-induced inhibition of neurons (Badanich et al., 2013). Recently, it has been shown that taurine, a glycine receptor partial agonist, modulates GABAA mediate evoked synaptic transmission in central amygdala (CeA) of naïve rats, without affecting the acute alcohol-induced facilitation of GABAergic responses. Additionally, preapplication of

the glycine receptor-specific antagonist strychnine blocked the EtOH-induced increase in GABA responses in CeA neurons from naïve rats. In CeA neurons from dependent rats, taurine no longer influenced evoked responses, but now blocked the EtOH-induced increases (Kirson et al., 2020). The relative influence of effects on synaptic versus extrasynaptic channels in producing this inhibition remains to be determined.

Acute EtOH exposure potentiates the function of 5-HT_3 receptors that contain an intrinsic cation channel (Lovinger 1991, Machu and Harris 1994). It is yet to be determined if this action alters pre- or postsynaptic mechanisms activated by this receptor.

1.1.2. Ionotropic Glutamate Receptors—The ionotropic glutamate receptors (iGluRs) constitute the second class of neurotransmitter activated LGICs. Three major classes of iGluRs exist, the AMPA receptors (AMPARs, gene name GRIA give proper iGluR name, made by GluRs1-4), the NMDA receptors (NMDARs1-3, gene name GRIN), and the kainate receptors (KARs, made by GluRs5-7 and KAs1-2, gene name GRIK). These receptors are now thought to be tetrameric and each subunit contains a large n-terminal domain and an extracellular loop domain that together participate in ligand binding via a "venus fly-trap" motif (Gouaux 2004). The subunits have three membrane spanning domains and a re-entrant pore-loop that forms the ion conduction pathway, as well as intracellular loops and a large intracellular c-terminal domain. The iGluRs are all cation-permeable, with varying ratios of Na^+, K^+ and Ca^{2+} selectivity. These receptors are present on all CNS neurons, where they mediate fast synaptic transmission and activation of intracellular signalling.

Ethanol has consistent inhibitory actions on iGluRs (although see Lu and Yeh 1999) (Figure 1C,D). Inhibition of NMDARs at EtOH concentrations associated with intoxication is the best characterized of these effects (Criswell et al. 2003, Dildy and Leslie 1989, Hoffman et al. 1989, Lima-Landman and Albuquerque 1989, Lovinger et al. 1989). The synaptic responses mediated by NMDARs are also reduced by EtOH (Lovinger et al. 1990, Morrisett and Swartwelder 1993, Roberto et al., 2004b, Weitlauf and Woodward 2008, Wang et al. 2007).

Functional NMDARs always contain an obligatory NR1 subunit in combination with at least one NR2 or NR3 subunit. While EtOH inhibits all NMDAR subtypes, differences in the sensitivity to inhibition have been observed for recombinant with receptors containing different subunit compositions. The most common observation is that EtOH is less potent at receptors containing the NR1/2C composition in comparison to those containing NR1/2A or NR1/2B (Masood et al., 1994, Chu et al. 1995, but see Kuner et al. 1993, Lovinger 1995). There are several splice variants of the NR1 subunit, and a recent comprehensive study by Woodward and co-workers showed that the NR1 splicing status, in combination with the identity of the co-assembled NR2 subunit, has small but reliable effects on EtOH sensitivity (Jin and Woodward 2006). This NR1 splice variant effect could account for the previous difference in reports of low EtOH sensitivity of NR2C-containing receptors. Receptors containing the NR3 subunit are relatively insensitive to inhibition by EtOH, but inclusion of the NR2B subunit enhances the EtOH inhibitory action on NR3-containing receptors (Jin et al. 2008). In addition, Mg^{2+} enhances EtOH inhibition of several NR1/2

and N1/2/3 receptor combinations, especially when NR2B is present (Jin et al. 2008). This finding may account for the larger effect of EtOH on NR2B containing NMDARs seen in some neuronal preparations (e.g. Fink and Gothert 1996, Lovinger 1995).

Recent studies indicate that portions of the transmembrane domains and c-terminal domain of different NMDAR subunits contribute to ethanol sensitivity of the receptor (Honse et al. 2004, Ren et al. 2003, 2007, 2012, 2013, 2017, Salous et al. 2009, Smothers et al. 2013, 2016, Wu et al. 2019, Zhao et al. 2015, 2016).

Ethanol also inhibits the function of AMPARs, and effects can be seen at concentrations as low as 10 mM (Akinshola 2001, Akinshola et al. 2003, Dildy-Mayfield and Harris 1992, Moykkynen et al. 2003, Nieber et al. 1998, Wirkner et al. 2000). In neurons from the brain, EtOH generally shows lower potency for inhibition of AMPARs in comparison to NMDARs (Frye and Fincher, 2000, Lovinger et al. 1989, Lovinger 1995). The ethanol sensitivity of recombinant AMPAR receptors is not greatly altered by changing the receptor subunit composition (Lovinger 1993), although the potency of EtOH is slightly higher for inhibition of GluR1-containing in contrast to GluR3-containing GluRs in Xenopus laevis oocytes (Akinshola 2001). In addition, recombinant AMPA receptors containing GluRs 2 and 3 exhibits slightly decreased EtOH sensitivity in comparison to those containing GluRs1, 2 and 3 or 3 alone (Akinshola et al. 2003). Recent studies suggest that this EtOH action involves increased receptor desensitization (Moykkynen et al. 2003, 2009), and thus the drug has little impact on AMPAR-mediated synaptic responses at most synapses given that desensitization does not contribute to the amplitude or time course of excitatory postsynaptic currents (EPSCs) (Lovinger 1990, Ariwodola et al. 2003, but see Nie et al., 1993, Roberto et al., 2004b, Mameli et al., 2005; Zhu et al. 2007; Logrip et al., 2017; Herman et al., 2016b). It is notable that a recent study indicates that EtOH enhances AMPAR-mediated EPSCs in the VTA via an indirect mechanism involving nicotinic ACh receptors (Engle et al. 2015).

Inhibition of KAR-mediated responses has been observed at quite low EtOH concentrations (Costa et al., 2000; Lack et al., 2008, Valenzuela et al., 1998a; Weiner et al., 1999). However, direct examination of KAR-mediated ion current has yielded mixed results, at least for the receptor constructs examined to date (Dildy-Mayfield and Harris, 1992, Valenzuela et al. 1998a). Thus, it is not yet clear if EtOH inhibition of KAR function involves a direct effect on protein function or a more indirect action. Ethanol inhibition of iGluRs is generally thought to dampen neuronal excitability in many brain regions by reducing excitatory synaptic drive and inhibiting synaptic plasticity that requires iGluR activation.

1.1.3. Purinergic LGICs—The third major subtype of LGIC is the P2X purinergic receptor subclass. The P2X receptors are trimeric (Mio et al. 2005) with each subunit containing an n-terminal ligand binding domain, 2 membrane-spanning domains linked by an extracellular ligand binding domain, and a c-terminal intracellular domain of moderate length. The 2nd membrane-spanning domain appears to serve as the lining for the ion conduction pathway. Ethanol inhibits the function of most P2X receptor subtypes, with some effects reported at concentrations associated with intoxication (Davies et al. 2002, Li et al. 1993). The P2X4 receptor appears to be the most sensitive to inhibition by EtOH, while

P2X3 receptors exhibit EtOH-induced potentiation (Davies et al. 2002, 2005). At present, the physiological consequences of P2X inhibition are unclear.

1.2. G Protein-Coupled Receptors and roles in EtOH Effects

The majority of neurotransmitter receptors are members of the G protein-coupled receptor (GPCR) superfamily. These receptors are specialized for binding a neurotransmitter, and this binding stimulates rearrangement of the protein to favor activation of intracellular signaling proteins known to bind GTP and GDP. In the GTP-bound state, the G protein is activated. Several forms of intracellular signaling proteins are affected by activated G proteins, including proteins that generate small molecule 2nd messengers, as well as protein kinases and ion channels. Thus, G protein activation can affect neurophysiology fairly directly by altering ion channel function, and can have a long-lasting influence on neuronal function by altering intracellular signaling and even gene expression.

Receptor-activated G proteins are heterotrimeric, consisting of α, β and γ subunits. The β and γ subunits form a tight complex, but when the G protein is activated the α subunit affinity for the β/γ complex is reduced. The result is that two signaling elements arise from the G protein activation and can act on different intracellular targets. The GPCRs act predominantly on three G protein subclasses; Gi/o, Gq-like and Gs-like (Wickman and Clapham 1995). The Gi/o G protein class has net inhibitory effects on neuronal function, through actions of both the α and β/γ protein subunits. For example, the α subunit inhibits the enzyme adenylyl cyclase (AC) that normally generates the 2nd messenger cAMP. The β/γ subunits activate potassium channels that inhibit neuronal activity (the so-called G protein-activated inward rectifier, GIRK, potassium channels). The β/γ subunits also inhibit the function of voltage-gated calcium channels, leading to inhibition of neurotransmitter release, and also appear to have more direct effects on vesicle fusion (Dolphin 2003; Elmslie 2003, Miller, 1998; Wu and Saggau, 1997). The Gq-like α subunits activate protein and lipid signaling pathways that activate ion channels that excite neurons, inhibit potassium channels, and increase neurotransmitter release. Thus, activation of the Gq subclass generally has a net excitatory effect on neuronal activity and synaptic transmission. The proximal effects of Gs-like G-protein activation are not always clear. The α subunit of these G proteins stimulates AC/cAMP formation which can enhance synaptic transmission and inhibits some potassium channels. The effects on ion channel function of the different G-proteins are outlined in detail in previous review articles (Luo et al. 2022; Mochida 2019; Proft and Weiss 2015; Wickman and Clapham 1995).

Direct effects of acute EtOH on the function of GPCRs and G proteins are generally weak. Furthermore, the physiological impact of these actions is not always clear. However, there are mechanisms involving these molecules that are influenced by EtOH. Studies beginning in the 1980s showed that EtOH can stimulate cAMP formation (Luthin and Tabakoff 1984, Rabin and Molinoff 1981). This may be due to direct EtOH actions on AC, but other proteins that influence GPCRs and their signaling might play roles in the neural actions of EtOH (Bjork et al. 2008; Bjork et al. 2013; Meinhardt et al., 2022). The physiological consequences of this AC activation have long been unclear. However, recent studies indicate that acute EtOH exposure can increase neurotransmitter release (described in greater detail

later in this review, Figure 1), and activation of AC is a strong candidate to mediate these effects (Kelm et al. 2008).

In heterologous expression systems, EtOH has been shown to inhibit responses to activation of GPCRs that couple to Gq-like G proteins. These findings mostly involve demonstrations that pharmacologically-relevant concentrations of EtOH reduce the ability of the GPCRs to activate a calcium-dependent chloride current in the Xenopus laevis oocyte preparation (Minami et al. 1997a,b, 1998). Among the GPCRs that have been examined in this context are metabotropic glutamate receptors (mGluRs), muscarinic ACh receptors and serotonin type 2 receptors. The observation that these receptor effects are all three inhibited despite differences in the structures of the receptor molecules themselves, indicates that the EtOH target site is likely downstream of the receptor itself. Indeed there is some evidence for involvement of protein kinase C, at least in the inhibition of muscarinic AChR (mAChR)-induced responses (Minami et al. 1997b).

Ethanol can also potentiate the function of GIRK-type potassium channels (Aryal et al. 2009, Kobayashi et al. 1999, Lewohl et al. 1999). This effect occurs at concentrations associated with intoxication and involves binding to a region of the channel implicated in phospholipid actions (Bodhinatan and Slesinger, 2013; Glaaser and Slesinger, 2017). The net effect of GIRK activation is to inhibit neuronal activity. This action of EtOH was originally observed in heterologous expression systems and in cerebellar granule neurons (Kobayashi et al. 1999, Lewohl et al. 1999), and subsequent studies have indicated similar actions in midbrain dopaminergic neurons (Federici et al. 2009). Ethanol effects on this G-protein target may contribute to intoxication. Studies by Blednov et al. (2001) indicate that loss of the GIRK2 channel subunit alters acute EtOH actions, while Tipps and coworkers (2016) showed enhanced ethanol conditioned place preference in mice lacking the GIRK2 subunit. The analgesic effects of ethanol are lost in mice carrying a missense mutation in GIRK2 (Kobayashi et al., 1999). Furthermore, constitutive deletion of GIRK3 in knockout (KO) mice selectively increased ethanol binge-like drinking, without affecting ethanol metabolism, sensitivity to ethanol intoxication, or continuous-access drinking (Herman et al., 2015). Notably, virally mediated expression of GIRK3 in the VTA reversed the phenotype of GIRK3 KO mice and further decreased the intake of their wild-type counterparts. In addition, GIRK3 deletion prevents ethanol-induced activation of VTA neurons and ethanol-induced release of dopamine in the nucleus accumbens (Herman et al., 2015). There is certainly a need for additional studies of how GIRK activation might contribute to intoxication.

1.3. Presynaptic Effects of EtOH

Ethanol potentiation of GABAergic synaptic inhibition is now known to result from both pre- and postsynaptic actions. As discussed in the section on LGICs, the postsynaptic effects result from potentiation of $GABA_A$/anion channels. A large literature indicates that EtOH also acts to enhance GABA release from presynaptic terminals, and that this action contributes to enhanced synaptic inhibition (reviewed in Siggins et al. 2005) (Figure 1). Increases in fast GABAergic synaptic transmission during EtOH treatment have been observed in cerebellum, hippocampus, VTA, hypoglossal nucleus, and amygdala, both

basolateral and central nuclei (Ariwodola and Weiner 2004, Ming et al. 2006, Kelm et al. 2007, Theile et al. 2008, Zhu and Lovinger 2006, Roberto et al. 2003, Sebe et al. 2003, Ziskind-Conhaim et al. 2003). These studies have been carried out mostly in brain slices and isolated brain neurons. Examination of spontaneous and miniature GABAergic IPSCs allows investigators to determine if the frequency of synaptic events is altered (a likely presynaptic change), or if the amplitude is affected (likely a postsynaptic change). Such analyses have consistently shown that the frequencies of spontaneous inhibitory postsynaptic currents (sIPSC) activated by spontaneous GABA release, and miniature inhibitory postsynaptic currents (mIPSC) activated by action potential-independent release of GABA quanta are increased at EtOH concentrations associated with intoxication, at least in the amygdala, cerebellum, hippocampus and VTA (Ariwodola and Weiner 2004, Zhu and Lovinger 2006, Theile et al. 2008, Roberto et al. 2003, Kelm et al. 2007; Jimenez et al., 2019; Herman et al., 2013b; Khom et al., 2020a,b; Kirson et al., 2021). These effects are rapid in onset and rapidly reversible following EtOH removal from tissue.

At present, little is known about the mechanisms underlying EtOH potentiation of GABA release. The increase in mIPSC frequency suggests that the site of EtOH action is downstream of action potential generation and calcium entry into the presynaptic terminal. Experiments in the cerebellum and VTA suggest that EtOH interacts with mechanisms involved in intracellular calcium release, perhaps increasing calcium concentrations in the presynaptic terminal (Kelm et al. 2007, Theile et al. 2009). It would be helpful to know if EtOH increases calcium concentrations in the relevant population of GABAergic presynaptic terminals. However, this is difficult to determine given the small size (<1μM diameter) of terminals, and the diversity of subtypes of terminals found on any given neuron. More recently, L-type voltage-gated calcium channels (LTCCs) have been implicated in the EtOH-induced increases in CeA action-potential dependent activity (neuronal firing rates and GABA release) in naïve rats, and ethanol dependence reduces CeA LTCC membrane abundance (Varodayan et al., 2017b). Notably, nifedipine, an LTCC antagonist, prevents ethanol induced GABA release and firing in naïve CeA, but not in dependent rats where a CRF1 antagonist (R121919) did. This switch from an LTCC- to a CRF1-based mechanism with alcohol dependence is accompanied by a shift from a role for inositol triphosphate receptor (IP3R) mediated calcium-induced calcium release to the involvement of ryanodine receptors (RyRs) (Varodayan et al., 2017b). Furthermore, P/Q-type voltage-gated calcium channels mediate ethanol-induced CeA vesicular GABA release in a PKA and PKC dependent manner in both naïve and dependent rats (Varodayan et al., 2017c; Cruz et al., 2011).

In fact, the role of intracellular signaling pathways in this potentiating EtOH effect has also been examined. It is well established that activation of AC or PKC potentiates transmission at synapses throughout the nervous system (see Leenders and Sheng 2005, Nguyen and Woo 2003 for review). Thus, it is logical to speculate that these signaling molecules might play a role in the acute alcohol action. Potentiation of GABA release onto cerebellar Purkinje neurons and principal neurons in the basolateral amygdala is eliminated in the presence of AC and protein kinase A (PKA) inhibitors (Kelm et al. 2008; Talani and Lovinger 2015), and is also affected by compounds targeting phospholipase C and PKC (Kelm et al. 2010). The potentiating effect of EtOH is impaired in the CeA in mice that lack PKCε (Bajo

et al. 2008). Thus, PKC is implicated in both the pre- and postsynaptic effects of EtOH at GABAergic synapses. It is notable that GABA release appears to be increased in the PKCε knockout mice prior to EtOH exposure, and thus the effect in this case may be more akin to occlusion rather than blockade of the drug action. Recently, a new class of PKCε inhibitors designed on the Rho-associated protein kinase (ROCK) inhibitor Y-27632, displayed selectivity for PKCε over other kinases, and prevented ethanol-stimulated GABA release in the mouse CeA slices (Blasio et al., 2018). Nevertheless, it remains to be determined if the effects of EtOH on these signaling molecules are direct or indirect. Indeed, several studies indicate that EtOH interacts with neuromodulators such as CRF and endocannabinoids to alter GABA release (Ariwodola and Weiner 2004, Nie et al., 2004, Talani and Lovinger 2015, Roberto et al. 2010a,b; Varodayan et al., 2015; Varodayan et al., 2016).

Inhibition of GABA transmission by acute EtOH exposure has also been observed (Blomeley et al. 2011, Wilcox et al., 2014, Patton et al., 2016). Experiments in striatal brain slices support a presynaptic mechanism of decreased GABAergic transmission onto the medium spiny projection neurons (MSNs) (Wilcox et al., 2014, Patton et al., 2016). Using an optogenetic technique in which channel rhodopsin (ChR2) was expressed in parvalbumin-containing fast-spiking striatal GABAergic interneurons (FSIs), Patton and colleagues (2016) found that ethanol inhibited transmission at this synapse. This inhibition involves presynaptic inhibition of GABA release due to activation of delta opiate receptors, presumably secondary to increased extracellular enkephalin.

In contrast to the effects on GABA release, the vast majority of studies indicate that acute EtOH either has no effect or inhibits release of glutamate (reviewed in Siggins et al. 2005), although increases have been observed in some brain regions (Eggers and Berger 2004, Gioia et al. 201y, Herman et al. 2016b, Silberman et al. 2015, Xiao et al., 2009; Herman et al., 2016b). The vesicle-associated Munc 13 proteins are implicated in EtOH inhibition of glutamate release in the basolateral amygdala (Gioia et al. 2017) and neurotransmitter release in Drosophila melanogaster (Xu et al. 2018). These findings suggest differences between GABAergic and glutamatergic terminals in most brain regions that may be useful in determining what factors contribute to EtOH sensitivity of release.

1.4. Monoamines and Neurotransmitter Transport

Acute EtOH effects on neurotransmitter transport have been investigated using brain tissue and heterologous expression systems. *In vivo* studies indicate that EtOH increases monoamine levels in brain (reviewed in Deehan et al., 2016; Gonzales et al. 2004, LeMarquand et al. 1994, Thielen et al. 2001), and there is also evidence for EtOH-induced increases in human ventral striatum (Aalto et al., 2015). However, most studies of neurotransmitter transporters show them to be relatively insensitive to EtOH. However, increased cell surface expression of the dopamine transporter (DAT) was observed when this protein was heterologously expressed (Mayfield et al. 2001, Maiya et al. 2002). This effect would most likely decrease striatal dopamine during acute *in vivo* EtOH exposure in rodents, and thus does not help to explain the findings from *in vivo* studies. However, there is some controversy as to whether EtOH has potent effects on dopamine uptake measured in brain

tissue using voltammetric techniques (Jones et al. 2006, Mathews et al. 2006, Robinson et al. 2005, Yavich and Tiihonen 2000). The EtOH-induced increase in striatal DA levels is unperturbed in DAT knockout mice, suggesting that the drug action responsible for this effect does not involve the transporter (Mathews et al. 2006). Furthermore, studies using *in vitro* voltammetry and *in vivo* microdialysis to measure dopamine levels indicate that direct infusion of EtOH into striatum does not alter DA levels (Mathews et al. 2006, Yan 2003, Yim et al. 1998). Thus, the physiological impact of alterations in DAT function is not yet clear. Ethanol decreases DA release in striatal brain slices, albeit only at high concentrations (Budygin et al., 2001; Schilaty et al., 2014), but DAT has not been implicated in this effect. Interestingly, acute EtOH (44 mM) also decreases DA release, without impacting noradrenaline, in CeA slices of naive rats (Hedges et al., 2020).

Examination of EtOH effects on the brain serotonergic system has yielded interesting findings. In addition to potentiating 5-HT_3 receptor function, as mentioned in the previous section on ligand-gated ion channels, inhibition of 5-HT1c by EtOH has also been reported (Sanna et al. 1994) although it is not clear if this inhibition results from a direct effect on the receptor or on downstream signaling mechanisms. Exposure to acute EtOH also increases extracellular 5-HT levels in brain (LeMarquand et al., 1994, Thielen et al. 2001), and a recent report indicates that reduced 5-HT uptake may contribute to this effect as well as to the acute intoxicating effects of EtOH (Daws et al. 2006). A recent study showed that alcohol dependence and protracted withdrawal did not alter either 5-HT1A-mediated decrease of CeA GABA release or Htr1a expression but disrupted 5-HT2C-signaling without affecting Htr2c expression (Khom et al., 2020b). Collectively, those results provide detailed insights into modulation of CeA activity by the 5-HT system and unravel this system to chronic EtOH exposure. Thus, EtOH effects on serotonin and other monoamines require further examination.

1.5. Acetylcholine

Acute EtOH exposure has mixed effects on cholinergic synaptic transmission. As noted above, EtOH potentiates the function of some nicotinic ACh receptors, while inhibiting others. In addition, the Gq-coupled mAChRs are inhibited by acute EtOH (Candura et al. 1992, Kovacs et al. 1995, Larsson et al. 1995, Sanna et al. 1994; Smith, 1983).

Early studies in the neuromuscular junction indicated that EtOH enhances and prolongs cholinergic synaptic transmission (Gage et al. 1975). These effects appeared to involve EtOH actions on the postjunctional (muscle) side of the synapse but were only observed at concentrations that would be near-lethal or lethal. This conclusion was supported by evidence that high concentrations of EtOH enhance responses to ACh directly applied to muscle (Bradley et al. 1980).

Mixed effects of acute EtOH on cholinergic synaptic function have been observed in different brain regions. In the striatum, the majority of ACh is provided by large, tonically active cholinergic neurons that ramify extensively and innervate many other striatal neuronal subclasses (Goldberg and Wilson 2017). Ethanol inhibits the tonic firing of these neurons, and this inhibition relieves tonic mAChR actions on striatal MSNs (Blomeley et al. 2011). The medial septum contains both cholinergic and non-cholinergic neurons. Acute EtOH

application enhances the firing rate of both neuronal subtypes (Ericson et al. 1984), and the increase observed in non-cholinergic neurons is prevented by an mAChR antagonist. In the hippocampal CA1 region, acute EtOH potentiates a slow postsynaptic current mediated by mAChRs (Madamba et al. 1995) and enhances responses to applied ACh measured *in vivo* (Mancillas et al. 1986). These acute effects in medial septum and hippocampus appear to be due to increased ACh tone.

Ethanol has also been shown to decrease ACh release in brain slices, including studies in cortex (Carmichael and Israel 1975, Kalant and Grose 1967) and striatum (Darstein et al. 1997). *In vivo* studies have also shown decreased ACh levels during acute alcohol exposure in brain regions including parietal cortex and the reticular system (Erickson and Graham 1988), as well as in hippocampus (Henn et al. 1998). In contrast, ACh levels in hypothalamic slices were increased following a single *in vivo* exposure to alcohol (Kaneyuki et al. 1995). Increased ACh has also been observed *in vivo* in the striatum, brainstem and VTA during acute exposure (Hunt and Dalton 1976; Larsson et al., 2005). It is not yet clear if the differential effects in different brain regions are due to molecular, cell-type or circuit differences in responses to EtOH. It should also be noted that these studies were performed using techniques with low temporal resolution, and thus it will be interesting to revisit alcohol effects on ACh release using newer approaches with subsecond resolution (Jing et al. 2018). More direct measurement of ACh release in brain slices would help to clarify the presynaptic effects of EtOH at cholinergic synapses in different brain regions.

1.6. EtOH and Synaptic Plasticity

Long-lasting changes in the efficacy of synaptic transmission are thought to contribute to brain development, learning and memory, and addiction (Hyman et al. 2006, Kauer and Malenka 2007). The most commonly studied forms of long-lasting synaptic plasticity are long-term potentiation (LTP), a persistent increase in synaptic transmission, and long-term depression (LTD), a persistent decrease in transmission. These types of plasticity are usually brought about by repetitive patterned activation of afferent inputs to a given postsynaptic neuron.

Effects of EtOH on LTP have been studied in different brain regions (Zorumski et al. 2014; Lovinger and Kash 2015), but the majority of information comes from studies of the Schaffer collateral inputs to the CA1 pyramidal neurons of the hippocampal formation (Blitzer et al. 1990, Morrisett and Swartzwelder 1993, Mulkeen et al. 1987, Sinclair and Lo 1986). Acute EtOH exposure generally suppresses the induction of LTP at this and other synapses (Yin et al. 2007, Blitzer et al. 1990, Givens and McMahon 1995, Morrisett and Swartzwelder 1993, Mulkeen et al. 1987, Sinclair and Lo 1986, Wayner et al. 1993, Weitlauf et al. 2004). Effects occur at EtOH concentrations associated with intoxication, and in some studies at surprisingly low concentrations (Blitzer et al. 1990, Fujii et al. 2008). While inhibition of NMDAR function has been implicated in EtOH-induced LTP reduction (Blitzer et al. 1990, Schummers and Browning 2001), other mechanisms, including enhanced GABAergic transmission, corticosterone, acetaldehyde and neurosteroid production have also been implicated (Izumi et al. 2007, 2015, Ramachandran et al. 2015, Schummers et al.

1997, Tokuda et al. 2013). Ethanol also inhibits LTP induced by kainate receptor activation in the basolateral amygdala (Lack et al., 2008).

There is not as much information regarding EtOH effects on LTD. Two prominent subtypes of LTD can be elicited in the hippocampal CA1 region. The most widely studied form of LTD is induced by repetitive low-frequency synaptic activation, and requires activation of NMDA receptors (Dudek and Bear 1992, Mulkey and Malenka 1992). In the hippocampal CA1 region LTD is enhanced by exposure to EtOH at a concentration associated with strong intoxication (Hendricson et al. 2002), although this observation has not been consistent (Izumi et al. 2005). In the nucleus accumbens (NAc), acute EtOH inhibits NMDAR-dependent LTD (Jeanes et al., 2011, 2014). Short-term *in vivo* exposure to EtOH prevents this LTD, and instead LTP is induced following low frequency stimulation (Jeanes et al., 2011).

Other forms of LTD observed in hippocampus and elsewhere involve activation of mGluRs (reviewed in Luscher and Huber 2010). One report indicates that EtOH, at concentrations associated with severe intoxication, prevents mGluR-LTD at hippocampal synapses (Overstreet et al. 1997). At glutamatergic synapses onto cerebellar Purkinje neurons mGluR-LTD involves decreased surface expression and function of AMPARs (Ito 2001). Acute EtOH exposure inhibits this cerebellar LTD (Belmeguenai et al. 2008, Su et al. 2010), most likely due to inhibition of voltage-gated calcium channels and mGluR function. This finding is intriguing given that acute EtOH is known to impair motor coordination, and cerebellar function has been implicated in these effects. In the dorsal striatum, LTD involving these receptors also requires endocannabinoid (EC) signaling from the post- to the presynaptic neuron (retrograde EC signaling) and subsequent activation of CB1 cannabinoid receptors (Gerdeman et al. 2002). The expression of this form of LTD appears to be on the presynaptic side of the synapse. Acute EtOH increases the expression of this EC-dependent mGluR-LTD in dorsal striatum (Yin et al. 2007). It is not presently clear what mechanisms contribute to this effect of EtOH.

2. Chronic EtOH Actions

2.1. Chronic EtOH Effects on Glutamatergic Transmission and Glutamate Roles in Synaptic Plasticity

Chronic EtOH treatment in animals provides critical information relevant to central changes that take place during long-term alcohol abuse in humans (Cui et al., 2013). Persistent ethanol exposure produces both tolerance and dependence. Tolerance is manifested as a decreased behavioral response to EtOH that implies a decrease in the intoxicating effects and other responses to the drug. Therefore, higher amounts of EtOH are required to achieve the same intoxicating effects seen with acute drug administration. Ethanol dependence is generally described by symptomology elicited during and following withdrawal from EtOH (Heilig et al. 2010). These effects include anxiety, dysphoria and increased seizure susceptibility, hyperalgesia and disruption of sleep states (Enoch 2008; Grobin et al. 1998; Kumar et al. 2009). Chronic EtOH treatment is known to induce many neuroadaptative changes in the CNS involving both glutamatergic and GABAergic synaptic transmission (reviewed in Roberto and Varodayan, 2017).

The majority of work on chronic EtOH effects on glutamatergic transmission has focused on changes in glutamate receptors, particularly in light of the sensitivity of these receptors to acute EtOH actions (see previous discussion). Chronic EtOH exposure or intake generally produces an increase in the function of NMDARs and in NMDAR-mediated glutamatergic synaptic transmission (Cebere et al. 1999, Cheng et al., 2017; Grover et al. 1998, Gulya et al. 1991, Lack et al., 2007, Ma et al., 2017; Smothers et al. 1997) (Figure 1D), although decreases were observed in the medial prefrontal cortex (Holmes et al., 2012). Initial studies examined effects of receptor activation on neuronal calcium and nitric oxide signals either in preparations made from EtOH-exposed animals or in cultured neurons treated with ethanol in the medium (Grover et al., 1998; Gulya et al., 1991, Chandler et al. 1997, Iorio et al. 1992, Smothers et al. 1997). Exposure to EtOH for days to weeks increased NMDAR agonist-induced increases in intracellular calcium. These effects could be observed at EtOH concentrations that did not alter neuronal viability and did not affect baseline intracellular calcium levels. Furthermore, changes in responses to NMDAR activation were consistently larger than changes in the effects of activation of other ionotropic glutamate receptors (Chandler et al. 1997, Gulya et al. 1991, Smothers et al. 1997). Direct examination of ion current through the NMDAR pore has revealed effects consistent with a chronic EtOH-induced upregulation of NMDAR function (Floyd et al., 2003, Grover et al. 1998). An increase in the component of current mediated by NR2B-containing receptors has also been observed (Floyd et al. 2003, Kash et al. 2009, Roberto et al. 2004b, Roberto et al., 2006). However, in the Nucleus accumbens core an increase in synaptic receptors containing the NR2C subunit contributes to changes in glutamatergic transmission and drinking despite adverse consequences (Seif et al., 2013). Interestingly, acute EtOH inhibition of NMDARs in most brain regions is still intact or even increased after chronic *in vivo* exposure (Floyd et al. 2003, Roberto et al., 2006; Roberto et al., 2004b), although a small decrease in inhibition was observed in medial septum/diagonal band neurons (Grover et al. 1998). Evidence of tolerance to EtOH inhibition during acute exposure has also been observed in hippocampal slices (Grover et al. 1994, Miyakawa et al. 1997). Overall, it appears that NMDAR function is still suppressed during intoxication even after prolonged EtOH exposure, and thus the increase in NMDAR function is likely to be dramatic after EtOH withdrawal following chronic exposure. In the mouse mPFC (Layer 5), chronic intermittent ethanol (CIE) and abstinence from CIE leads to enduring increases in synaptic glutamatergic transmission and long-term synaptic plasticity (Kroener et al., 2012). Consistent with the Kroener's report, CIE exposure (for 15 days) increased the baseline amplitude of evoked NMDA currents in layer V pyramidal neurons of mPFC of rats examined either 1 week or 4 weeks into withdrawal (Trantham-Davidson et al., 2014). Glutamatergic transmission was also enhanced in layer 2/3 mPFC of 48 hr. withdrawn CIE mice compared to control mice (Pleil et al., 2015b). While this study did not separate out NMDAR- and AMPAR-mediated currents, neurons from the infralimbic of CIE mice had larger sEPSC amplitudes, indicating altered postsynaptic receptor expression/function. Also, this enhancement of glutamatergic transmission in mPFC was accompanied by a reduction in sEPSC amplitudes in the CeA of the CIE mice. A recent study investigated the concomitant alterations in basal synaptic function and neuronal excitability in the rat mPFC and dentate gyrus of the hippocampus during CIE, protracted abstinence from CIE, and re-exposure to one ethanol vapor session during protracted abstinence (Avchalumov al., 2021a,b). Chronic ethanol

consistently increased excitability of layer 2/3 pyramidal neurons in the mPFC and granule cell neurons in the DG. In the DG, this effect persisted during 21 day of abstinence. Re-exposure did not enhance excitability, suggesting resistance to vapor-induced effects. Western blotting demonstrates enhanced phosphorylation of $Ca2^{+}$/calmodulin-dependent protein kinase II (CaMKII), and reduced phosphorylation of NMDA receptor (N2A/2B subunits) (Avchalumov et al., 2021b; Natividad et al., 2018). One consequence of the increase in NMDAR-mediated calcium influx appears to be an increase in susceptibility to excitotoxic effects of NMDA (Chandler et al. 1993, Iorio et al. 1993), although enhanced NMDAR-mediated neuroprotection can also be observed in young cerebellar granule neurons (Pantazis et al. 1998). It has thus been postulated that excitotoxicity during EtOH withdrawal contributes to alcohol-related neuronal loss in the brain. Cortical NMDARs in appear to contribute to EtOH drinking in mice (Radke et al., 2017a), and this may be related to regulation of subunit expression and receptor function after chronic intake (Radke et al., 2017b).

The mechanisms underlying the increase in NMDAR function are still under investigation, but several interesting facets of the story have already emerged. Analysis of receptor function and pharmacology, as well as examination of receptor subunit expression and location, indicate that receptors containing the NR2B subunit are the subtypes most strongly affected by chronic EtOH exposure (Carpenter-Hyland et al. 2004, Floyd et al. 2003, Kash et al. 2009, Roberto et al., 2004b) (Figure 1D). The molecular basis of increased NR2B function is less clear. While some investigators have reported increases in NR2B mRNA expression following chronic alcohol exposure *in vitro* (Hu et al. 1996, Snell et al., 1996), and *in vivo* (Follesa and Ticku 1995, Kash et al. 2009, Roberto et al. 2006) such increases have not been observed in every brain region (Cebere et al. 1999, Floyd et al. 2003, Lack et al., 2005). Increases in NR2B, and to a lesser extent NR2A, protein expression have also been observed using immunological techniques after both *in vitro* and *in vivo* EtOH exposure (Kash et al. 2005, Obara et al. 2009, Snell et al. 1996, Staples et al. 2015; Avchalumov et al., 2021). However, other investigators did not observe increased expression of this protein. Changes in expression of proteins that associate with NR2B may also contribute to chronic EtOH effects on transmission (Swartzwelder et al. 2016, Wills et al. 2017). Increased expression of mRNA and protein for other NR subunits and particular NR1 splice variants has been observed in some brain regions following chronic EtOH exposure (Raeder et al. 2008, Trevisan et al. 1994, Roberto et al., 2006, Winkler et al. 1999, but see Morrow et al. 1994), but there is less evidence for increased receptor function as a result of these increases. Thus, it is not clear if increased subunit expression is the driving force behind increased receptor function, and if so, what mechanisms underlie the increase in expression or trafficking.

Changes in subcellular distribution of receptors may also contribute to altered NMDAR function following chronic EtOH exposure. In cultured hippocampal neurons, exposure to EtOH leads to increased NMDAR expression in dendritic spines, the location of glutamatergic synapses (Carpenter-Hyland et al., 2004). This increased trafficking to spines is accompanied by an increase in the contribution of NMDARs to glutamatergic transmission, but does not appear to involve increased NMDAR protein expression. The synaptic NMDARs observed following chronic EtOH exposure appear to contain the NR2B

subunit. Increases in the contribution of NMDARs to glutamatergic synaptic transmission have also been observed following subacute (10s of seconds or min) EtOH exposure, and NR2B-containing receptors also appear to contribute to these increases (Wang et al. 2007, Yaka et al. 2003). Tyrosine phosphorylation by a Fyn-like kinase has been implicated in these rapid increases in the function of NR2B-containing receptors (Wang et al. 2007), but it is yet to be determined if this mechanism plays a role in chronic EtOH effects on the receptor.

Chronic EtOH effects on AMPA and kainate receptors have been examined, with variable results. Increases in AMPA receptor subunit mRNA have been observed in hippocampus following chronic EtOH exposure (Bruckner et al., 1997). Expression of AMPAR subunit proteins was also induced by chronic exposure in primary cortical cultures (Chandler et al., 1999), while increased AMPAR binding was observed in cortical membranes from EtOH-exposure animals, and AMPA receptor binding in cortical membranes (Haugbol et al., 2005). Evidence of increased AMPAR function has also been reported following chronic EtOH exposure, as measured with intracellular calcium signals in cerebellar Purkinje neurons (Netzeband et al., 1999), and AMPA receptor-mediated synaptic responses are increased in basolateral amygdala (Lack et al. 2007). This latter effect was observed following during withdrawal but not just after the end of chronic EtOH exposure. However, other studies have reported that AMPAR expression and function are not altered following chronic EtOH exposure (e.g. Smothers et al. 1997). Chronic ethanol up-regulates neuronal activity via pentraxin (Narp) levels as well as increases in levels of the AMPAR subunits in the mouse NAcc (Ary, 2012). Additionally, Marty and Spigelman (2012) reported that the amplitude and conductance of AMPAR-mediated miniature EPSCs were increased in CIE-treated rats due to an increase in a small fraction of functional postsynaptic GluA2-lacking AMPA receptors (Marty & Spigelman, 2012). Similarly, CIE induced a significant increase in baseline AMPAR-mediated signaling in D1+ but not D1- MSNs in the rat NAcc (Renteria et al., 2017). The factors that underlie this variability in findings may include the type of preparation examined, the duration and pattern of EtOH exposure, and whether assays were performed just after the end of drug exposure or after withdrawal had been allowed to proceed. Increased glutamatergic transmission involving both AMPA and NMDA receptors is observed at prefrontal cortex synapses in the dorsomedial striatum following chronic alcohol consumption (Ma et al., 2017), while increased AMPAR-mediated transmission was observed in ventral hippocampus and medial prefrontal cortex (Ewin et al. 2019; Varodayan et al. 2018; Avchalumov et al., 2021b). With respect to kainate receptors, Chandler and collaborations (Chandler et al., 1999) observed no change in receptor expression in cultured cortical neurons following chronic EtOH exposure. In contrast, enhancement of both subunit protein and kainate receptor function was found in cultured hippocampal neurons (Carta et al., 2002), and chronic intermittent EtOH increased KAR-mediated synaptic transmission in basolateral amygdala (Lack et al. 2009).

Chronic alcohol has also been associated with functional upregulation of mGluR2/3 receptor signaling in the CeA and bed nucleus of the stria terminalis (BNST) (Kufahl et al. 2011), as opposed to the downregulation observed in mPFC (Meinhardt et al., 2013, 2022). Furthermore, chronic ethanol self-administration (alcohol-deprivation model) also increased sEPSC rise times indicative of compromised CeA glutamatergic receptor function (Suarez

et al. 2019). Additionally, chronic intermittent ethanol treatment did not alter evoked CeA glutamate but decreased both spontaneous vesicular glutamate (mEPSCs) release and postsynaptic glutamate receptor function at rat CeA synapses (Varodayan et al. 2017a).

Chronic EtOH intake has also been shown to enhance intracellular signaling associated with mGluRs, particularly mGluR5, in the NAc (Cozzoli et al. 2009). While chronic EtOH drinking can induce increases in mGluR1 and mGluR5 protein expression in NAc and amygdala (Szumlinski et al. 2008, Obara et al. 2009), changes in mGluR5 signaling in NAc are not always associated with an increase in the protein itself (Szumlinski et al. 2008). In cultured cerebellar Purkinje neurons, exposure to EtOH for 11 days produced a decrease in mGluR-induced dendritic calcium signals (Netzeband et al. 2002). Clearly, more work is needed to determine how signaling by the many mGluR subtypes changes with long-term EtOH exposure and drinking.

Measurements of extracellular glutamate levels in brain have consistently shown increases produced by chronic EtOH exposure, especially after withdrawal or repeated cycles of withdrawal (Dahchour and DeWitte 1999, 2003, Pati et al. 2016, Rossetti and Carboni 1995; Roberto 2004b). However, reduced glutamate levels were observed following chronic ethanol drinking in mPFC (Meinhardt et al., 2021). These findings have generally been derived from measurements using *in vivo* microdialysis in brain. However, microdialysis measures of this type must be interpreted carefully, as both synaptic and nonsynaptic sources of glutamate contribute to the extracellular pool of this amino acid. Indeed, there is mounting evidence that changes in the cystine/glutamate exchanger generate increases in extracellular glutamate produced by some drugs of abuse (Kalivas 2009). Evidence of increased synaptic glutamate release has been observed in amygdala and hippocampus following chronic EtOH treatment (Chefer et al., 2011; Christian et al., 2013; Lack et al. 2007, Zu and Pan 2007; Roberto et al., 2004b). Increased glutamatergic transmission onto MSNs involving presynaptic mechanisms has also been observed following chronic EtOH consumption. Amygdala inputs to dorsomedial striatum exhibit increases in glutamate release following chronic drinking (Ma et al., 2017). Presynaptic effects may be stronger at D1 receptor-expressing MSNs related to those that express D2 receptors (Cheng et al., 2017). Decreases in glutamate uptake have also been noted following chronic EtOH exposure (Melendez et al. 2005). Examination of effects of pharmacological treatments that alter extracellular glutamate levels indicate that increased glutamate in the NAc contributes to increased EtOH intake (Griffin et al., 2014), and glutamate uptake mechanisms may thus be a target for treatment of AUD (Rao et al. 2015). The mGlu2 metabotropic receptors provide feedback reduction of glutamate release, and dysfunction of these receptors appears to contribute to increased release following chronic EtOH exposure (Adermark et al., 2011a; Johnson et al., 2020; Meinhardt et al., 2013). Enhancing this feedback function may be useful in reducing excessive EtOH consumption (Griffin et al., 2014; Meinhardt et al., 2013). However, mechanisms independent of glutamate transport and group II mGluRs have also been implicated in the increase in extracellular glutamate in the NAc (Pati et al. 2016). There may be multiple factors that contribute to increased extracellular glutamate levels and increased or decreased glutamatergic transmission following chronic EtOH exposure and withdrawal.

Despite the evidence that NMDAR function and extracellular glutamate levels are increased following chronic EtOH exposure, studies of hippocampal LTP indicate that this form of synaptic plasticity is decreased under the same conditions (Drissi et al. 2018, Durand and Carlen 1984, Roberto et al. 2002, Talani et al. 2014, although see Fujii et al. 2008, Stragier et al. 2015). Altered function of NMDA receptors containing the 2A and 2B subunits, resulting from related from changes in histone acetylation has been implicated in impaired LTP (Drissi et al., 2018). Similar results have been obtained in the amygdala (Stephens et al., 2005). In the NAc, NMDAR-dependent LTP is also impaired by repeated EtOH exposure, and this alteration is associated with sensitization to the locomotor stimulating effects of the drug as well as increased EtOH intake (Abrahao et al., 2013). In a subsequent study, loss of LTP in NAc was only observed in D2 receptor expressing MSNs following binge drinking (Ji et al., 2017). It is not yet clear what factors underlie the decrease in LTP, but mechanisms occurring downstream of NMDAR activation in the LTP induction process may play a role. However, mice expressing ethanol-resistant NMDARs show enhanced sensitization and consumption (den Hartog et al., 2013, 2017), implicating this receptor in altered sensitization perhaps related to loss of LTP. In the NAc changes in dopaminergic transmission involving D1 receptors may play a role in LTP impairment (Ji et al., 2017). Loss of LTD in the hippocampal CA1 region has been observed in mice that are resistant to locomotor sensitization, suggesting that resilience to plasticity of glutamatergic transmission may contribute to lack of this increased response to EtOH (Coune et al., 2017). Hippocampal LTD is also impaired following two high-dose ethanol exposures, and this is associated with impaired novel object recognition (Silvestre de Feron et al., 2015). In a recent rat study, ethanol self-administration and chronic intermittent ethanol exposure (6–7 weeks) did not alter the degree of LTP compared to naïve controls in mPFC of both females and males, and this form of LTP was dependent on both NMDA and AMPA receptors activation (Avchalumov et al., 2021b).

Disruption of mGluR-dependent hippocampal LTD has also been observed following chronic intermittent EtOH exposure (Wills et al., 2017). This change in plasticity is associated with altered expression of a number of proteins associated with the NR2B NMDAR subunit, including the ARC and Homer proteins that also interact with group I mGluRs. These proteins may thus mediate cross-talk between NMDA- and mGluR-based LTD mechanisms that are altered by EtOH and contribute to impaired plasticity.

It should be noted that LTP is enhanced following chronic EtOH exposure in some brain regions. For example, glutamatergic synapses in the prefrontal cortex show enhanced LTP in chronic EtOH-exposed mice (Kroener et al., 2012; Nimitvilai et al., 2016). Recent studies have shown that chronic ethanol drinking produces increased AMPAR function in the medial part of the dorsal striatum resembling that seen in LTP, particularly at medial PFC inputs to this striatal subregion, and synapses onto the striatal projection neurons that express D1-type dopamine receptors (Wang et al. 2012, 2015, Ma et al. 2017). A similar effect has been observed at glutamatergic synapses in the NAc and can appear after the first session of EtOH self-administration (Beckley et al. 2016). Inducing LTP and LTD in the dorsomedial striatum alters ethanol drinking (Ma et al., 2018). In the BNST, enhanced LTP of glutamatergic synapses is observed following chronic intermittent ethanol exposure (Wills et al., 2012).

Chronic EtOH exposure also alters LTD in striatal brain regions. In the NAc, chronic EtOH-induced changes in LTD vary according to neuronal subtype. In D1 receptor-expressing direct pathway MSNs, LTD appears after chronic exposure, while it is lost, and even converted to LTP in D2-expressing, indirect pathway MSNs (Jeanes et al. 2014, Renteria et al. 2017, 2018). In dorsal striatum, chronic EtOH exposure reduces or eliminates endocannabinoid-dependent LTD (Adermark et al., 2011b; Cui et al., 2011; DePoy et al., 2013). Impairment of the dampening of cortical glutamatergic inputs may contribute to enhanced activation of dorsolateral striatum and altered decision making (DePoy et al., 2013, 2015). Impairment of a form of LTD in the BNST driven by activation of alpha1 adrenergic receptors is observed following chronic EtOH exposure (McElligott et al. 2010).

In recent years it has become apparent that chronic EtOH exposure or drinking reduces presynaptic modulation by a number of G protein-coupled receptors. The affected receptors are generally those that couple to Gi/o-type G proteins and reduce glutamate release (Ding et al. 2016, Johnson and Lovinger 2016, 2020, Muñoz et al. 2018; Roberto and Varodayan, 2017). Activation of these receptors often results in a presynaptically-expressed form of LTD (Atwood et al. 2014). Activation of the presynaptic Gi/o-coupled mGlu2 receptor produces LTD, and mutations that lead to loss of receptor function in alcohol preferring rats contributes to their increased EtOH consumption (Zhou et al., 2013).

2.2. Chronic EtOH and GABAergic transmission: Postsynaptic effects

Chronic EtOH treatment is known to induce many neuroadaptative changes in the CNS. Over the past 20 years, it has been widely demonstrated that GABAergic transmission is sensitive to EtOH in distinct brain regions and is clearly involved in ethanol tolerance and dependence (Eckardt et al. 1998; Grobin et al. 1998). Chronic EtOH exposure often results in the development of tolerance to many GABAergic effects of the drug including the anxiolytic, sedative, ataxic, and positive reinforcing effects (Kumar et al. 2004; Kumar et al. 2009). Substantial evidence suggests that these behavioral and neural adaptations involve marked changes in the expression profile of specific $GABA_A$ receptor subunits (Grobin et al. 1998) and in the pharmacological properties of $GABA_A$ receptors (Kang et al. 1998b) (Figure 1).

Chronic EtOH administration differentially altered the expression of distinct $GABA_A$ receptor subunit mRNAs and peptide levels in various brain regions. In the cerebral cortex, both mRNA and peptide levels for $GABA_A$ receptor α1, α2 and α3 subunits were decreased (Devaud et al. 1997; Devaud et al. 1995). In contrast, both α4, β1, β2, β3, γ1 and γ2 subunit mRNA and peptide levels were increased (Devaud et al. 1997; Devaud et al. 1995). These alterations in the subunit expression affect the $GABA_A$ receptor assemblage and consequently, also affect receptor function and binding. It has been reported that recombinant $GABA_A$ receptors with α4β2γ2 subunits are less sensitive to GABA and benzodiazepines compared to α1β2γ2 receptors (Whittemore et al. 1996). Therefore, these alterations may account for the decreased sensitivity to GABA in cerebral cortical synaptoneurosomes (Morrow et al. 1988) and benzodiazepines in cortical membrane vesicles (microsacs) (Buck and Harris 1990). Following chronic EtOH exposure, acute ethanol did not facilitate the GABA or muscimol-stimulated Cl- uptake in cortex (Morrow et al.

1988) and in cerebellum (Allan and Harris 1987). Recently, Morrow and collaborators have reported in cultured rat cortical neurons two distinct populations of synaptic and extrasynaptic α4-containing $GABA_A$Rs [1,2] that are altered after chronic EtOH treatment.

In the cerebellum, chronic EtOH exposure decreased $GABA_A$ receptor α1 subunit mRNA and increased α6 subunit mRNA (Mhatre and Ticku 1992; Morrow et al. 1992). Chronic EtOH administration also decreased the polypeptide levels of the δ subunit of $GABA_A$ receptors in the rat cerebellum and hippocampus, whereas there were no changes in the δ subunit polypeptide levels in the rat cerebral cortex (Marutha Ravindran et al. 2007). Furthermore, chronic EtOH administration caused a down-regulation of native δ subunit-containing $GABA_A$ receptor assemblies in the rat cerebellum as determined by [(3)H]muscimol binding to the immunoprecipitated receptor assemblies (Marutha Ravindran et al. 2007).

The alterations in $GABA_A$ receptor gene expression are regionally and temporally dependent. For example, chronic EtOH consumption produced a significant increase in the level of $GABA_A$ receptor α4 subunit peptide in the hippocampus following 40 days but not 14 days exposure (Matthews et al. 1998). The relative expression of hippocampal $GABA_A$ receptor α1, α2, α3, β(2/3), or γ2 subunits was not altered by either period of chronic EtOH exposure (Charlton et al. 1997; Matthews et al. 1998). Hippocampal α1 subunit immunoreactivity and mRNA content were also significantly reduced after 12 weeks of treatment, but not after 4 weeks of exposure. In contrast, α5 mRNA content was increased in this brain region. In marked contrast, chronic EtOH consumption for both 14 (Devaud et al. 1997) and 40 (Devaud et al. 1997; Matthews et al. 1998) days significantly increased the relative expression of cerebral cortical $GABA_A$ receptor α4 subunits and significantly decreased the relative expression of α1 subunits (Devaud et al. 1997; Matthews et al. 1998). These findings indicate that chronic EtOH consumption alters $GABA_A$ receptor gene expression in the hippocampus but in a different manner from that in either the cerebral cortex or the cerebellum (Kaplan et al., 2016, for review see Valenzuela & Jotty (2015)). In addition, these alterations are dependent on the duration of EtOH exposure (Grobin et al. 1998).

The Olsen and Spigelman groups have developed a chronic intermittent EtOH treatment paradigm in which rats are given a 5- to 6-g/kg dose of ethanol on alternate days for 60 treatments (120 days). This chronic administration of EtOH to rats on an intermittent regimen, for 60 repeated intoxicating doses and repeated withdrawal episodes, increases levels of α4 subunit mRNA in hippocampus with no significant change in the mRNAs for the α5 subunit (Mahmoudi et al. 1997). Similarly, rats that were exposed to intermittent episodes of intoxicating EtOH and withdrawal showed increased hippocampal α4 subunit peptide expression (Cagetti et al. 2003) and alteration in the pharmacological responses of $GABA_A$ receptors to benzodiazepine agonists and inverse agonists (Cagetti et al. 2003). The mRNA levels for the γ2S and γ1 subunits were also elevated. In CA1 pyramidal slices from chronic intermittent EtOH exposed rats, the baseline decay time of $GABA_A$R-mediated mIPSCs was decreased, and the positive GABA receptor modulation of mIPSCs was also reduced compared with control rats. However, mIPSC potentiation by the α−preferring

benzodiazepine ligand bretazenil was maintained, and mIPSC potentiation by Ro15–4513 was increased (Cagetti et al. 2003; Liang et al. 2009).

In the VTA, levels of α1 subunit immunoreactivity were significantly decreased after 12 weeks but not 1–4 weeks of treatment (Charlton et al. 1997). Papadeas et al., (Papadeas et al. 2001) found that in the amygdala, α1 and α4 subunit expression was significantly decreased after two weeks of chronic EtOH consumption. In the nucleus accumbens (NAC), α4 subunit expression was decreased, but α1 subunit expression was not altered. In the VTA, there were no changes in α1 and α4 subunit expression. Muscimol-stimulated Cl- uptake was enhanced in the extended amygdala, but not the NAC of EtOH-dependent rats. These results suggest that chronic EtOH exposure alters $GABA_A$ receptor expression in the amygdala and NAC and that decreased expression of α4 subunits is associated with increases in $GABA_A$ receptor function in the amygdala but not the NAC (Papadeas et al. 2001).

Alterations in subunit assembly could induce alterations in the functional properties of $GABA_A$ receptors without alterations in the total number of receptors (Devaud et al. 1995; Kumar et al. 2009; Morrow et al. 1992). The expression of $GABA_A$ receptors involves a highly regulated process of synthesis, assembly, endocytosis, and recycling or degradation. Changes in the expression and composition of various $GABA_A$ receptors could result from selective endocytosis, recycling, and/or trafficking of newly synthesized receptors to the cell surface. $GABA_A$ receptor trafficking on the cell surface following EtOH consumption is thought to contribute to the development of EtOH dependence (Kumar et al. 2004). It has been reported by Kumar et al (Kumar et al. 2003) that chronic EtOH exposure selectively increases the internalization of α1 GABAA receptors with no change in the internalization of α4 GABAA receptors into clathrin coated vesicles of the cerebral cortex. There is also a decrease in α1 $GABA_A$ receptors and a significant increase in α4 subunit peptide in the synaptic fraction following chronic EtOH exposure. These results suggest that the regulation of intracellular trafficking following chronic EtOH administration may alter the subtypes of $GABA_A$ receptors on the cell surface and may account for changes in the pharmacological properties of $GABA_A$ receptors (Kumar et al. 2004) (Figure 1).

Clathrin and the adaptor complex (AP) play a crucial role in the internalization of $GABA_A$ receptors following chronic EtOH administration. Notably, in the intracellular fraction, the clathrin-α1-$GABA_A$ receptor complex is increased following chronic EtOH administration (Kumar et al. 2004). Specific $GABA_A$ receptor subunits (β2 and/or γ2) are required for recognition of the receptor by the AP-2 that precedes clathrin dependent endocytosis (Herring et al. 2003; Kittler et al. 2008). Chronic EtOH exposure induces an increase in the expression of α4-, β2-, and β3- $GABA_A$ receptor subunits in the cerebral cortex and all of these subunits contain consensus phosphorylation sites for PKC. In contrast, α1, α2, and α3 $GABA_A$ receptor subunits are decreased in the cortex and these subunits do not contain consensus phosphorylation sites for PKC. Hence, it has been hypothesized that PKC may phosphorylate the $GABA_A$ receptor subunits and/or AP-2 following chronic EtOH administration, altering the recognition and endocytosis of $GABA_A$ receptors by blocking AP-2 binding (Macdonald 1995; Mohler et al. 1996). A single dose of EtOH also increases the internalization of $GABA_A$ receptor α4 and δ subunits (Liang et al. 2007).

In rat hippocampus, chronic EtOH exposure induces a decrease in the tyrosine kinase phosphorylation of α1 subunits, an increase of β2 subunits and no alteration in γ2 subunits (Marutha Ravindran et al. 2007).

$GABA_A$ receptor trafficking is regulated by many protein kinases, including PKC, PKA and fyn. However, to date, the role of these protein kinases has not yet been studied in the trafficking of $GABA_A$ receptors, especially following EtOH exposure. Chronic EtOH consumption decreases association of PKCγ with α1 $GABA_A$ receptors and increases association of PKCγ with α4 GABAA receptors, accompanied by a decreased expression of the α1 subunit and an increased expression of α4 at the cell surface in cerebral cortex (Kumar et al. 2002). However, there were no alterations in the association of PKCγ with GABAA receptors in the α1 subunit expression following chronic EtOH administration in the hippocampus (Kumar et al. 2004). The increased association of PKCγ with α4 $GABA_A$ receptors may phosphorylate $GABA_A$ receptor subunits and prevent recognition of the receptor by AP-2, thus preventing its internalization. Indeed, phosphorylation of $GABA_A$ receptor subunits reduced the binding of receptors with AP- 2 and subsequent internalization (Kittler et al. 2008). Moreover, reduced PKC-dependent $GABA_A$ receptor phosphorylation increases receptor binding to the AP-2 and promotes receptor endocytosis (Terunuma et al. 2008). Chronic activation of PKA in cerebellar granule cells increases cell surface expression of $GABA_A$ receptor α1 subunit (Ives et al. 2002). Ethanol exposure alters expression and translocation of PKA (Diamond and Gordon 1994; Newton and Messing 2006) suggesting that PKA is likely also involved in the trafficking of $GABA_A$ receptors following EtOH exposure. Future studies will determine the specific role of various protein kinases in $GABA_A$ receptor trafficking following chronic EtOH administration. Post-translational modifications such as phosphorylation and glycosylation of $GABA_A$ receptors may play a role in the development of EtOH dependence. In particular, phosphorylation of $GABA_A$ receptors has been demonstrated to modulate receptor function. In Xenopus oocytes and isolated mouse brain membrane vesicles (microsacs), PKC and PKA phosphorylation of $GABA_A$ receptors decreases receptor activation (Kellenberger et al. 1992; Krishek et al. 1994; Leidenheimer et al. 1992). Phosphorylation by CAM kinase II or tyrosine kinase enhances $GABA_A$ receptor function (Churn et al. 2002; Valenzuela et al. 1995). As discussed previously, acute EtOH induces changes in $GABA_A$ receptor function that may be dependent on phosphorylation of particular proteins. Chronic EtOH exposure might be expected to result in long term changes in second messenger systems, including kinase activity. However, the heterogeneity of $GABA_A$ receptors expressed *in vivo* has precluded definitively answering this question and none of these studies have directly demonstrated that phosphorylation is involved in EtOH modulation of $GABA_A$ receptor function. The exact mechanisms involved in the alteration of $GABA_A$ receptor function following chronic EtOH exposure still remain to be determined.

From the preceding 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 $GABA_A$ receptors themselves. Some of the disparity in the findings across laboratories on postsynaptic sites of EtOH action may reflect the differences in the chronic EtOH treatment duration and protocol, brain region examined, and methods of assessing receptor function. Most of these studies were generally

in agreement that chronic EtOH exposure and withdrawal did not result in dramatic decreases in the number of $GABA_A$ receptors in most brain regions. However, many of these studies reported marked alterations in the expression of specific $GABA_A$ receptor subunits and hypothesized that those changes in the subunit composition of the $GABA_A$ receptors may account for the physiological and pharmacological alterations in GABAergic signaling associated with chronic EtOH administration (Grobin et al. 1998).

Of particular clinical importance is the development of tolerance and dependence to EtOH, and it is likely that adaptive changes in synaptic function in response to ethanol's actions on $GABA_A$ receptors play a role in this process. Indeed, it is well known that chronic EtOH treatment can lead to 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). Few studies have reported the effects of long-term EtOH exposure on GABAergic synaptic transmission looking at both postsynaptic and presynaptic mechanisms using *in vitro* brain slice methods.

As described above, the adaptive changes in $GABA_A$ receptor expression are thought to lead to a pronounced hypofunction of GABAergic neurotransmission and possibly the development of tolerance to the *in vitro* acute effects of EtOH on these synapses. In the hippocampus, there is a decrease in the threshold for seizure induction by the $GABA_A$ receptor antagonist pentylenetetrazole (Kokka et al. 1993) and a decrease in $GABA_A$ receptor activity in hippocampal slices that also lasts for at least 40 days after the last EtOH dose (Cagetti et al. 2003; Kang et al. 1996; Liang et al. 2004; Liang et al. 2009). Using analysis of tetrodotoxin (TTX)-resistant mIPSCs recorded from CA1 pyramidal neurons of chronic EtOH exposed and control rats, this group demonstrated a significant decrease in the amplitude and decay of these responses (Cagetti et al. 2003) possibly reflecting the observed alteration in the expression of α1 and α4 subunits. The mIPSC frequency is also slightly decreased, suggesting that chronic EtOH exposure may also be associated with a presynaptic decrease in GABA release at these synapses (see later section). Importantly, the pharmacological alterations in the properties of GABAergic synapses were consistent with the observed changes in subunit expression. For example, diazepam and the neurosteroid alphaxalone did not have any effect on mIPSCs in slices from chronic EtOH exposed rats (Cagetti et al. 2003), possibly reflecting the loss of α1 and γ-subunits, respectively.

On the other hand, drugs with some selectivity for α4-subunits (e.g., RO 15–4513 and DMCM) showed an increased modulation of mIPSCs possibly reflecting the increase in α4 subunit expression (Kang et al. 1998a; Kang et al. 1996; Kang et al. 1998b). Interestingly, the evoked IPSCs were still sensitive to alphaxalone (Kang et al. 1998b) suggesting differences in the populations of $GABA_A$ receptors that underlie evoked and mIPSCs. In addition, the acute effect of EtOH on evoked IPSCs was significantly increased in 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 receptor functions in CA1 neurons. These investigators found similar

alterations in the synaptic mIPSCs and the tonic extrasynaptic $GABA_A$ receptor-mediated conductance associated with chronic EtOH exposure. Both mIPSCs and the tonic current show profound tolerance to α1-containing $GABA_A$ receptor selective doses of diazepam and zolpidem (Cagetti et al. 2003). As previously demonstrated (Grobin et al. 2000), chronic EtOH exposure results in a decrease in BZP-sensitive α1-subunits and an increase in BZP-insensitive α4-subunits at synaptic receptors. Thus, THIP (a high affinity and efficacy agonist of the α4-containing $GABA_A$ receptors and a partial agonist at most other $GABA_A$ receptor assemblies) activated the tonic GABA current in slices from control-untreated rats and had little effect in slices from chronic EtOH exposed rats (Liang et al. 2004). However, THIP depressed mIPSCs in control-untreated rats but strongly increased mIPSCs in chronic EtOH treated rats. In addition, the chronic EtOH treated rats show a modest tolerance to the soporific effects of THIP and no change in its anxiolytic effects (Liang et al. 2004). In the last decade, significant progress has been made in understanding tonic conductance in the CeA of rodents using electrophysiology and immunohistochemistry (Herman et al., 2013a; Herman et al., 2016a). Two types of tonic conductance expressed in a cell-type-specific manner were also observed in rat CeA (Herman and Roberto 2016). One type is mediated by the α1-GABAA receptor subunit and is insensitive to acute ethanol exposure and the other type is mediated by the δ-GABAA receptor subunit and can be activated by increasing the ambient GABA concentration or by acute ethanol exposure. Notably, chronic ethanol exposure produces a functional switch in ongoing tonic signaling in the CeA in the specific cell-populations, however there is no change in the ability of THIP and acute ethanol to further augment tonic conductance in these neurons, suggesting that these receptors are either not maximally activated or that THIP or ethanol is able to displace the ambient GABA to produce similar levels of activation as seen in naïve rats. Collectively, the presence of cell-type-specific tonic signaling in the CeA provide support for the complex mechanisms of actions of acute and chronic ethanol in inhibitory circuitry in this brain region (Herman and Roberto 2016; Herman et al., 2016a).

In the last decade, non-human primates (Cynomolgus macaques) have been a powerful model to study the effects of long-term EtOH consumption (Vivian et al. 2001). Ongoing research in the Weiner lab has provided the first evidence of neuroadaptations in the GABAergic synapses in monkey hippocampus (Weiner and Daunais 2005). In this paradigm of EtOH-self administration, cynomolgus macaques are trained to self-administer a 4% EtOH solution on an operant panel and then given 22 hr. daily access to the ethanol solution. Control subjects were age- and sex-matched animals that had free access to food and water but were not exposed to the operant panels. The preliminary *in vitro* electrophysiological findings revealed a significant increase in paired-pulse facilitation (PPF) of $GABA_A$ IPSCs in dentate granule cells in slices prepared immediately following the last day of 18 months of daily EtOH drinking. Their finding is consistent with a decrease in GABA release probability (see section 2.3 on presynaptic ethanol effects at GABAergic synapses) and agrees with the decrease in mIPSC frequency observed in rats following chronic intermittent EtOH exposure (Cagetti et al. 2003). Interestingly, there was lack of tolerance for both the acute facilitatory effect of EtOH and flunitrazepam on evoked $GABA_A$ IPSCs (Weiner and Daunais 2005). Using the same paradigm of EtOH self-administration, whole-cell patch clamp recordings on acutely dissociated amygdala neurons from ethanol-exposed

cynomolgus macaques showed a decrease in the effect of flunitrazepam on the currents gated by exogenous GABA application compared with amygdala neurons from 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 ethanol consumption. In addition, mRNA expression levels for the β, γ, and δ subunits in total amygdala RNA isolated from control and EtOH-drinking animals were measured. Chronic EtOH significantly reduced amygdala β1 and γ2 subunit expression. Overall, these finding demonstrate that chronic EtOH self-administration reduces the benzodiazepine sensitivity of amygdala $GABA_A$ receptors and this reduced sensitivity may reflect decreased expression of the γ subunit.

Electrophysiological studies in the monkey striatum indicate that chronic alcohol consumption decreases GABAergic synaptic transmission onto projection neurons (Cuzon Carlson et al., 2011, 2018). This effect was especially prominent in the putamen striatal subregion, and the decrease was larger in putamen of monkeys that began EtOH drinking as adolescents compared to those who started later (Cuzon Carlson et al., 2018).

Early work by Roberto et al., (Roberto et al. 2004a) assessed whether GABAergic synaptic changes occur with EtOH-dependence in CeA slices. To obtain dependent rats, these investigators used an EtOH vapor inhalation method (Rogers et al. 1979). In this study, male Sprague–Dawley rats were exposed to a continuous EtOH vapor for 2–3 weeks with a targeted blood alcohol level of 150–200 mg/dL while control rats were maintained in similar chambers without EtOH vapor. On experiment days, the chronic EtOH-treated rats were maintained in the ethanol vapor chamber until preparation of the CeA slices, and recordings of GABAergic transmission were made in EtOH-free solution 2–8 hours after cutting the slices (Roberto et al. 2004a). In CeA neurons from EtOH-dependent rats, both evoked IPSCs and mean baseline amplitude of mIPSCs were significantly increased compared to naïve rats, suggesting a postsynaptic effect of chronic ethanol (Roberto et al. 2004a). However, possible changes in the expression of $GABA_A$ receptor subunits were not characterized. It was also found that the baseline PPF ratio of IPSCs was significantly decreased and the mIPSC frequency was higher in neurons of EtOH-dependent rats compared to naïve rats, suggesting that GABA release was augmented in chronic ethanol treated rats (Roberto et al. 2004a) (see later section on presynaptic change). In addition, acute EtOH (44 mM) increased IPSCs, decreased the PPF ratio of IPSCs and increased the mIPSCs frequency to the same extent in ethanol-dependent rats and naïve rats, suggesting a lack of tolerance for the acute ethanol effects (Roberto et al. 2004a). These results have been replicated by several recent studies from the same group (Herman and Roberto 2016; Khom et al., 2020a,b; Varodayan et al., 2017c; Kirson et al., 2020; 2021; Tunstall et al., 2019) and one of the most consistent findings is the lack of tolerance for the acute potentiating effect of EtOH on GABAergic synapses in rodents after chronic ethanol exposure (up to 2 weeks of ethanol withdrawal). These studies suggest that GABAergic mechanisms may not be associated with the tolerance that is known to develop with some of the behavioral effects of EtOH (e.g., ataxia, sedation). Additional studies will be needed to determine the molecular mechanisms responsible more carefully for these adaptive changes in different brain regions and length/duration of EtOH exposure required to induce such neuroadaptations in GABAergic synapse. Moreover, these data also suggest that, as with the acute effects of EtOH, long-term exposure to ethanol

results in both pre- and postsynaptic alterations and these changes may differ between brain regions (Siggins et al. 2005; Weiner and Valenzuela 2006; Roberto and Varodayan, 2017).

In contrast to the rodents, in the monkey amygdala, acute ethanol application significantly increased the frequency of sIPSCs in controls, but not in abstinent drinkers, suggesting a tolerance to ethanol-enhanced GABA release in abstinent rhesus monkeys with a history of chronic ethanol self-administration and repeated abstinence drinkers (Jimenez et al., 2019). It is important to note that the loss of an acute effect of ethanol in the CeA in abstinent monkeys may be due to the extended (28-day) ethanol-abstinent protocol, which it has not tested in rodent models (for review see Roberto et al., 2020).

2.3. Chronic EtOH and GABAergic transmission: Presynaptic effects

There are only a few studies reporting that chronic EtOH exposure can alter GABAergic transmission by effects on GABA release. Short *in vitro* chronic EtOH exposure (one day) induced a transient decrease in mIPSC duration in cultured cortical neurons. Chronic EtOH exposure did not change mIPSC frequency nor did it produce a substantial cross-tolerance to a benzodiazepine in cortical neurons (Fleming et al. 2009). The results suggest that EtOH exposure *in vitro* has limited effects on synaptic $GABA_AR$ function and action potential–independent GABA release in cultured neurons. This group also investigated the effect of chronic EtOH exposure on GABA release in cultured hippocampal neurons (Fleming et al. 2009). These investigators found that chronic EtOH exposure did not alter mIPSC kinetics and frequencies in hippocampal neurons (Fleming et al. 2009). These results suggest that EtOH exposure in cultured cortical and hippocampal neurons may not reproduce all the effects that occur *in vivo* and in acute brain slices.

In fact, more results generated using *in vitro* brain slices show a stronger effect of EtOH on GABA release, as discussed earlier in this review (Figure 1). *In vitro* brain slice preparations provide a number of highly sensitive experimental strategies that can be employed to detect presynaptic changes in transmitter release (for reviews of these approaches, see Siggins et al. 2005; Weiner and Valenzuela 2006; Roberto and Varodayan, 2017).

Studies in the hippocampus show that chronic EtOH exposure decreased long-term potentiation (LTP) by increasing the electrically-stimulated (but not basal) release of tritiated GABA pre-loaded in CA1 hippocampal slices (Tremwel et al. 1994). The GABA uptake or $GABA_AR$ function was not altered, and this effect may be due to alterations in the mAChR regulation of GABA release at presynaptic terminals (Hu et al. 1999). In addition, studies using the $GABA_B$ receptor agonist baclofen to reduce release of tritiated GABA suggest that a change in $GABA_B$ autoreceptors on GABAergic terminals may also contribute to this effect of chronic EtOH exposure on LTP (Peris et al. 1997) (see later $GABA_B$ paragraph). 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). More recent studies also reported that chronic EtOH consumption induces tolerance to the impairing effects of acute ethanol treatment on induction of LTP in rat CA1 slices (Fujii et al. 2008). In CA1 slices from control rats, stable LTP was induced by tetanic stimulation, and LTP induction was blocked if the tetanus was delivered in the presence of 8.6 mM EtOH or muscimol. A decrease in the stimulation threshold for inducing LTP was found

in hippocampal slices from chronic EtOH treated rats. In addition, application of EtOH or muscimol did not affect LTP induction in these cells, suggesting that the effects of chronic ethanol exposure on LTP induction are mediated by a reduction in GABAergic inhibition in hippocampal CA1 neurons (Fujii et al. 2008).

Weiner et al. (Weiner 2004) have found that voluntary EtOH drinking is associated with a significant increase in paired-pulse plasticity at GABAergic synapses in dentate gyrus neurons from the hippocampal formation of monkeys (cynomolgus macaques), consistent with a reduction in GABA release probability. In addition, a lack of tolerance to the facilitating effects of both acute EtOH and flunitrazepam on the $GABA_A$ IPSCs was reported.

In contrast, Melis et al. (Melis et al. 2002) reported that a single EtOH exposure *in vivo* induces a long-lasting facilitation of GABA transmission in the VTA of ethanol-preferring C57BL/6 mice. These investigators observed that evoked $GABA_A$ IPSCs in dopaminergic neurons of EtOH-treated animals exhibited paired-pulse depression (PPD) compared with saline-treated animals, which exhibited PPF (Melis et al. 2002). An increase in frequency of mIPSCs was also observed in the EtOH-treated animals. Moreover, the $GABA_B$ receptor antagonist, CGP35348, shifted PPD to PPF, indicating that presynaptic $GABA_B$ receptor activation, likely attributable to GABA spillover, might play a role in mediating PPD in the EtOH-treated mice (see later $GABA_B$ paragraph). In a more recent study, the same group (Wanat et al. 2009) demonstrated that EtOH exposure also increased GABA release onto VTA dopamine neurons in ethanol non-preferring DBA/2 mice. However, a single EtOH exposure reduced glutamatergic transmission and LTP in VTA dopamine neurons from the ethanol non-preferring DBA strain but not ethanol-preferring C57BL/6 mice (Wanat et al. 2009). *In vivo* recordings in VTA indicate that acute EtOH reduces the activity of putative GABAergic neurons, while increased firing of putative dopaminergic neurons occurs on a faster time scale (Burkhardt and Adermark, 2014). These findings indicate that both direct effects and indirect disinhibitory effects may contribute to EtOH-induced increases in DA release.

Additional data from Roberto and coworkers (Roberto et al. 2010a; Roberto et al. 2004a) further suggest that chronic EtOH exposure can affect CeA GABA release, perhaps via an action on GABAergic terminals. Baseline $GABA_A$ IPSCs were significantly higher, and baseline PPF of $GABA_A$ IPSCs was significantly smaller in CeA neurons from EtOH-dependent rats compared to non-dependent rats, suggesting that evoked GABA release was augmented after chronic ethanol exposure. These investigators also reported an increase in the baseline frequency of mIPSCs in CeA neurons from EtOH dependent rats compared to that of naïve controls. Acute superfusion of EtOH significantly enhanced $GABA_A$ IPSCs, decreased the PPF ratio of IPSCs and increased the mIPSC frequency to the same extent in CeA slices from ethanol-dependent rats and naïve rats, suggesting a lack of tolerance to the presynaptic acute EtOH effects (Roberto et al. 2004a). In addition, these investigators estimated the interstitial GABA levels in CeA using microdialysis in freely moving rats. In agreement with the *in vitro* electrophysiological results, the *in vivo* data showed a 4-fold increase of baseline dialysate GABA concentrations in CeA of EtOH-dependent rats compared to naïve rats. Moreover, local administration of EtOH by dialysis increased

the dialysate GABA levels in CET rats. These findings again indicate a lack of tolerance to presynaptic acute EtOH effects on GABA release in CeA of CET rats (Roberto et al. 2004a). These studies strengthen the possibility that chronic as well as acute EtOH may alter the function of the GABAergic synapses acting at both the postsynaptic site and presynaptic terminals. As mentioned above, recent studies have also consistently replicated the increased GABA release in the CeA of rodents using the same and/or slightly different chronic ethanol exposure in rodents (Herman and Roberto 2016; Khom et al., 2020a,b; Varodayan et al., 2017c; Kirson et al., 2020; Tunstall et al., 2019). Interestingly, the data obtained in abstinent rhesus monkeys with a history of chronic ethanol self-administration and repeated abstinence agree with the rodent studies showing increased GABA release in the CeA following chronic ethanol exposure at early (2–10 h) withdrawal, and late [5–7 days (Herman et al., 2016a) and 14 days (Khom et al., 2020a,b)] withdrawal. Furthermore, a recent study showing decreased GABA transporter (GAT-3) levels and impaired GABA clearance in the CeA of alcohol-preferring rodents and in humans (Augier et al., 2018) support an elevation of GABA level. . together, these data suggest that long-term exposure to EtOH causes changes at GABAergic synapses that may differ between brain regions and with the duration of chronic exposure. Further studies will be needed to more carefully determine the specific exposure durations required to elicit these changes in GABAergic synapses, the molecular mechanisms responsible for these adaptive changes, as well as their behavioral consequences with respect to withdrawal and dependence.

Evidence of decreased GABA release following chronic alcohol ingestion has also been observed in mouse striatum (Wilcox et al., 2014). In this study, mice drank alcohol in the drinking in the dark schedule that produces binge like consumption. The frequency of action potential-independent miniature IPSCs was decreased in both dorsolateral and dorsomedial striatum in the alcohol-drinking mice.

In summary, a growing area in which action of EtOH on GABA function has been implicated is withdrawal from chronic ethanol. Withdrawal results in an increased sensitivity to induction of seizures (Allan and Harris 1987; Frye et al. 1983). Several functional and behavioral studies on benzodiazepines and other drugs with GABAmimetic action reduced such withdrawal-related hyper-excitability (Breese et al. 2006; McCown et al. 1985; Roberto et al. 2008; Ticku and Burch 1980; Herman et al., 2016a; Khom et al., 2020a,b). Collectively, these results offer strong support for the hypothesis that at least a part of the action of EtOH was mediated by effects on neural functions associated with GABA transmission and that these effects play an important role in the maintenance of addictive drinking behavior.

The molecular basis of chronic EtOH effects on presynaptic function is just beginning to be explored, and early findings implicated changes vesicle-associated proteins (see Das 2020 for review). In rhesus macaque monkeys, chronic alcohol consumptions alters expression of the vesicle-associated SNAP-25 protein (Alexander et al. 2018, Nimitvilai et al. 2017). and Das et al. 2013, Ghosh et al. 2017). Increased expression of Munc13–1, another vesicle associated protein has also been observed following chronic EtOH exposure in both mouse and monkeys (Alexander et al. 2018, Ghosh et al. 2017). These findings are particularly interesting as alcohol directly interacts with Munc13–1 (Das et al. 2013) and this protein has

been implicated in acute EtOH effects on synaptic transmission (Gioia et al. 2017). It will be interesting to determine if chronic EtOH effects on neurotransmitter release depend on these proteins and/or other proteins involved in vesicle fusion.

2.4. $GABA_B$ Receptors and chronic EtOH Actions

Several studies demonstrated $GABA_B$ receptor involvement in the effects of EtOH. For instance, $GABA_B$ receptor antagonists enhance the ability of acute EtOH to facilitate GABA transmission in the hippocampus (Ariwodola and Weiner 2004; Wan et al. 1996; Wu and Saggau 1994) and NAc (Nie et al. 2000). Ariwodola and Weiner (2004) suggested that the effect of EtOH to facilitate GABA transmission is limited because of GABA feedback on presynaptic $GABA_B$ receptors (Figure 1). The presence of $GABA_B$ receptors accounted for the difference in sensitivity to EtOH influences on GABA transmission in specific subfields of the hippocampus (Weiner et al. 1997). On the other hand, $GABA_B$ receptors did not influence GABA release from neurons in the CeA (Roberto et al. 2003). Thus, the involvement of $GABA_B$ receptors on GABA release in various brain regions may not be universal, suggesting that the presence or absence of presynaptic $GABA_B$ receptors may be an important determinant for the regional specificity of ethanol to affect GABA transmission (Ariwodola and Weiner 2004).

As mentioned above, Peris et al., (Peris et al. 1997) showed that chronic EtOH treatment, sufficient for decreasing LTP in rats, also increased 3H-GABA release from hippocampal slices in these same animals. These investigators characterized presynaptic autoreceptor modulation of 3H-GABA release in hippocampal slices from control and EtOH-dependent rats. Effects of a $GABA_B$ receptor agonist (baclofen) and antagonist [2-hydroxy (OH)-saclofen] on electrically stimulated 3H-GABA release from superfused hippocampal slices were examined. Baclofen decreased stimulated release in a dose-dependent manner and the antagonist 2-OH-saclofen increased release consistent with the presence of presynaptic $GABA_B$ autoreceptors in hippocampus. The $GABA_A$ antagonist bicuculline did not significantly modulate basal or stimulated release. Presynaptic modulation of release by baclofen and 2-OH-saclofen was decreased in animals 48 hr. after withdrawal from EtOH. Using quantitative autoradiographic techniques, the density of 3H-baclofen binding sites in the hippocampus was not affected by chronic EtOH exposure, whereas the density of 3H-bicuculline binding sites was increased by 28% in EtOH-treated rats. These data may explain how chronic EtOH treatment increases presynaptic regulation of GABA release from hippocampus that may contribute to the decrease in LTP seen in rats after chronic ethanol exposure (Peris et al. 1997).

Another study assessed the impact of EtOH on postsynaptic $GABA_B$ receptors via baclofen-induced hyperpolarization of hippocampal CA1 and CA3 pyramidal neurons. These receptors activate outward K+ currents via a pertussis toxin-sensitive G protein cascade to reduce membrane potential during the slow inhibitory postsynaptic potential and may play a role in EtOH intoxication and withdrawal excitability. In both types of pyramidal neurons, baclofen applied consecutively in increasing concentrations caused concentration dependent hyperpolarization. There were no significant differences in resting membrane potential, input resistance, maximum baclofen-induced hyperpolarization or EC_{50} between CA1 and

CA3 neurons, although slope values were significantly smaller in the former neurons. These parameters were not significantly changed in the presence of EtOH 10–100 mM. Chronic EtOH treatment (12 days) did not shift sensitivity or maximum response to baclofen in CA1 neurons. These results suggest that $GABA_B$ receptors in this model were essentially insensitive to ethanol (Frye and Fincher 1996).

Melis et al. (Melis et al. 2002) linked the long-lasting potentiation of GABAergic synapses on dopaminergic neurons in the VTA by systemic EtOH to an effect on presynaptic $GABA_B$ receptors. Moreover, the frequency (but not the amplitude) of mIPSCs was also significantly higher in VTA neurons of EtOH-treated animals compared to controls, further supporting an increased probability of presynaptic GABA release independent of neuronal discharge in VTA neurons treated with ethanol. Interestingly, the $GABA_B$ receptor antagonist, CGP35348, shifted PPD to PPF in EtOH-treated animals by increasing the amplitude of the second evoked $GABA_A$ IPSC and without affecting $GABA_A$ IPSC in the saline-treated animals. In addition, both the frequency and the amplitude of mIPSCs were unaffected by CGP35348 in both groups of mice. Thus, the PPD observed in the EtOH treated mice could result from an increased probability of GABA release, which might in turn lead to activation of presynaptic $GABA_B$ receptors and decrease the second IPSC. These results further support the hypothesis that GABA levels are increased after EtOH exposure, leading to spillover onto presynaptic $GABA_B$ receptors, whose activation leads to inhibition of release (Hausser and Yung 1994; Melis et al. 2002).

In a recent study, Roberto et al., (Roberto et al. 2008) reported neuroadaptations in $GABA_B$ receptors in CeA after chronic EtOH exposure. The sensitivity of GABA IPSCs to the $GABA_B$ receptor antagonist CGP 55845A and agonist baclofen was decreased after chronic EtOH, suggesting downregulation of this system. Specifically, the $GABA_B$ receptor antagonist, CGP 55845A significantly increased the mean amplitude of evoked IPSCs in CeA from naïve rats. This increase in the IPSC amplitude was associated with a significant decrease in PPF, suggesting a tonic activation of presynaptic $GABA_B$ receptors in naïve rats. In contrast, in CeA from EtOH-dependent rats, CGP 55845A did not alter the mean evoked IPSCs and did not affect mean PPF. Baclofen markedly depressed evoked GABA IPSC amplitudes in neurons of naïve rats, with recovery during washout. The baclofen-induced inhibition of GABA IPSCs was significantly reduced in neurons of EtOH-dependent rats. In addition, in CeA neurons from EtOH-dependent rats, baclofen-induced depression was associated with a smaller increase of the PPF ratio of GABA IPSCs compared to that in neurons of naïve rats. These data suggest that the downregulation of the $GABA_B$ system associated with EtOH-dependence may explain in part the increased GABAergic tone reported in dependent rats (Roberto et al. 2008).

2.5. Glycine Receptor Roles in Chronic Alcohol Actions

In comparison to GABAergic transmission, much less is known about chronic EtOH effects on glycinergic synapses. However, there is increasing information about how glycine receptors in the CNS contribute to alcohol-related behaviors. Using mice in which an EtOH-insensitive mutated GlyR alpha1 subunit is substituted for the wildtype receptor, investigators have shown reduced sedative responses to acute EtOH (Aguayo et al., 2014).

These mice also show greater conditioned place preference for EtOH and greater EtOH intake upon first exposure to the drug (Muñoz et al., 2020). Mice carrying a similar mutation that renders the alpha2 GlyR subunit EtOH-insensitive show a similar pattern of shorter-duration of EtOH-induced sedation and increased EtOH consumption (Gallegos et al., 2021). Mice lacking alpha2 GlyR subunits show reduced EtOH intake and preference, and increased aversive responses to the drug while mice lacking the alpha3 subunit show increased intake and preference but a decrease in conditioned EtOH taste aversion (Blednov et al., 2015). Thus, glycinergic synaptic effects appear to have roles in acute EtOH actions and regulation of EtOH drinking. As mentioned above, agonism of the glycine receptor impacts GABAergic transmission in CeA of naïve rats, without affecting the acute alcohol-induced facilitation of GABAergic responses, and this effect is lost in neurons from alcohol-dependent rats (Kirson et al., 2020). Glycine transport in the prefrontal cortex appears to play a role in impulsivity during abstinence following chronic EtOH exposure (Irimia et al., 2017). This may involve GlyRs or glycine-sensitive NMDARs. It will be interesting to determine how chronic alcohol affects glycine release and glycine receptors.

Chronic alcohol drinking also alters the expression of a number of genes related to glycinergic transmission (Vengeliene et al., 2010). Some of these changes can be reversed by treatment with a glycine transporter antagonist that also reduced compulsive-like drinking in rat (Vengeliene et al., 2010).

2.6 Changes in Dopaminergic Transmission Induced by Chronic Alcohol

There are conflicting reports of chronic alcohol effects on DA release and extracellular DA. Several studies indicate that increases in extracellular DA concentrations persist throughout chronic EtOH exposure and intake in self-administration paradigms and also become associated with conditions that predict drug availability (Bassareo et al., 2017, Doyon et al. 2003, Hirth et al., 2016) (Figure 2A). Sensitization to the dopamine-increasing effects in NAc of EtOH microinjection into the VTA has also been observed following chronic EtOH consumption (Ding et al., 2016). Examination of striatal tissue from AUD patients indicates decreased DAT expression, possibly indicating a hyperdopaminergic state in these individuals (Hirth et al., 2016) (Figure 2A). A combined analysis of extracellular tortuosity and modeling suggested the changes dopamine diffusion could contribute to increased availability of the neurotransmitter after chronic ethanol exposure (De Santis et al., 2020). Altogether These findings suggest that the ability of EtOH to enhance accumbal NAc either does not show tolerance with repeated exposure or undergoes adaptations that maintaince high dopamine levels, and some mechanisms may even be enhanced under these circumstances.

However, other studies indicate that dopamine release is reduced following chronic EtOH consumption or forced exposure. For example, Karkhanis et al. (2016) found that acute EtOH stimulation of DA release switched to inhibition in NAc following chronic exposure. Decreased DA release in the NAc core subregion was observed in adult rats following adolescent EtOH exposure (Zandy et al., 2015). Enhanced function of kappa opiate receptors and D2 dopamine autoreceptors may contribute to the decreased DA release both in mice and monkeys (Rose et al., 2016, Siciliano et al., 2015, 2016), although decreased D2

autoreceptor function has also been observed, a change that varies across sexes (Salinas et al., 2021) (Figure 2A).

Expression of D2 dopamine receptors is reduced in striatum in humans with AUD, as assessed with positron emission tomography imaging (Volkow et al., 2017) (Figure 2A). This decrease most likely reflects loss of the receptors expressed by MSNs, and may lead to a loss of one brake on striatal output. It remains to be determined if this receptor loss reflects a pre-existing state or an effect of long-term ethanol consumption.

2.6. Chronic Alcohol Effects on Cholinergic Systems

Decreases in the number of basal forebrain cholinergic neurons have been observed following chronic EtOH exposure in adult rat (Arendt et al. 1988, 1989; Smiley et al., 2021). However, Vetreno and coworkers did not observe a similar loss of neurons following EtOH exposure (Vetreno et al., 2014). Evidence for decreases in the number of axon terminals made by these neurons in the dentate gyrus and hippocampal gyri was also observed (Cadete-Leite et al., 1995; Pereira et al. 2016). These losses were reversed by treatment with nerve growth factor that is known to be trophic for these cells (Lukoyanov et al., 2003; Pereira et al., 2016). The numbers of cholinergic neurons in the pedunculopontine and laterodorsal tegmental areas was also decreased following chronic EtOH consumption and withdrawal (Pereira et al., 2020).

Mixed effects of chronic EtOH on ACh levels and release have been reported (Figure 2B). Decreased ACh levels following chronic EtOH exposure were originally reported in several brain regions (Hunt and Dalton, 1976). However, subsequent studies reported increased ACh concentration in the rat striatum 1–3 days after the cessation of a four day EtOH treatment (Hunt et al., 1979), and mixed results were observed in other studies examining a variety of brain regions (Parker et al., 1978; Smyth and Beck, 1969). The activity of enzymes involved in ACh synthesis as well as the high-affinity choline uptake system have also been examined following chronic EtOH exposure (Norberg and Wahlstrom, 1992). Activity of the synthetic enzyme choline acetyltransferase (ChAT) is increased after a few days of EtOH exposure (Ebel et al., 1979), but decreased after weeks of exposure (Smyth and Beck, 1969; Pelham et al., 1980). High affinity choline uptake is increased a few days after withdrawal from a relatively short exposure to EtOH (Hunt et al., 1979, Hunt and Majchrowitz, 1979). It must be noted that the subject of ACh levels and enzyme expression/function has not be revisited with newer research approaches, and thus additional study is warranted.

Reduced preparations have also been used to examine effects of chronic EtOH exposure on ACh release. Using slices of Nucleus accumbens and dorsal striatum, Nestby et al. (1997) found that 15 days of exposure to a moderate ethanol treatment enhanced electrically-evoked ACh release. No changes in ACh release from cortical or hippocampal synaptosomes were observed following chronic EtOH consumption (Sabriá et al., 2003).

Subsequent studies examined changes in ACh release *in vivo* following chronic ethanol exposure using microdialysis. As is the case for acute EtOH exposure, chronic EtOH in rat also generally decreases hippocampal ACh levels (Casamenti et al., 1993; Imperato et al., 1998). Decreased ChAT activity appears to be associated with the decreased release

(Casamenti et al., 1993). Recovery of release was observed following four weeks of abstinence subsequent to three months of EtOH drinking, but less recovery was observed with abstinence after six months of drinking (Casamenti et al., 1993). In anesthetized rat, increased hippocampal ACh was observed following a 4-day EtOH exposure and subsequent withdrawal (Imperato et al., 1998). In another set of studies, mixed results were obtained within the same laboratory. Decreased hippocampal ACh was observed following nine months of drinking in Sprague-Dawley rats, and this was correlated with impaired passive avoidance performance (Melis et al., 1996). However, these effects were not observed in the Sardinian alcohol-preferring rat (Fadda et al., 1999). Chronic exposure to EtOH can alter the initial acute drug effects. Increased ACh in the VTA during EtOH intake appears to subside with continued drinking (Larsson et al., 2005). Acute ethanol-induced increases in ACh levels in hypothalamus changed to decreases following several days of administration (Kaneyuki et al., 1995).

As is the case for acute EtOH actions on ACh and cholinergic synapses, effects of chronic EtOH exposure are mixed and depend on ACh receptor subtype and anatomical region (Figure 2B). Radioligand binding studies were initially employed to identify changes in receptor numbers and affinity. The most direct measures of changes in nAChRs were conducted in cell lines in which binding can be examined independent of changes in cell type or circuitry. In the PC12 neuroblastoma cell line, exposure to EtOH in the medium for 48–96 hours increased the binding of epibatidine, a ligand for the α4β2 subunit-containing nAChR subtype (Dohrman and Reiter 2003). Nicotine stimulation of binding was also increased by this treatment. In an M10 cell line engineered to express α4β2-containing receptors, exposure to EtOH for 12–48 hours had the opposite effect, decreasing epibatidine binding, but a slight increase was observed after 96 hours of exposure (Dohrman and Reiter 2003). It is not clear why different effects were observed in these different cell lines, but differences in intracellular signaling are likely to be involved. The decrease in binding in the M10 cells was blocked by a protein kinase C inhibitor, but this was not tested in PC12 cells.

Mixed effects on nAChR radioligand binding have also been observed in brain tissue from animals chronically treated with EtOH *in vivo*. In the rat hippocampus, hypothalamus and thalamus decreased binding of nicotine was observed (Yoshida et al., 1982). However, hippocampal nicotine binding was decreased just after voluntary EtOH drinking in rat (Robles and Sabriá 2006), but increased after withdrawal from drinking (Robles and Sabriá 2008). Examination of binding of αBungarotoxin, a ligand for α7-type nAChRs, revealed differential effects of chronic EtOH drinking in the inbred Long-sleep (decreased hippocampal and increased thalamic binding) and Short-sleep mice (increased binding in cerebellum and superior colliculus) (Booker and Collins 1997). Evidence for decreased binding to α4β2-containing receptors was also observed in rhesus monkey cortex after chronic alcohol drinking (Hillmer et al. 2014).

Radioligand binding and molecular biological approaches have been used to examine chronic alcohol effects on mAChR expression. Exposing human neuroblastoma cells for several days led to an increase in mAChR-induced inositol phosphate production (Larsson et al., 1996). This effect was accompanied by increased radioligand binding that implicated M1-type mAChRs in the potentiation. A similar increase in mAChR binding was also

observed following 2 days of EtOH exposure in NG108-15 neuroblastoma x glioma cells (Hu et al., 1993). In general, *in vivo* chronic EtOH exposure has been shown to increase mAChR binding sites in cerebral cortex, hippocampus, mammillary body, and striatum, although mixed effects have been observed in different studies (reviewed in Nordberg and Wahlstrom, 1992, Pick et al., 1993; Pietzak et al., 1988; Rothberg et al., 1996; Tabakoff et al., 1979). In general, the increases were largest after exposure periods of a few days and during withdrawal following longer exposure periods. The M1 mAChR is one subtype that appears to be upregulated after chronic EtOH exposure (Pietzak et al., 1988; Hoffman et al., 1986; Muller et al., 1980). Expression of the five different mAChR subtype proteins in hippocampus was also examined with immunoprecipitation, but no effect of chronic EtOH exposure was observed (Rothberg et al., 1993).

Effects of chronic EtOH exposure on the functional consequences of mAChR activation have also been examined, using both receptor-mediated inositol phosphate generation and electrophysiological changes as the functional readouts. A reduction in the ability of EtOH to inhibit mAChR-mediated stimulation of inositol triphosphate formation in mouse brain tissue and synaptosomes was observed following chronic EtOH consumption (Hoffman et al., 1986; Smith, 1983). It is unclear if this tolerance is due to decreased mAChR expression or downstream signaling mechanisms, but given the general finding of increased receptor binding it is probable that decreased downstream signaling is involved. Activation of mAChRs enhances the population spike (PS) and inhibits the field excitatory postsynaptic potential (fEPSP) during extracellular field potential recordings in the hippocampal CA1 subregion. Chronic EtOH exposure reduces the population spike facilitation but does not alter fEPSP inhibition (Rothberg and Hunter, 1991; Rothberg et al., 1993). Frye and coworkers also found no chronic EtOH-induced change in mAChR inhibition of hippocampal fEPSPs, as well as no change in inhibition of the afterhyperpolarization by mAChRs (Frye et al., 1995). The differential EtOH effects on these responses likely result from the fact that different mAChRs mediate the different physiological effects, with those coupled to Gi/o-type G-proteins in involved in fEPSP inhibition and G-q G-protein coupled receptors mediating the other responses.

Cholinergic neuron numbers and mAChR binding have also been examined in postmortem samples from humans with Alcohol Use Disorder. Decreased basal forebrain cholinergic neuron numbers were observed in humans diagnosed with Korsakoff syndrome, as a consequence of prolonged alcohol drinking (Arendt et al., 1983). Redioligand binding revealed evidence of decreases in mAChRs in older AUD patients (Freund and Ballinger, 1989a,b; Hellstrom-Lindahl et al., 1993; Nordberg et al., 1983; Nordberg and Wahlstrom, 1992). It is unclear if the receptor loss is a result of the decrease in cholinergic neuron number mentioned previously, although Freund and Ballinger (1991) did not observe evidence of neurodegeneration in the brains in which they observed decreased mAChR binding sites. Activity of the ChAT enzyme is also reduced in postmortem brain samples from individuals with AUD (Antuono et al., 1980).

3. Neuropeptide Roles in Acute and Chronic Alcohol Actions

Neuropeptides are potent neuromodulators in the CNS whose actions are mediated via GPCRs. In contrast to classical neurotransmitters, neuropeptides are released in a frequency-dependent fashion and often have a longer half-life of activity after release. These factors, among others, enable neuropeptides to produce long-lasting effects on cellular functions such as excitatory and inhibitory synaptic transmission, neuronal excitability and gene transcription (Gallagher et al. 2008). Thus, a long-lasting dysregulation of neuropeptides could have significant effects on the activity of neurons and consequently, behavior. Thus, several neuropeptideric system in different brain circuits have received a lot of attention particularly in the development of AUD (Koob and Volkow, 2016).

3.1. Corticotropin-Releasing Factor

Corticotropin-releasing factor (CRF) is a 41-amino acid polypeptide that has a major role in coordinating the stress response of the body by mediating hormonal, autonomic, and behavioral responses to stressors. CRF (originally called corticotropin-releasing hormone, although the International Union of Pharmacology designation is CRF) was identified through classic techniques of peptide sequencing (Vale et al. 1981). Subsequently, genes encoding three paralogs of CRF – urocortins 1, 2, and 3 (Ucn 1, Ucn 2, Ucn 3), were identified by modern molecular biological approaches. Ucn 2 and Ucn 3 are also referred to as stresscopin-related peptide and stresscopin, respectively. CRF and the urocortins have been implicated in the modulation of multiple neurobiological systems, including those that regulate feeding, anxiety and depression, hypothalamic–pituitary–adrenal (HPA) axis signaling, and EtOH consumption (Hauger et al. 2006; Heilig and Koob 2007; Ryabinin and Weitemier 2006; Smith and Vale 2006). CRF and the Ucn peptides produce their effects by binding to the G-protein-coupled CRF type 1 (CRF1R) and CRF type 2 (CRF2R) receptors. CRF binds to both receptors, but has greater affinity for the CRF1R (Bale and Vale 2004; Fekete and Zorrilla 2007; Hauger et al. 2006; Pioszak et al. 2008).

CRF1R and CRF2R are GPCRs that are predominantly positively linked to the activation of AC (Figure 1), and recent reports also implicate other second messenger systems such as inositol triphosphate and PKC (Blank et al. 2003; Grammatopoulos et al. 2001). Using corticotrophins, Antoni and coworkers (Antoni et al. 2003) demonstrated a coupling of CRF1R to AC9 and AC7. The switch in coupling from AC9 to AC7 results in a more robust cAMP signal when CRF binds to the CRF1R (Antoni 2000; Antoni et al. 2003). It should be emphasized that AC7 is localized both postsynaptically (striatum, hippocampus) and presynaptically (nucleus accumbens, amygdala) (Mons et al. 1998a; Mons et al. 1998b), and is anatomically positioned to receive signals from GPCRs on both dendrites and axon terminals.

Pharmacological and transgenic studies show that brain and pituitary CRF1Rs mediate many of the functional stress-like effects of the CRF system (Heinrichs and Koob 2004). CRF and the Ucn peptides have a wide distribution throughout the brain, but there are particularly high concentrations of cell bodies in the paraventricular nucleus of the hypothalamus, the basal forebrain (notably the extended amygdala), and the brainstem (Swanson et al. 1983). Ucn1 binds with equal affinity to CRF1R and CRF2R, and Ucn2 and Ucn3 are CRF2R

agonists (Hauger et al. 2006; Pioszak et al. 2008). CRF and the Ucn peptides exert their behavioral and neuroendocrine actions through central hypothalamic and extrahypothalamic pathways (Hauger et al. 2006; Heilig and Koob 2007; Heinrichs and Koob 2004; Koob and Le Moal 2008).

Increasing evidence implicates CRF and its receptors in the synaptic effects of EtOH. Ethanol induces release of CRF from the hypothalamus that initiates the activation of the HPA axis (Ogilvie et al. 1998). Ethanol also modulates the extra-neuroendocrine CRF system involved in behavioral stress responses, particularly in the amygdala. Ethanol withdrawal induces an increase in CRF levels in the amygdala (Merlo Pich et al. 1995) and in the BNST (Olive et al. 2002).

The central administration of a CRF antagonist attenuates both EtOH self-administration and the anxiety-like response to stress observed during alcohol abstinence, (Valdez et al. 2002) and administration of a CRFR antagonist into the CeA reverses the anxiogenic-like effect of alcohol (Rassnick et al. 1993). Rats tested 3–5 weeks post alcohol withdrawal showed an anxiogenic-like response provoked by a mild restraint stress only in rats with a history of alcohol dependence. This stress-induced anxiogenic-like response was reversed by a competitive CRF1R antagonist (Valdez et al. 2003). The increased self-administration of alcohol observed during protracted abstinence also was blocked by a competitive CRF1R antagonist (Valdez et al. 2003). Gehlert et al., (2007) also described that a novel CRF1R antagonist, the 3-(4-Chloro-2-morpholin-4-yl-thiazol-5-yl)-8-(1-ethylpropyl)-2,6-dimethyl-imidazo[1,2-b]pyridazine (MTIP) has advantageous properties for both clinical development and in preclinical models of Alcohol Use Disorder (AUD). MTIP dose-dependently reversed anxiogenic effects of ethanol withdrawal, and blocked excessive alcohol self-administration in Wistar rats with a history of dependence (Gehlert et al. 2007). CRF also contributes to increased alcohol consumption in dependent animals, because increased EtOH self-administration is reduced by CRF1R antagonists in dependent animals but not in non-dependent animals (Funk et al. 2007, Overstreet et al. 2004) and by CRF1R deletion (Chu et al. 2007; Sillaber et al. 2002). More recently, it has been reported that chronic CRF1R antagonist treatment blocked withdrawal-induced increases in alcohol drinking by dependent rats, and tempered moderate increases in alcohol consumption (Roberto et al. 2010a). In addition, inactivation of the CeA CRF+ neurons prevents recruitment of this neuronal ensemble, decreases the escalation of alcohol drinking, and decreases the intensity of somatic signs of withdrawal (de Guglielmo et al., 2019). These results have led to the hypothesis that negative emotional states (including anxiety-like states) contribute to the compulsive alcohol intake associated with AUD via negative reinforcement mechanisms (Koob 2008; Zorrilla et al., 2013; Zorrilla and Koob, 2010; Zorrilla et al., 2014; Gilpin and Roberto, 2012).

Several recent reviews (Lowery and Thiele 2010; Zorrilla et al., 2013; Zorrilla and Koob, 2010; Zorrilla et al., 2014; Spierling and Zorrilla, 2017; Quadros et al. 2016) provide a comprehensive overview of preclinical evidence from rodent studies that suggest a promising role for CRFR antagonists in the treatment of alcohol abuse disorders. In contrast, few other reviews emphasize the preclinical results that hinder the translational of CRF pharmacology to the clinic (Pomrenze et al., 2017; Cannella et al., 2019, Agoglia et al.,

2020; Roberto et al., 2017). These reviews point to the lack of preclinical studies performed in female rodents (as most the studies have been performed in male rodents) and that would strongly suggest sex differences in the ability of CRF/CRF1-directed therapies to functionally regulate alcohol drinking in the clinical setting.

CRFR antagonists protect against excessive EtOH intake resulting from ethanol dependence without influencing ethanol intake in non-dependent animals. Similarly, CRFR antagonists block excessive binge-like ethanol drinking in non-dependent mice but do not alter ethanol intake in mice drinking moderate amounts of ethanol (Lowery and Thiele 2010). CRFR antagonists also protect against increased EtOH intake and relapse-like behaviors precipitated by exposure to a stressful event. Additionally, CRFR antagonists attenuate the negative emotional responses associated with EtOH withdrawal. The protective effects of CRFR antagonists are modulated by CRF1R. Finally, recent evidence has emerged suggesting that CRF2R agonists may also be useful for treating alcohol abuse disorders for review see (Lowery and Thiele 2010; Spierling and Zorrilla, 2017; Roberto et al., 2017).

Low CRF concentrations can influence neuronal properties in the CNS (see (Aldenhoff et al. 1983; Siggins et al. 1985). CRF decreases the slow afterhyperpolarizing potential in hippocampus (Aldenhoff et al. 1983) and CeA (Rainnie et al. 1992), and enhances R-type voltage-gated calcium channels in rat CeA neurons (Yu and Shinnick-Gallagher 1998). These and other data (Liu et al. 2004; Nie et al. 2004; Nie et al. 2009; Roberto et al. 2010a; Ungless et al. 2003) also suggest that CRF plays an important role in regulating synaptic transmission in CNS. For example, in VTA dopamine neurons, CRF potentiates NMDA-mediated synaptic transmission via CRF_2 activation (Ungless et al. 2003), and we recently found that CRF augments GABAergic inhibitory transmission in mouse CeA neurons via CRF1 activation (Figure 1).

3.1.1. CRF Actions in the VTA.—The VTA receives CRF inputs from a number of sources including the limbic forebrain and the paraventricular nucleus of the hypothalamus (Rodaros et al. 2007). These CRF inputs form symmetric and asymmetric synapses, mostly onto dendrites, that co-release either GABA or glutamate, respectively (Tagliaferro and Morales 2008). VTA dopamine neurons express both types of CRF receptors, CRF1R and CRF2R (Ungless et al. 2003), and approximately 25% of VTA dopamine neurons, express the CRF binding protein (CRF-BP);(Wang et al. 2005; Wang and Morales 2008). CRF regulates dopamine neurons through a subtle interplay of effects at CRF1R, CRF2R and CRF-BP. CRF increases action potential firing rate in VTA dopamine neurons via CRF1R and involves a PKC-dependent enhancement of I_h (a hyperpolarization-activated inward current) (Wanat et al. 2008). CRF enhanced the amplitude and slowed the kinetics of IPSCs following activation of D2-dopamine and $GABA_B$ receptors. This action is postsynaptic and dependent on the CRF1R. The enhancement induced by CRF was attenuated by repeated *in vivo* exposures to psychostimulants or restraint stress (Beckstead et al. 2009).

CRF can induce a slowly developing, but transient, potentiation of NMDAR-mediated synaptic transmission (Ungless et al. 2003). This effect involves the CRF2R and activation of the protein kinase C pathway and the requirement of CRF-BP. However, the effect of

CRF is restricted to a subset of dopamine neurons expressing large I_h currents (Ungless et al. 2003).

In addition to fast, excitatory glutamate-mediated synaptic transmission, dopamine neurons also express metabotropic glutamate receptors (mGluRs) which mediate slower, inhibitory synaptic transmission (Fiorillo and Williams 1998). The rapid rise and brief duration of synaptically released glutamate in the extracellular space mediates a rapid excitation through activation of ionotropic receptors, followed by inhibition through the mGluR1 receptor (Fiorillo and Williams 1998). CRF can enhance these mGluRs via a CRF2R-PKA pathway that stimulates release of calcium from intracellular stores (Riegel and Williams 2008). The CRF modulation of VTA synaptic activity is very complex because CRF has diverse actions on dopamine neurons that are excitatory and inhibitory. Furthermore, desensitization of D2 receptors induced by dopamine or CRF on DAergic VTA neurons is associated with increased glutamatergic signaling in the VTA (Nimitvilai et al., 2014). In summary, the excitatory effects of CRF on dopamine neurons appear to affect fast events (e.g., action potential firing rate and NMDAR-mediated synaptic transmission), whereas the inhibitory effects involve slow forms of synaptic transmission. Another important aspect is that CRF1R-mediated effects do not involve interactions with the CRF-BP, whereas CRF2R-mediated effects do. Recently, the CRF-BP has been considered a potential target for its role in AUD (Haass-Koffler et al. 2016; Ketchesin et al. 2016), and its role in the escalation of alcohol drinking may involve its interaction with CRF2 (Albrechet-Souza et al. 2015; Quadros et al. 2016).

It is speculated that these effects on short-term plasticity phenomena may modulate longer-lasting forms of plasticity. For example, NMDAR activation is required for the induction of long-term potentiation in VTA dopamine neurons (Bonci and Malenka 1999; Borgland et al. 2010).

3.1.2. CRF Actions in the Central Amygdala.—The CeA contains CRF receptors and abundant CRF-containing fibers (De Souza et al. 1984; Uryu et al. 1992); CRF itself is generally co-localized in CeA neurons together with GABA (Eliava et al. 2003; Asan et al. 2005). Acute EtOH augments evoked $GABA_A$ receptor-mediated inhibitory postsynaptic currents (IPSCs) by increasing GABA release in both mouse (Bajo et al. 2008; Nie et al. 2004) and rat CeA neurons (Roberto et al. 2003; Roberto et al. 2004).

CRF1Rs mediate the EtOH-induced augmentation of IPSCs in mouse CeA (Nie et al. 2004; Nie et al. 2009) via the PKCε signaling pathway (Bajo et al. 2008; Nie et al. 2004). Both CRF and EtOH augment evoked IPSCs in mice CeA neurons, and CRF1R (but not CRFR2) antagonists blocked both CRF and ethanol effects. In addition, CRF and EtOH augment IPSCs in wild-type and CRF2R knockout mice, but not in CRF1R knockout mice (Nie et al. 2004) or with CRF1 antagonism (Nie et al., 2009).

Electrophysiological data showed that CRF, like EtOH, also enhances GABAergic transmission in the rat CeA (Roberto et al. 2010a). As in mice, CRF and EtOH actions involve presynaptic CRF1R activation at the CeA GABAergic synapses. Interestingly, the interactions between the CRF and GABAergic systems in the CeA may play an important

role in alcohol reward and dependence (Roberto et al. 2010a). These results suggest that the presynaptic effect of EtOH on GABA release in rodent CeA involves CRF1R and perhaps release of CRF itself. Furthermore, both CRF and EtOH decreased PPF of IPSCs in mouse and rat neurons, and the effects of both were selectively blocked by CRF1R antagonists. In addition, both EtOH and CRF increase the frequency of GABAR-mediated mIPSCs, and this effect is blocked by CRF1R antagonists (Nie et al. 2004; Nie et al. 2009; Roberto et al. 2010). Thus, EtOH probably enhances the release of GABA by activating CRF1R on GABAergic terminals (Nie et al. 2009; Roberto et al. 2010a). Conversely, CRF1R antagonists directly increased PPF of IPSCs and decreased mIPSC frequencies, consistent with decreased GABA release, thus opposing EtOH effects. Because GABA and CRF are often co-localized in CeA neurons, the EtOH-elicited GABA release may involve release of the CRF peptide itself, perhaps even from the terminals synapsing on autoreceptors on the same cell bodies or on collaterals from other GABAergic interneurons. Thus, this example raises the possibility of involvement of other, secondary messengers in EtOH effects on GABAergic terminals.

Chronic EtOH exposure produces functional adaptation of the CRF system in CeA (Hansson et al. 2006; Hansson et al. 2007; Sommer et al. 2008; Weiss et al. 2001). Interestingly, in CeA of dependent rats, the ability of maximal (200 nM) and a submaximal (100 nM) concentrations of CRF to augment evoked IPSCs was significantly enhanced compared to naïve CeA. A greater effect of CRF1R antagonists on basal IPSCs of dependent rats was also reported. The greater effect of CRF and CRF1R antagonists may reflect increased tonic release of endogenous CRF, constitutive CRF1R activation, increased receptor number, and/or sensitization of CRF1R in CeA of dependent rats This is supported by increased CRF and CRF1 mRNA levels seen in the CeA of alcohol-dependent rats, and by reversal of dependence–induced elevations in amygdalar GABA dialysate by a CRF1 antagonist (Roberto et al. 2010a). Thus, these combined findings suggest an important EtOH-CRF interaction on GABAergic transmission in the CeA that markedly increases during development of ethanol dependence (Roberto et al. 2010a).

In other studies using adult mice, one and six cycles of the drinking in the dark paradigm (DID) increases CeA CRF immunoreactivity, suggesting that the CRF system is recruited during early binge-like drinking episodes (Lowery-Gionta et al. 2012). Notably, the synaptic effects of CRF on CeA GABAergic transmission are reduced after repeated bouts of binge-like drinking (Lowery-Gionta et al. 2012).

Given the critical role of the CRF/CRF1 system and the cellular heterogeneity in the CeA, several recent studies have used a transgenic mouse line expressing the green fluorescent protein (GFP) under the Crhr1 promoter (CRF1:GFP) to readily identify neurons expressing CRF1 (CRF1+) (Justice et al. 2008; Herman et al. 2013a; Herman et al. 2016a) to unveil unique molecular, morphological and functional properties that distinguish CeA CRF1+ neurons from their CRF1 non-expressing (CRF1−) neighbors. CRF1+ neurons are mainly located in the medial subdivision of the CeA and exhibit an ongoing tonic GABAergic conductance driven by action potential-dependent GABA release. In contrast, CRF1− neurons do not display tonic inhibition (Herman et al. 2013a). As described above, chronic ethanol induced functional adaptations on phasic and tonic inhibition and cell firing in

CRF1+ and CRF1− CeA neurons (Herman et al. 2016a). In particular, a loss of tonic currents and a significantly higher basal firing rate was observed in CRF1+ CeA neurons projecting to the BNST of CIE vs. control mice (Herman et al. 2016a). Recent work from the Herman laboratory has shown that CRF1+ CeA neurons exhibit sex differences in sensitivity to the effects of acute alcohol, as well as CRF1 agonists and antagonists (Agoglia et al., 2020; 2021). Furthermore, chronic alcohol drinking produced neuroadaptations in CRF1+ neurons that increased the sensitivity of GABAA receptor-mediated sIPSCs to the acute effects of alcohol, CRF and the CRF1 antagonist R121919, but these adaptations were more pronounced in male versus female mice. The CRF1 antagonism reduced voluntary alcohol drinking in both sexes and abolished sex differences in alcohol drinking. The minimal alcohol-induced changes in the female CRF1 system may be related to the elevated alcohol intake displayed by female mice and could contribute to the ineffectiveness of CRF1 antagonists in female AUD patients (Agoglia et al., 2020; 2021).

Retson and colleagues (2016) have reported similar results supporting clear sex differences in CeA CRF in rats. They found that alcohol drinking activated CeA CRF neurons and enhanced the response of these neurons to stress selectively in male but not female rats (Retson et al., 2016). Further investigation of these sex differences is necessary to clarify the contributions of CRF activity to alcohol use in both males and females.

Overall those studies have yielded significant insight into cell type-specific effects of acute and chronic alcohol in local and downstream CRF-CeA circuits. In parallel molecular studies have also assessed expression of subpopulation markers and neuropeptides, dendritic spine density and morphology, and glutamatergic transmission in CeA CRF1+ vs. CRF1− neurons (Wolfe et al. 2019). In brief, CeA CRF1+ neurons are GABAergic, but do not segregate with calbindin, calretinin, or PKCδ. Co-expression analysis using in situ hybridization revealed Crhr1 had highest co-expression with Penk and Sst and least with neuropeptide Y (NPY). Additionally, CeA CRF1+ neurons do not display differences in mature spines and accordingly no difference in basal CeA glutamate transmission. CRF application enhances overall glutamate release onto both CRF1+ and CRF1− neurons but increases postsynaptic glutamate receptor functions selectively in CRF1+ neurons (Wolfe et al. 2019).

CRF-related peptides serve as hormones and neuromodulators of the stress response and play a role in affective disorders. It has been shown that excitatory glutamatergic transmission is modulated by two endogenous CRF-related peptide ligands, CRF rat/human (r/h) and Ucn I, within the CeA and the lateral septum mediolateral nucleus (LSMLN) (Liu et al. 2004). Activation of these receptors exerts diametrically opposing actions on glutamatergic transmission in these nuclei. In the CeA, CRF(r/h) depressed excitatory glutamatergic transmission through a CRF1R-mediated postsynaptic action, whereas Ucn I facilitated synaptic responses through presynaptic and postsynaptic CRF2R-mediated mechanisms. Conversely, in the lateral septum mediolateral nucleus (LSMLN), CRF induced a CRF1R-mediated facilitation of glutamatergic transmission via postsynaptic mechanisms, whereas Ucn I depressed EPSCs by postsynaptic and presynaptic CRF2R-mediated actions. Furthermore, antagonists of these receptors also affected glutamatergic neurotransmission, indicating a tonic endogenous modulation at these synapses (Liu et al. 2004). These data

show that CRF receptors in CeA and LSMLN synapses exert and maintain a significant synaptic tone and thereby regulate excitatory glutamatergic transmission. In fact, studies on CIE-induced changes in the modulation of rat glutamatergic synapses by CRF (Varodayan et al. 2017a) reveleaed that CRF also decreased rat CeA locally- or basolateral amygdala (BLA)-evoked glutamatergic responses. In contrast to the evoked data CRF increased mEPSC frequency similarly in naive and CIE neurons, suggesting increased vesicular glutamate release (Varodayan et al. 2017a; Herman et al., 2016b). Those studies also revealed that CRF-induced facilitation of glutamate release is mediated by CRF1 receptors, but the mechanisms are complex and may involve both CRF1 and CRF2 receptors with opposite receptor subtype effects on glutamate release (Varodayan et al. 2017a). These rat studies agree with mouse studies showing that, acute bath application of EtOH significantly increased sEPSC frequency in a concentration-dependent manner in CeA neurons, and this effect was blocked by pretreatment of co-applied CRFR1 and CRFR2 antagonists (Silberman et al., 2015).

3.1.3. CRF Actions in the Bed Nucleus of the Stria Terminalis.—The BNST, a brain region associated with anxiety, has enriched expression of CRF (Ju and Han 1989) and CRFRs (Van Pett et al. 2000). A component of the extended amygdala, the BNST is anatomically well-situated to integrate stress and reward-related processing in the CNS, regulating activation of the hypothalamic-pituitary-adrenal (HPA) axis and reward circuits. The oval nucleus is a rich source of CRF neurons and terminals which may originate from local CRF neurons or from CRF neurons projecting from the CeA (Morin et al., 1999; Sakanaka et al., 1986; see also Kash et al., 2015 for review). Much evidence supports the role of CRF signaling in the BNST in general anxiety (Gafford et al., 2012; Sink et al., 2013 see also Kash et al., 2015 for review), and anxiety-like behaviors induced by ethanol withdrawal (Huang et al., 2010).

Pharmacological studies suggest that CRF signaling in the BNST is involved in anxiety (Lee and Davis 1997) and stress-induced relapse to cocaine self-administration (Erb and Stewart 1999). Moreover, a stimulus that promotes anxiogenic responses, the withdrawal of rodents from chronic EtOH exposure, produces rises in extracellular levels of CRF in the BNST (Olive et al. 2002). However, in another study, following 2 weeks of binge-like alcohol intake, adolescent rats display decreases in CRF cell number in the CeA and no changes in BNST (Karanikas et al., 2013). Interactions between CRF and GABAergic transmission in BNST were reported to play a role in regulating stress and anxiety (Kash and Winder 2006). In this study the actions of CRF on GABAergic transmission in the ventrolateral region of the BNST were examined. This region projects to both the VTA (Georges and Aston-Jones 2002) and the PVN of the hypothalamus (Cullinan et al. 1993), thus providing a point of access to both reward and stress pathways. Using whole-cell recordings in a BNST slice preparation, Kash and Winder (2006) found that CRF enhances GABAergic transmission. Their pharmacological and genetic experiments suggest that CRF and urocortin CRF enhance postsynaptic responses to GABA through activation of the CRF1R. CRF1-R signaling in the BNST also enhances glutamatergic drive on neurons projecting to the VTA in a presynaptic fashion (Silberman et al., 2013). Thus, CRF can

enhance both inhibitory and excitatory transmission in the BNST, albeit through distinct signaling mechanisms.

Kash and coworker (Kash et al. 2008) also showed the action of dopamine on cellular and synaptic function in the BNST using an *ex vivo* slice preparation. These investigators demonstrated a rapid and robust dopamine-induced enhancement of excitatory transmission in the BNST. This enhancement is activity-dependent and requires the downstream action of CRF1R, suggesting that dopamine induces CRF release through a local network mechanism. Furthermore, it was found that both *in vivo* and *ex vivo* cocaine induced a dopamine receptor and CRF1R-dependent enhancement of a form of NMDA receptor-dependent short-term potentiation in the BNST. These data highlight a direct and rapid interaction between dopamine and CRF systems that regulates excitatory transmission and plasticity in a brain region key to reinforcement and reinstatement. Because a rise in extracellular dopamine levels in the BNST is a shared consequence of multiple classes of drugs of abuse, this suggests that the CRF1R-dependent enhancement of glutamatergic transmission in this region may be a common key action of substances of abuse (Kash et al. 2008). Subsequent studies from the Kash laboratory revealed a complex interaction between CRF and NPY in the BNST in the regulation of binge alcohol drinking in both mice and monkeys (see section below and Pleil et al., 2015a).

Francesconi et al., (Francesconi et al. 2009a; Francesconi et al. 2009b) investigated the effects of protracted withdrawal from alcohol in the juxtacapsular nucleus of the anterior division of the BNST (jcBNST). The jcBNST receives robust glutamatergic projections from the BLA, the postpiriform transition area, and the insular cortex as well as dopamine inputs from the midbrain. In turn, the jcBNST sends GABAergic projections to the medial division of the central CeA as well as other brain regions. These investigators described a form of long-term potentiation of the intrinsic excitability (LTP-IE) of neurons of the jcBNST in response to high-frequency stimulation (HFS) of the stria terminalis that was impaired during protracted withdrawal from alcohol (Francesconi et al. 2009b). Administration of the selective CRF1R antagonist (R121919), but not of the CRF2R antagonist (astressin 2B), normalized jcBNST LTP-IE in animals with a history of alcohol dependence (Francesconi et al. 2009b). In addition, repeated, but not acute, administration of CRF itself produced a decreased jcBNST LTP-IE. These investigators also showed that dopaminergic neurotransmission is required for the induction of LTP-IE of jcBNST neurons through dopamine D1 receptors (Francesconi et al. 2009b). Thus, activation of the central CRF stress system and altered dopaminergic neurotransmission during protracted withdrawal from alcohol and drugs of abuse may contribute to the disruption of LTP-IE in the jcBNST. Furthermore, the jcBNST also shows marked reductions in excitability after protracted withdrawal from CIE (Szücs et al., 2012). Overall, the impairment of this form of intrinsic neuronal plasticity in the jcBNST could result in inadequate neuronal integration and reduced inhibition of the CeA, contributing to the negative affective state that characterizes protracted abstinence in post-dependent individuals (Francesconi et al. 2009a; Francesconi et al. 2009b).

It is important to mention that NE is another key interface in the BNST-CRF with stress and chronic ethanol. Studies have examined the effects of NE on BNST CRF neuron activity

and determine if these effects may be modulated by CIE exposure or a single restraint stress (Snyder et al., 2019). Stress and CIE enhance BNST CRF neuron activity via similar β-AR dependent mechanisms. Surprisingly, stress and CIE do not appear to alter NE-induced inhibition of glutamatergic inputs onto BNST CRF neurons, an effect previously shown to be α-AR dependent 2 (Fetterly et al., 2019). Together, these results indicate that stress and chronic EtOH target the activity of β-ARs on BNST CRF neurons without altering α-AR modulation of these neurons, thereby altering the α/β-AR balance within this circuitry. Thus, maintaining α/β-AR balance in BNST CRF circuits may be an important target for novel treatments for stress-related disorders and stress-induced reinstatement to alcohol seeking behaviors (Snyder et al., 2019).

3.1.4. CRF Actions in the Basolateral Amygdala.—

Liu et al., (Liu et al. 2004) demonstrated that CRF and its related family of peptides act differentially at CRF1 vs. CRF2 synaptic receptors to facilitate or depress excitatory transmission in CeA and lateral septum mediolateral nucleus. Notably, the effects of CRF and its ligands occurred without any apparent direct action on membrane potential or membrane excitability, suggesting that the role of CRF at these limbic synapses is that of a ‘neuroregulator’. The investigators suggested pre- and post-synaptic loci for CRF1 and CRF2 receptors within the glutamatergic CeA and LSMN synapses. Although both synapses exhibit a comparable pre- and post-synaptic location of CRF1 and CRF2 receptors, their functions (facilitation vs. depression of glutamatergic transmission) are opposite within each synapse (Gallagher et al. 2008). Liu et al., (Liu et al. 2004) also demonstrated that endogenous CRF ligands induce a tonic effect on excitatory glutamatergic transmission at synapses within both of these nuclei since application of competitive, selective CRF1 or CRF2 receptor antagonists resulted in an enhancement or depression of glutamatergic EPCS. A similar tonic endogenous action of CRF ligands was not observed under control conditions in the medial prefrontal cortex (Orozco-Cabal et al. 2006). This latter result further emphasizes that CRF effects are different depending upon the CNS synapse being investigated. Most of these studies in the Gallagher group aimed to investigate the action of CRF on glutamatergic synapses in relation to cocaine administration. There is very poor data on EtOH-CRF-glutamate interaction.

Taken together these data suggest that a dysregulation of the extrahypothalamic CRF function is a major determinant of vulnerability to high alcohol intake and maintenance of alcohol and drug dependence and other aspects of AUD.

3.2. Neuropeptide Y

The inhibitory NPY peptide is produced in abundance in the hypothalamus, and phylogenetically conserved across species (Allen et al. 1986). NPY is involved in regulation of food and water intake. It has recently been ascribed its prominent role in the aversive aspects of alcohol withdrawal and relapse via their actions in the CeA. Endogenous NPY reduces anxiety via actions in the amygdala (Heilig et al. 1993; Sajdyk et al. 2002) and suppresses alcohol drinking in rats (Gilpin et al. 2003) via its actions in CeA (Gilpin et al. 2008a; Gilpin et al. 2008b; Thorsell 2008). More specifically, NPY microinjection into the

CeA exhibits an enhanced ability to suppress alcohol drinking in certain subpopulations of drinkers, including rats that are made dependent on alcohol via vapor inhalation.

NPY is generally co-localized with GABA in inhibitory interneurons. NPY mediates its actions by interacting with a family of G-protein coupled receptors (GPCRs), at least 5 of which have been cloned and designated Y1, Y2, Y4, Y5, and Y6. These receptors are widely distributed throughout the brain. NPY also has been shown to be a regulator of neuronal excitability in hippocampus, where its cellular actions have been most extensively studied (Colmers et al. 1991). In the amygdala, NPY has anxiolytic effects that are mediated via activation of Y1 receptors (Heilig et al. 1993). NPY neurons in the amygdala project to the BNST (Allen et al. 1984), which also contains Y1 receptors and Y1 and Y2 receptor mRNA. Further, the CeA receives NPYergic input from the nucleus of the solitary tract, arcuate nucleus, and the lateral septum (see (Kask et al. 2002) for a review). Y1, Y2 and Y5 receptors, and receptor mRNA are found in the amygdala, and each of these receptor subtypes has been implicated in anxiety (Kask et al. 2002). Y2 receptors are thought to act presynaptically as autoreceptors providing negative feedback to NPYergic nerve terminals, whereas Y1 receptors appear to act postsynaptically (Kask et al. 2002; Wolak et al. 2003).

Many *in vivo* studies point to the involvement of NPY in mediating some of the behavioral effects of EtOH (Caberlotto et al. 2001; Cippitelli et al. 2010; Rimondini et al. 2005). NPY KO mice show increased EtOH preference but blunted behavioral responses to ethanol, while NPY overexpressors show a lower preference and increased sensitivity to ethanol (Thiele et al. 1998). Likewise, increased NPY expression in the CeA was noted in two independent strains of alcohol-preferring rats (Hwang et al. 1999). There were increased levels of NPY in the paraventricular nucleus of the hypothalamus (PVN) and arcuate nucleus of EtOH-preferring rats and decreased NPY levels in the CeA of ethanol-preferring rats, suggesting an inverse relationship between NPY levels in the CeA and EtOH consumption. Additionally, alcohol-preferring rats show significant decreases in both cAMP-responsive element-binding protein (CREB) and NPY levels in the CeA and medial amygdala, but not the basolateral amygdala (Pandey et al. 2005). Further, virally-mediated alterations in NPY levels in the CeA differentially affect EtOH consumption in rats with low and high basal levels of anxiety (Primeaux et al. 2006). Also, recent genetic and pharmacological evidence indicates that C57BL/6J mice have low NPY levels in CeA compared to DBA/2 mice, suggesting that NPY contributes to the high EtOH consumption characteristic of C57BL/6J mice (Hayes et al. 2005).

Electrophysiological findings suggest that NPY and EtOH have a similar profile of actions (Ehlers et al. 1998a; Ehlers et al. 1998b; Ehlers et al. 1999). Increased sensitivity to NPY and CRF was observed in cortex and amygdala after chronic EtOH exposure, as measured by EEG activity and event-related potentials (Slawecki et al. 1999). Modulation of amygdala EEGs by NPY differs in naïve P and NP rats, suggesting that NPY has different neuromodulatory effects in these two strains (Ehlers et al. 1998a). Furthermore, NPY antagonizes the effects of CRF in the amygdala (Ehlers et al. 1998a).

At the cellular level NPY interactions with EtOH have been characterized in the CeA and other brain regions (for review see (Gilpin et al., 2015; Robinson & Thiele, 2017). Gilpin

and colleagues (Gilpin et al., 2011) found that NPY in rat CeA prevents acute alcohol-induced increases in evoked and spontaneous GABA release. Pharmacological manipulation with antagonists confirm the presynaptic site of action and suggest that NPY blocks alcohol effects via presynaptic Y2Rs. NPY also normalizes alcohol dependence-induced increases in GABA release in CeA, suggesting that chronic exposure causes neuroadaptations in NPY systems that affect inhibitory transmission. Notably, in mice, central infusion of NPY, a NPY Y1 receptor (Y1R) agonist, and a Y2R antagonist significantly blunted binge-like ethanol drinking in C57BL/6J mice (Sparrow et al., 2012). Binge-like ethanol drinking reduced NPY and Y1R immunoreactivity in the CeA, and 24 h of ethanol withdrawal increased Y1R and Y2R immunoreactivity. Binge-like ethanol drinking also increased the ability of NPY to inhibit GABAergic transmission. Thus, binge-like ethanol drinking in C57BL/6J mice promoted alterations of NPY signaling in the CeA (Sparrow et al., 2012), and administration of exogenous NPY compounds protected against binge-like drinking. Overall, these results in the CeA of rats and mice align with findings on NPY modulation of GABA transmission in BNST (Kash and Winder 2006) and suggest that Y2Rs function as autoreceptors regulating NPY release. NPY and CRF have opposing effects on stress and anxiety as well as on synaptic activity in BNST (Heilig et al. 1994; Kash and Winder 2006). Kash and Winder (2006) found that NPY and CRF inhibit and enhance GABAergic transmission, respectively: NPY depresses GABAergic transmission through activation of the Y2 receptors, whereas CRF and urocortin enhance GABAergic transmission through activation of CRF1 receptors. Further, NPY appears to reduce GABA release, whereas CRF enhances postsynaptic responses to GABA, suggesting potential anatomical and cellular substrates for the robust behavioral interactions between NPY and CRF in the extended amygdala. A recent study employed physiological, pharmacological and chemogenetic approaches to identify a precise neural mechanism in the BNST underlying the interactions between NPY and CRF in the regulation of binge alcohol drinking in both mice and monkeys (Pleil et al., 2015a). The results showed that Y1R activation in the BNST suppressed binge alcohol drinking by enhancing inhibitory synaptic transmission specifically in CRF neurons via a previously unknown Gi-mediated, PKA-dependent postsynaptic mechanism. In addition, chronic alcohol drinking altered Y1R function in the BNST of both mice and monkeys, highlighting the enduring, conserved nature of this effect across mammalian species (Pleil et al., 2015a).

Chronic restraint stress also alters the NPY system (Pleil et al., 2012). Specifically, increases NPY and Y2R expression in the BNST and reduces the Y2R-mediated effect of NPY on inhibitory synaptic transmission in a stress-susceptible mouse strain (DBA/2J), but not a stress-resilient strain (C57BL/6J) (Pleil et al., 2012). Notably, deletion of neuropeptide Y2 receptors from GABAergic neurons in the extended amygdala differently affected affective and alcohol drinking behaviors in male and female mice (McCall et al., 2013). Specifically, females displayed greater basal anxiety, higher levels of ethanol consumption, and faster fear conditioning than males, and that knockout mice exhibited enhanced depressive-like behavior in the forced swim test. Together, these finding support higher expression of negative affective and alcohol drinking behaviors in females than males, and they highlight the importance of Y2R function in GABAergic systems in the expression of depressive-like behavior (McCall et al., 2013).

3.3. Orphanin FQ/nociceptin (OFQ/N)

Nociceptin (known also as orphanin FQ) is the most recently discovered member of the endogenous opioid peptide family, albeit nearly 15 years ago. Nociceptin mediates or influences many behavioral, psychological and neurobiological processes, including memory, anxiety, stress and reward (Economidou et al. 2008; Martin-Fardon et al. 2010; Murphy 2010). The heptadecapeptide nociceptin is the endogenous ligand of the nociceptin opioid receptor (NOR), previously referred to as opiate receptor-like1 (ORL1). NOR is a GPCR that belongs to the opioid receptor family (Mogil et al. 1996; Mogil and Pasternak 2001). In rodents, moderate to high levels of NOR mRNA are detected in cerebral cortex, nucleus accumbens, amygdala, dorsal raphe nucleus and hippocampus (Harrison and Grandy 2000). Nociceptin has a high structural homology with opioid peptides, especially dynorphin A (Meunier et al. 1995; Reinscheid et al. 1995), but nociceptin does not bind to MOR, DOR or KOR (μ, δ and κ-opioid receptors) and opioid peptides do not bind NOR (Lachowicz et al. 1995; Reinscheid et al. 1995). Nociceptin inhibits forskolin-stimulated cAMP formation (see (Harrison and Grandy 2000; Hawes et al. 2000), and protein kinase C (PKC), MAP kinases and phospholipase A2 have been linked to NOR (Fukuda et al. 1998; Hawes et al. 2000; Lou et al. 1998).

At the cellular level, nociceptin acts at NOR to augment K+ conductances in amygdalar (Meis and Pape 1998, 2001), hippocampal (Amano et al. 2000; Ikeda et al. 1997; Madamba et al. 1999; Tallent et al. 2001; Yu and Xie 1998) and thalamic neurons (Meis 2003; Meis et al. 2002), thus depressing cell excitability. Nociceptin has also been shown to decrease Ca^{2+} currents (Abdulla and Smith 1997; Calo et al. 2000; Connor et al. 1999; Henderson and McKnight 1997; Larsson et al. 2000) and to reduce the amplitude of both non-NMDA receptor-mediated excitatory postsynaptic currents (EPSCs) and IPSCs in rat lateral amygdala (Meis et al. 2002).

Roberto and Siggins (2006) found that nociceptin did not significantly alter resting membrane potential, input resistance or spike amplitude, in accord with results reported by others in CeA (Meis and Pape 1998) and for other brain regions (Ikeda et al. 1997; Madamba et al. 1999; Tallent et al. 2001). However, nociceptin dose-dependently reduced $GABA_A$-IPSCs. This inhibition of GABAergic transmission was reversible on washout (Roberto and Siggins 2006). Nociceptin also concomitantly increased the PPF of IPSCs, and decreased the frequency of mIPSCs, suggesting decreased GABA release. Thus, nociceptin decreases GABAergic transmission by reducing GABA release at CeA synapses (Roberto and Siggins 2006). Interestingly, nociceptin applied before EtOH completely prevented the ethanol-induced enhancement of GABAergic transmission in CeA opposing the enhancing action of ethanol on GABA release (Roberto and Siggins 2006). These investigators also found that the nociceptin-induced decrease of GABAergic transmission was larger in EtOH-dependent rats and might reflect neuroadaptations associated with ethanol-dependence. Notably, nociceptin completely blocked the CRF-induced increase of GABA release (Cruz et al., 2012), suggesting that nociceptin antagonized the effect of CRF. Moreover, the NOP receptor antagonist [Nphe1]nociceptin(1–13)NH2 blocked the nociceptin-induced diminution of GABA but not the CRF-induced augmentation of GABA release, indicating that nociceptin modulates both ethanol and CRF effects through the

NOP receptors. Nociceptin also blocked CRF-induced increases in GABAergic responses in CeA from ethanol-dependent rats (Cruz et al., 2012). Using a multidisciplinary approach, Ciccocioppo and collaborators (2014) found a selective upregulation of the nociceptin and downregulation of the CRF1 receptor transcripts in the CeA and BLA after stress restraint (Ciccocioppo et al., 2014a). Notably, intra-CeA injections of nociceptin reduced anxiety-like behavior in restrained rats in the elevated plus maze. Finally, in restraint stressed rats, baseline CeA GABAergic responses were elevated and nociceptin exerted a larger inhibition of GABA responses compared with non-restrained rats (Ciccocioppo et al., 2014a).

Nociceptin interaction on glutamatergic transmission and ethanol effects were also investigated (Kallupi et al., 2014a). Acute and chronic ethanol exposures significantly decrease glutamate transmission by both pre- and postsynaptic actions (Roberto et al., 2004b). Nociceptin diminished basal-evoked compound glutamatergic and spontaneous glutamate transmission by mainly decreasing glutamate release in the CeA of naive rats (Kallupi et al., 2014a). Nociceptin blocked the inhibition induced by acute ethanol and ethanol blocked the nociceptin-induced inhibition of glutamatergic responses in CeA neurons of naive rats. Like the GABAergic synapses, nociceptin antagonism revealed tonic inhibitory activity of NOP on CeA glutamatergic transmission only in alcohol-dependent rats. The antagonist also blocked nociceptin-induced decreases in glutamatergic responses but did not affect ethanol-induced decreases in evoked glutamate responses. Taken together, these studies implicate a potential role for the nociceptin system in regulating CeA glutamatergic and GABAergic synapses in both acute stress and alcohol dependence providing translational support for nociceptin as a “druggable” candidate system for medication development for the treatment of AUD. In support of this concept, it is important to continue to identify novel soluble non-peptidergic molecules, such as nociceptin agonists (Ciccocioppo et al., 2014b; Kallupi et al., 2014b) that decrease excessive drinking and act at the cellular level in brain regions such as the amygdala that are associated with ethanol dependence.

The functional interactions of neuropeptides (CRF, NPY, nociceptin) with inhibitory and excitatory systems in the brain may play major roles in the acute reinforcement effects of EtOH. Understanding the underlying mechanisms of these interactions may offer a possible avenue for restoring “normal” function following chronic drug exposure. The neuroadaptations induced by chronic EtOH on GABAergic and glutamatergic systems may represent homeostatic or compensatory mechanisms in response to the acute ethanol actions on these systems.

4. New Approaches to Determine *In Vivo* Roles of Ethanol Effects on Synaptic Transmission

From the foregoing discussion it should be clear that we now know a great deal about how acute and chronic EtOH exposure alters synaptic function. However, less is known about the roles played by these synaptic effects in the *in vivo* physiological and behavioral effects of the drug. New genetic, optical, pharmacological and physiological techniques allow for faster advancement in this research area.

Assessing alcohol effects on *in vivo* neural function has moved beyond traditional single- and multi-unit electrophysiological recordings. New systems such as the "Neuropixels" recording system allow investigators to measure the firing of 1000s of neurons in a single recording with excellent signal/noise ratios and discrimination of single neurons (Steinmetz et al., 2021). When combined with the proper analysis tools this approach has the potential to enhance our understanding of the alcohol impact of neurophysiology. Combining such recordings with genetic and pharmacological manipulations of synaptic proteins and synaptic function will allow investigators to determine how particular synapses contribute to EtOH-induced changes in neuron/circuit function and behavior.

An explosion of techniques for measurement of neuronal activity and neurotransmitter levels has taken place over the last decade. With the development of genetically-encoded fluorescent sensors for intracellular calcium, other second messengers and extracellular neurotransmitters, real-time *in vivo* measurements can now be made with imaging and optical fiber-based photometry (Jing et al., 2019; Liang et al., 2015; Labouesse and Patriarchi, 2021; Meng et al., 2018; Siciliano and Tye, 2019). Combining these approaches with behavioral analysis in awake animals is providing unprecedented analyses of how a variety of neuronal/synaptic functions are related to behavior. These techniques are already being applied to examine effects of EtOH on the function of specific afferent projections in the brain (Siciliano and Tye, 2019). Studies in the coming years are sure to reveal much more detailed evidence of EtOH effects on neurotransmitter levels that can be related to drinking or other behaviors.

As mentioned earlier in this chapter, optogenetic activation of specific afferent projections has now gained widespread usage in neuroscience and alcohol research. By expressing a light-activatable opsin that induces depolarization or hyperpolarization in a specific cell type, investigators can now interrogate how EtOH and other drugs alter synaptic transmission at a given synapse. Studies investigating EtOH effects on optogenetically-activated synaptic transmission in brain slices have already been discussed. This technique is also being used to examine how altered function of specific neurons affects EtOH-related behaviors (Juarez et al., 2019). Combining optogenetic approaches with other techniques outlined in this chapter should allow investigators to determine how EtOH exposure alters afferent and synaptic function *in vivo*.

The development of techniques for activation of non-native LGICs and GPCRs with ligands that are normally biologically inactive has revolutionized techniques for altering neuronal and synaptic function (Campbell et al., 2018; Vardy et al., 2015). These approaches allow investigators to examine how activation, inhibition and modulation of different neural cells contribute to circuit function and behavior. With regard to synaptic function, the Designer Receptor Exclusively Activated by Designer Drug (DREADD) technique is especially attractive. This technique uses genetically engineered GPCRs that can be inserted into neurons of interest and affect neuronal/synaptic function in numerous ways. For example, the DREADD variants that couple to Gi/o G-proteins inhibit neurotransmitter release (e.g. hM4Di) (Armbruster et al., 2007), as expected from other GPCRs with similar coupling. It is now possible to alter transmitter release at an identified synaptic terminal *in vivo*, especially when DREADD expression is combined with local injection of the designer drug

receptor agonist (Gremel et al., 2016; Mahler et al., 2014). This will allow investigators to interrogate how a particular presynaptic manipulation alters EtOH-related behaviors, and mimic effects of EtOH at identified synapses. Additional uses of this technique to alter pre- and postsynaptic function and interactions with EtOH will undoubtedly be used in the coming years.

5. Conclusions

In this review we have focused on acute and chronic EtOH actions on synaptic transmission. It is not possible to cover all aspects of this topic, and thus we have focused on describing the best established EtOH actions. As the review attests, EtOH affects numerous aspects of synaptic transmission both directly and indirectly, to alter brain function and behavior. Acute exposure to EtOH generally increases the function of cys-loop ligand-gated ion channels, with prominent effects of $GABA_A$ and glycine receptors. These actions increase synaptic and extrasynaptic inhibition and are thought to contribute to sedation and other aspects of intoxication. Ionotropic glutamate and P2X receptors are generally inhibited by acute EtOH exposure, with some noted exceptions. The inhibitory effect on ionotropic glutamate receptors is most prominent at NMDARs and on NMDAR-mediated synaptic responses, and this inhibitory action is thought to contribute to cognitive impairment produced by EtOH. At present the postsynaptic EtOH effects on neurotransmitter receptors appear to occur within the receptor molecules themselves, although more work is needed to elucidate the roles of posttranslation modification. On the presynaptic side, acute EtOH generally potentiates GABA release, contributing to the enhanced neuronal inhibition produced by the drug. The molecular mechanisms involved in EtOH potentiation of GABA release remain to be fully explored. Ethanol also alters other aspects of synaptic transmission involving amino acid transmitters and monoamines. The net result of the EtOH effects of transmission seems to be to dampen synaptic excitation in many brain regions and reduce most forms of synaptic plasticity (with noted exceptions).

Chronic exposure to EtOH, whether by forced administration or ingestion, generally enhances the function of NMDARs, most often those containing the NR2B subunit. Increases in glutamate release and responses to some other glutamate receptors are also observed following chronic exposure. The net effect of these increases in glutamatergic transmission appears to be a hyperexcitable CNS state during withdrawal that contributes to withdrawal symptoms and relapse. Excitotoxicity might be another result of this hyper-glutamatergic state. In general, acute EtOH effects on glutamate receptor function and glutamatergic transmission are intact even after subchronic or chronic ethanol exposure, suggesting that behavioral tolerance is not a simple function of loss of pharmacological effects at these synapses. At GABAergic synapses, chronic EtOH generally alters either the efficacy of inhibitory synaptic transmission or the types of receptors involved in transmission. Extrasynaptic $GABA_A$ receptor-mediated synaptic responses are also altered, leading to changes in tonic current in the postsynaptic neuron. The pattern of chronic EtOH effects on GABAergic transmission varies considerably across brain regions, making this subject a rich and important area for future investigation. The resultant alterations in patterns of GABAergic transmission in key brain regions may contribute to aspects of AUD including EtOH tolerance, dependence and drug intake. More work is needed to determine

the exact pattern of changes in GABAergic inhibition across brain regions, and how these changes contribute to aspects of alcohol use disorders including tolerance, dependence, and escalating intake.

The modulatory effects of neuropeptides have become subjects of intense investigation in the alcohol research field. Neuropeptides implicated in stress responses, such as CRF, appear to contribute to stress-EtOH interactions as well as drinking and relapse. Acute EtOH exposure alters the release of some neuropeptides, while others alter synaptic transmission in ways that interfere with the actions of ethanol. Chronic EtOH exposure also appears to alter neuropeptide modulatory actions. In addition to providing tools for investigation of mechanisms involved in ethanol actions, the neuropeptides may also provide new avenues for pharmacotherapies that could be used in the treatment of alcohol use disorders. Despite the great progress done and the promising results in understanding the mechanisms of action of numerous neuropeptides in well-established preclinical models of AUD, translating this knowledge to the clinical side has been ineffective. Similar issues hamper preclinical models of antidepressant activity and psychiatric domains in which neuropeptide-targeting compounds have yet to show clinical efficacy. Researchers have just begun to explore the alcohol-related actions of a few of the many neuropeptides found in brain. Thus, more work remains to fully define how peptides participate in alcohol the neural actions of alcohol.

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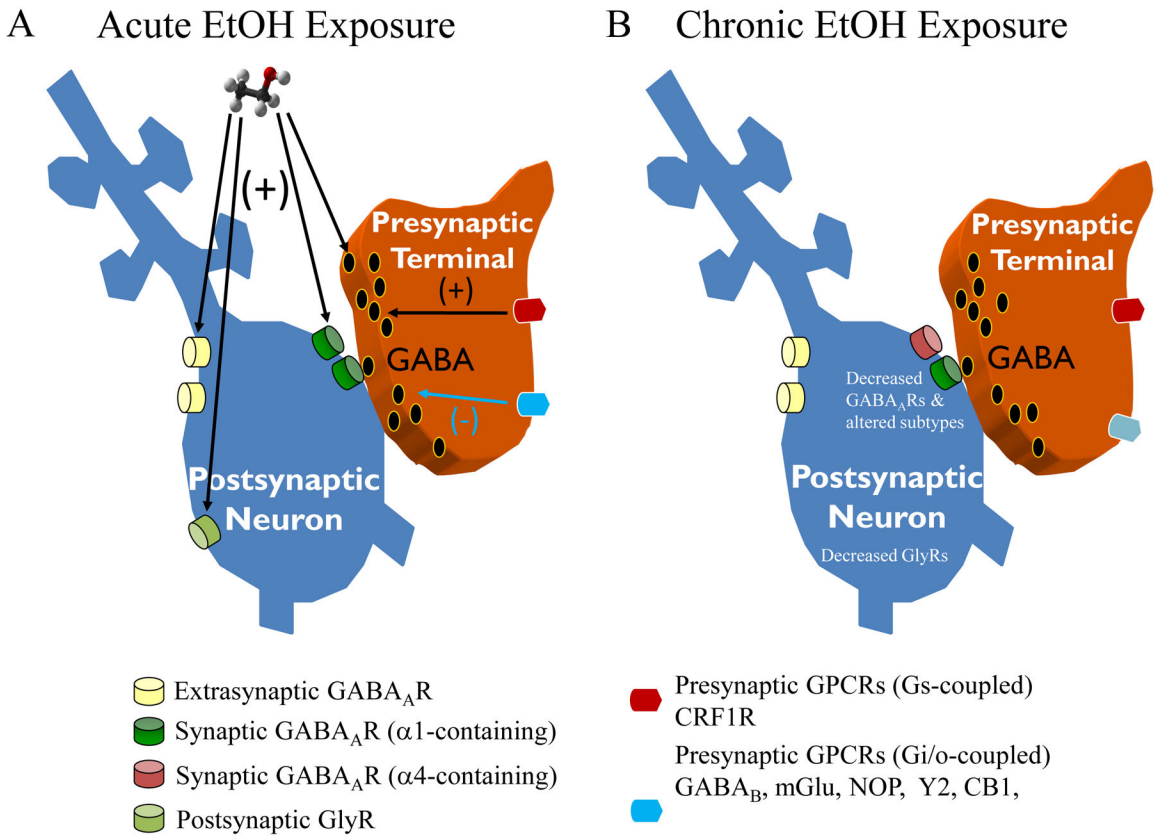

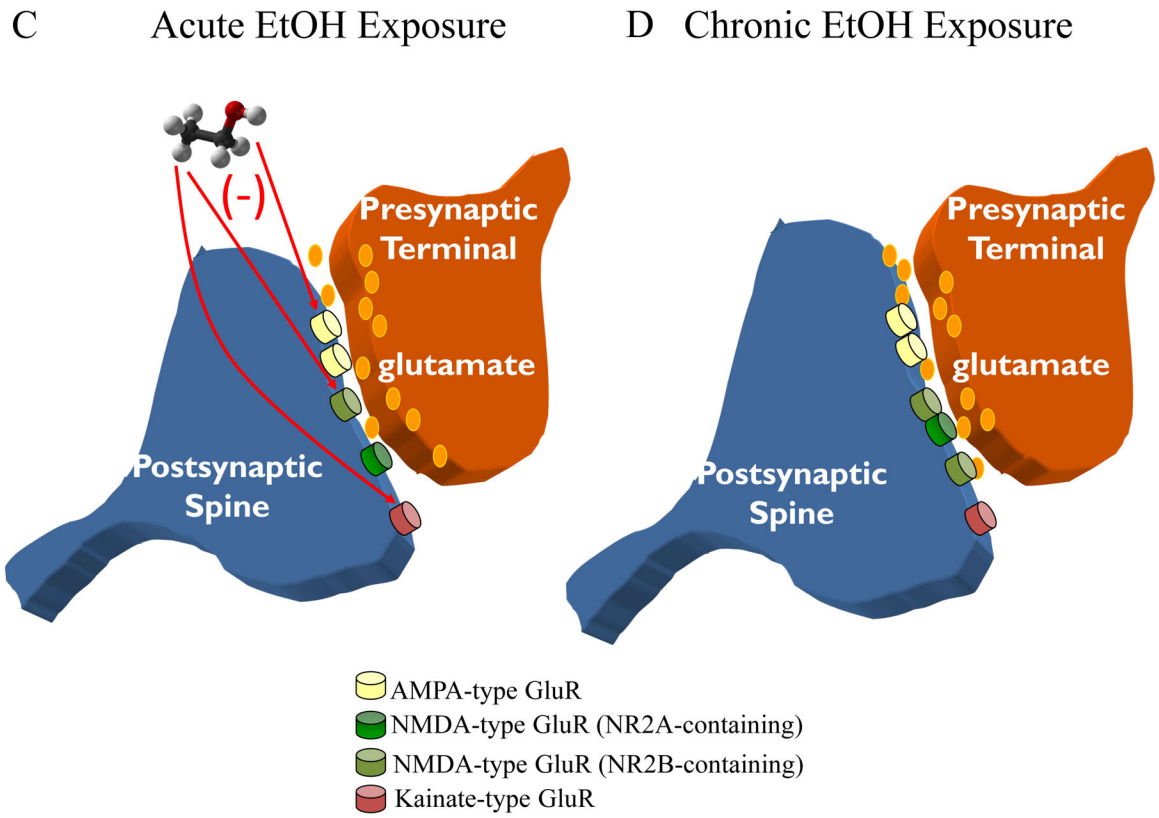

Figure 1.

Acute and chronic EtOH effects on GABAergic and glutamatergic synaptic transmission. A) Schematic diagram of an inhibitory CNS synapse, including presynaptic GPCRs that modulate neurotransmitter release, and postsynaptic ionotropic receptors (located both at synapses and extrasynaptically) that mediate fast synaptic transmission. The predominant presynaptic effect of acute EtOH is potentiation of GABA release (most likely by increasing the probability of vesicle fusion). This presynaptic potentiation may involve neuromodulators such as CRF, and activation of presynaptic GPCRs and downstream signaling pathways. Postsynaptically, EtOH potentiates ionotropic $GABA_A$ and glycine receptor function. Increases in synaptic $GABA_AR$ function prolong synaptic responses, while potentiation of extrasynaptic receptors increases tonic current that affects neuronal excitability. B) Changes in GABAergic synapses following chronic EtOH exposure. Presynaptically, the release of GABA is decreased. Alterations in levels of neuromodulators that act on GPCRs, as well as altered function of presynaptic GPCRs may contribute to these changes. Postsynaptically, the subunit composition of $GABA_ARs$ is altered, often including increased synaptic α4-containing receptors, and fewer α1-containing synaptic receptors. Synaptic α4–containing receptors may be less sensitive to acute EtOH, promoting tolerance to synaptic effect of the drug. C) Schematic diagram of a glutamatergic synapse on a dendritic spine, including postsynaptic ionotropic receptors that mediate fast synaptic transmission. The predominant effect of acute EtOH is to inhibit ionotropic glutamate receptor function, and all subclasses of these receptors are sensitive to EtOH inhibition. The most potent effects have been observed at kainate and NMDA receptor subtypes. D) Changes in glutamatergic synapses following chronic EtOH exposure. Presynaptically, the

release of glutamate is enhanced. Postsynaptically, NMDAR function is increased, most likely due to increased receptor density at the synapse. There is also evidence for increased numbers of NR2B-containing NMDARs, as well as evidence of increased dendritic spine volume.

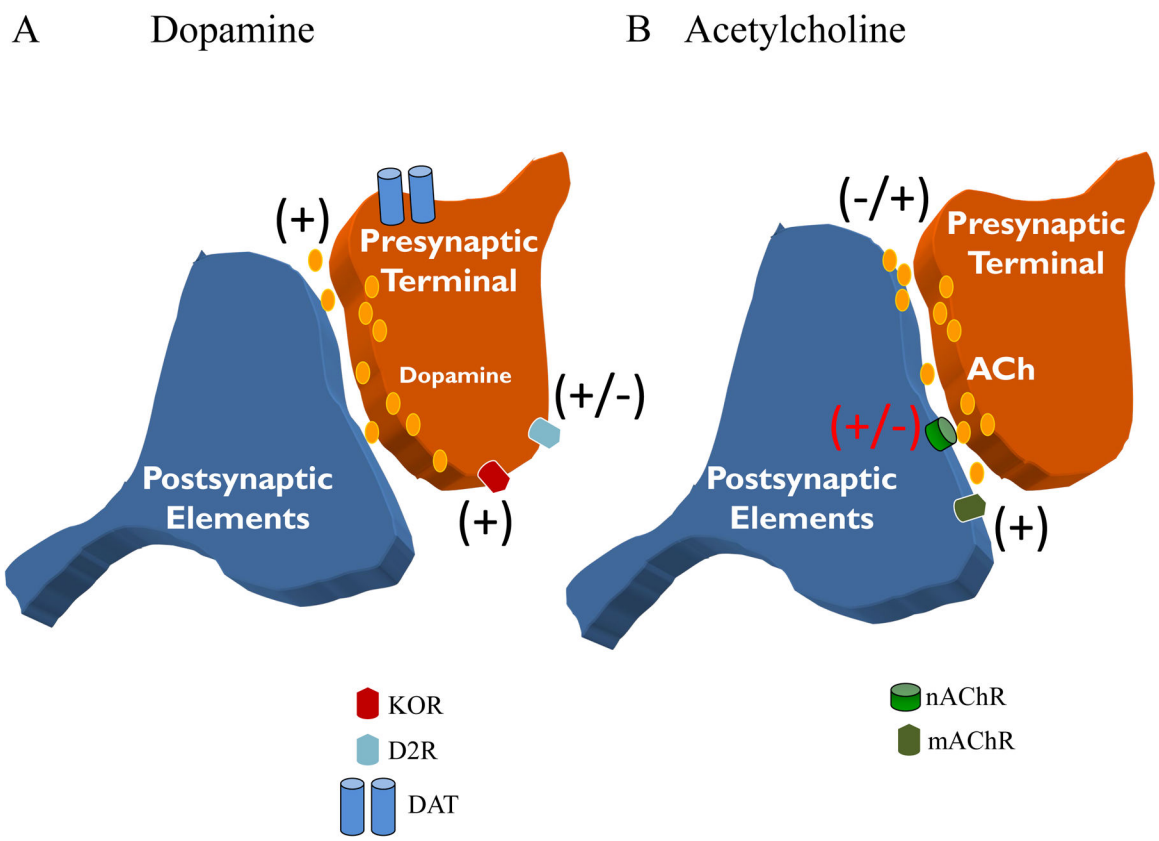

Figure 2.

Chronic EtOH effects on dopaminergic and cholinergic transmission. A) Chronic EtOH exposure generally enhances DA release, although decreases have also been observed. Decreased DAT expression may contribute to hyperdopaminergic conditions following chronic exposure. Enhanced potency of kappa opiate receptor agonist inhibition of DA release indicates either greater numbers or increased sensitivity of these presynaptic receptors. The number of D2 receptors is decreased in humans with AUD, while chronic EtOH-induced changes in D2 autoreceptor function vary in different species and sexes. B) Chronic EtOH exposure has mixed effects on ACh release, increasing release at some synapses, while decreasing it at others. Likewise, chronic EtOH effects on nAChR number and function are a mix of enhancement and reduction depending on receptor subtypes and cellular locus of the receptor. In contrast, chronic EtOH exposure generally increases mAChR function in the different preparations in which this has been examined.