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Persistent Adaptation by Chronic Alcohol is Facilitated by Neuroimmune Activation Linked to Stress and CRF

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Abstract

This review updates the conceptual basis for the association of alcohol abuse with an insidious adaptation that facilitates negative affect during withdrawal from chronic intermittent alcohol (CIA) exposure – a change that later supports sensitization of stress-induced anxiety following alcohol abstinence. The finding that a CRF1-receptor antagonist (CRF1RA) minimized CIA withdrawal-induced negative affect supported an association of alcohol withdrawal with a stress mechanism. The finding that repeated stresses or multiple CRF injections into selected brain sites prior to a single 5-day chronic alcohol (CA) exposure induced anxiety during withdrawal provided critical support for a linkage of CIA withdrawal with stress. The determination that CRF1RA injection into positive CRF-sensitive brain sites prevented CIA withdrawal-induced anxiety provided support that neural path integration maintains the persistent CIA adaptation. Based upon reports that stress increases neuroimmune function, an effort was undertaken to test whether cytokines would support the adaptation induced by stress/CA exposure. Twenty-four hours after withdrawal from CIA, cytokine mRNAs were found to be increased in cortex as well as other sites in brain. Further, repeated cytokine injections into previously identified brain sites substituted for stress and CRF induction of anxiety during CA withdrawal. Discovery that a CRF1RA prevented the brain cytokine mRNA increase induced by CA withdrawal provided critical evidence for CRF involvement in this neuroimmune induction after CA withdrawal. However, the CRF1RA did not

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block the stress increase in cytokine mRNA increases in controls. The latter data supported the hypothesis that distinct mechanisms linked to stress and CA withdrawal can support common neuroimmune functions within a brain site. As evidence evolves concerning neural involvement in brain neuroimmune function, a better understanding of the progressive adaptation associated with CIA exposure will advance new knowledge that could possibly lead to strategies to combat alcohol abuse.

Keywords

adaptation; kindling; chronic intermittent alcohol withdrawal; anxiety; stress; CRF; neuroimmune function; circuitry; signaling

Introduction

Ballenger and Post (1978) hypothesized that chronic intermittent alcohol (CIA) exposure and accompanying withdrawals over an extended period were responsible for seizures during withdrawal in alcoholics – a process proposed to depend upon “kindling”. These investigators further speculated that if the “kindling hypothesis” accounted for the incidence of seizures, this persistent adaptation induced by the CIA exposure also contributed to the maintenance of alcohol abuse (alcoholism) (Ballenger & Post, 1978). Somewhat later, McCown and Breese (1990) demonstrated that exposure of rats to a CIA protocol for 10 weeks facilitated kindling of seizures from the inferior colliculus – a change not duplicated in rats continuously exposed to this 10-week period of alcohol diet. This latter observation indicated that CIA cycling was critical for inducing the adaptation that supported the kindling of seizures (McCown & Breese, 1990). Subsequently, repeated CIA exposures were reduced to three to test the possibility that early stages of adaptation induced by repeated CIA exposures and withdrawals would worsen negative withdrawal symptoms by a “kindling” process (Overstreet, Knapp, & Breese, 2002). Whereas withdrawal from three cycles of CIA exposure facilitated anxiety-like behavior, continuous exposure to alcohol did not (Overstreet et al., 2002). This facilitation of anxiety during a shorter repeated exposure to CIA (Overstreet et al., 2002; Overstreet, Knapp, & Breese, 2004a, 2004b) but not following withdrawal from continuous alcohol provided a further conceptual foundation for an accumulated adaptation being induced by repeated CIA exposures that could contribute to the etiology of alcoholism (Ballenger & Post, 1978; Breese, Overstreet, & Knapp, 2005). Based upon this framework, the present review describes our current understanding of how such CIA exposures interact with CRF and neuroimmune neural mediation of stress to sustain this persistent adaptation that follows chronic repeated alcohol exposures.

CRF-receptor (CRFR) antagonism of CIA withdrawal-induced anxiety implicates a contribution of “stress”

The neural basis of the hypothesized kindling-like neuroadaptation that sensitized seizures (Ballenger & Post, 1978; McCown & Breese, 1990) and withdrawal-induced anxiety (Koob, 2003; Overstreet et al., 2002) first began to be elucidated more than a decade after the kindling hypothesis was advanced (Ballenger & Post, 1978). The finding that a corticotropin

releasing-factor 1 receptor antagonist (CRFR1A) prevented the anxiety that occurs following withdrawal from continuous alcohol exposure (Knapp, Overstreet, Moy, & Breese, 2004; Fig. 1A) provided part of the impetus to test drugs against sensitization arising during the repeated withdrawals (Overstreet et al., 2004a). In this latter testing, the CRF1RA given during each withdrawal in the repeated-withdrawal paradigm blocked the appearance of anxiety-like behavior during an untreated final (third) withdrawal (Fig. 1B) – a result that was particularly important in suggesting a basis for the neuroadaptation that accompanied alcohol-withdrawal anxiety. This finding was also consistent with the earlier finding by Baldwin et al. (1991) that CRF release during a final withdrawal contributes to the persistent adaptation that contributes to the anxiety linked to withdrawal from CA exposure (Baldwin, Rassnick, Rivier, Koob, & Britton, 1991; Rassnick, Heinrichs, Britton, & Koob, 1993). Additional studies confirmed and expanded on this pharmacological approach to manipulate the CIA effect on anxiety-like behavior (Breese, Knapp, & Overstreet, 2004; Knapp, Overstreet, Angel, Navarro, & Breese, 2007; Knapp et al., 2004; Overstreet et al., 2004a; Overstreet, Knapp, & Breese, 2005;Overstreet, Knapp, Moy, & Breese, 2003). These efforts provide further evidence that repeated CRF release during multiple withdrawals supports the developing adaptation associated with the withdrawal-induced anxiety following CIA exposure. A recent report that CIA exposure up-regulates CRF/CRF1R signaling supports this conclusion (Eisenhardt, Hansson, Spanagel, & Bilbao, 2014).

Subsequently, antagonists and agonists associated with a variety of neural systems were given during the initial withdrawals of the CIA protocol and found to prevent the sensitization of anxiety associated with the final withdrawal from CIA (Knapp, Overstreet, & Breese, 2005, 2007; Knapp et al., 2004; Overstreet et al., 2003, 2004a, 2005). In these extended evaluations, antagonists of receptors for 5-HT_{2C} and benzodiazepine receptors as well as a $GABA_B$ receptor agonist attenuated the CIA and chronic alcohol-withdrawal anxiety (Breese, Overstreet, Knapp, & Navarro, 2005; Knapp et al., 2004). Equally important was the finding that antagonists of NMDA, 5-HT_3, and CRF2 receptors did not affect the anxiety following withdrawal from chronic alcohol (CA) (Knapp et al., 2004; Overstreet et al., 2004a). Currently, it is not yet resolved how these other neural systems are implicated in the withdrawal anxiety that involves CRF. Regardless, these outcomes support the view that CRF as well as the other neural systems can contribute to the persistent adaptation that follows withdrawal from CA and CIA exposures.

Finding that a CRF1RA mitigated the sensitization of withdrawal anxiety induced by the CIA exposure (Knapp, Overstreet, Angel, et al., 2007; Knapp et al., 2004; Overstreet et al., 2003, 2004a, 2004b, 2005) and that a BZD inverse agonist (DMCM) substituted for the earlier withdrawals of CIA exposure (Knapp, Overstreet, Angel, et al., 2007) raised the possibility that the initial withdrawals from the CIA protocol induced a “stress-like” response that initiated CRF release (Bale & Vale, 2004; Dunn & Berridge, 1990a, 1990b; Imaki, Shibasaki, Hotta, & Demura, 1993; Vale, Spiess, Rivier, & Rivier, 1981). To determine whether activation of a neural “stress” pathway was a critical component in the CIA-induced anxiety during withdrawal (Overstreet et al., 2002), single bi-weekly stresses were substituted for the two initial weekly repeated withdrawals of CIA exposure. This strategy tested whether sensitization of negative symptoms (anxiety) would occur upon withdrawal from the single 5 days of alcohol exposure that alone induced no behavioral

change during withdrawal (Breese et al., 2004, 2008). In accord with the hypothesized stress induction of adaptation occurring during repeated alcohol withdrawals, the repeated weekly stresses prior to the single 5-day cycle of alcohol exposure sensitized anxiety during withdrawal (Breese et al., 2004; Fig. 2A). Further, this repeated stress sensitization of alcohol withdrawal anxiety was prevented by a CRF1RA (Huang et al., 2010; Knapp et al., 2011). Moreover, in related studies where repeated withdrawal preceded a stress challenge 3 days following the withdrawal, the CRF1RA applied either during the first two of the three repeated withdrawals or prior to the stress challenge were effective in limiting the magnitude of anxiety-like behavior (Breese, Overstreet, Knapp, et al., 2005; Fig. 2B, C). Together these findings suggested the possibility that stress in the recovering alcoholic could contribute to craving and relapse – an outcome that provides evidence for the idea that the brain's "neural stress system" is a critical component of the kindling process that supports the CIA persistent adaptation and the stress facilitation of anxiety following withdrawal (Overstreet et al., 2004a, 2005).

Humans have been observed to consume excessive amounts of alcohol under stressful conditions, and alcoholic patients have an increased susceptibility to stress – a response not observed in social drinkers (Breese, Chu, et al., 2005; Breese, Sinha, & Heilig, 2011; Gilman & Hommer, 2008; Gilman, Ramchandani, Davis, Bjork, & Hommer, 2008; Sinha, 2001). The Marlatt laboratory (Higgins & Marlatt, 1973, 1975; Marlatt & Gordon, 1985; Vujanovic, Bonn-Miller, & Marlatt, 2011) found that stressful circumstances consistently resulted in greater alcohol drinking. Subsequently, other research linked excessive alcohol use with stressful environments (Breese, Chu, et al., 2005; Breese et al., 2004; Brown et al., 1990; Brown, Vik, Patterson, Grant, & Schuckit, 1995; Kofeod, Friedman, & Peck, 1993; Koob, 2008; Sillaber & Henniger, 2004; Tate, McQuaid, & Brown, 2005; Uhart & Wand, 2009). Additionally, psychiatric disorders commonly susceptible to stress such as depression, generalized anxiety disorder, social phobia, and posttraumatic stress disorder (PTSD) have also been commonly identified with alcohol abuse (Anisman & Merali, 2003; Dunn, Swiergiel, & de Beaurepaire, 2005; Hammen, 2005; Hayley & Anisman, 2005; Kofoed et al., 1993; Miller, Maletic, & Raison, 2009; Pucak & Kaplin, 2005; Raison, Capuron, & Miller, 2006; Uddin et al., 2011). Because alcohol pharmacologically can be viewed as exerting an "anti-tension" action (Gilman et al., 2008), the alcohol tension-reduction hypothesis was proposed to explain the excessive use of alcohol associated with stress (Cappell & Herman, 1972; Conger, 1951, 1956). This latter concept presumably could also explain the behavior of drinking to reduce the negative symptoms accompanying alcohol withdrawal that have been linked to "stress" (Breese et al., 2008), and the consistent finding that previous CIA exposures enhance stress-induced anxiety during alcohol withdrawal (Breese, Overstreet, Knapp, & et al., 2005; Valdez, Zorrilla, Roberts, & Koob, 2003). It has not been determined whether stress-associated elevation in drinking in alcoholics is dependent on the developing adaptation associated with CIA exposure. The demonstration of a contribution of "stress" to the CIA cumulative adaptation (Breese et al., 2011; Heilig & Koob, 2007; Koob & Heinrichs, 1999) may provide a means to implicate neural systems that support "stress" in excessive alcohol ingestion.

Clinically, the stress that accompanies withdrawal from alcohol results in a susceptibility for craving – a reflection of the persistent neural dysregulation in alcoholics (Breese, Overstreet,

& Knapp, 2005; Breese et al., 2011; Brown et al., 1990; Cooney, Litt, Morse, Bauer, & Gaupp, 1997; Fox, Bergquist, Hong, & Sinha, 2007; Fox, Bergquist, Peihua, & Rajita, 2010; Fox, Hong, Siedlarz, & Sinha, 2008; Fox, Hong, & Sinha, 2008; Monti et al., 1987; Payne et al., 1992; Sinha, 2007; Sinha & O'Malley, 1999). Over time, the association of negative affect and craving that occurs during acute abstinence is accompanied by altered basal HPA axis function and a suppressed HPA cortisol response to stress in alcoholics compared with non-addicted counterparts (al'Absi, Hatsukami, & Davis, 2005; Badrick et al., 2008; Junghanns et al., 2003; Lovallo, Dickensheets, Myers, Thomas, & Nixon, 2000; Munro, Oswald, Weerts, McCaul, & Wand, 2005). The altered pattern of HPA and autonomic dysregulation in the alcoholic is similar to that documented in chronic high distress states such as PTSD (Ehlert, Gaab, & Heinrichs, 2001; Yehuda, Giller, Southwick, Lowy, & Mason, 1991). Furthermore, the negative emotional state that accompanies withdrawal from alcohol is accompanied by physiological dysregulation, including elevated blood pressure and heart rate. These reflections of adaptation can be accompanied by negative affect, sleep disturbances, and cognitive and behavioral changes – alterations which persist in the alcoholic for an extended period (Brower, 2001; Drummond, Gillin, Smith, & DeModena, 1998; Sinha et al., 2009). Distress and craving in response to stress cues are also observed in abstinent alcoholics but not in controls (Sinha et al., 2009, 2011; Seo et al., 2011). The Sinha Laboratory (Fox, Hong, & Sinha, 2008; Sinha, 2007; Sinha et al., 2009, 2011; see Breese et al., 2011) has pursued the possibility that the persistent maladapted neural changes associated with alcoholism contribute to high relapse rates. In spite of the strong evidence that a persistent adaptation develops over time in the alcoholic (Ballenger & Post, 1978), duration and timing of alcohol abuse required to have a persistent consequence is presently not known.

Multiple brain site involvement of CRF in stress substitution for CIA withdrawal sensitization of anxiety-like behavior

Merlo Pich et al. (1995) used microdialysis to demonstrate that extracellular CRF release increased in the amygdala of rats not only following restraint stress, but also during alcohol withdrawal. Consistent with this report, sheep exposed acutely to a predator (i.e., a stress) also exhibited an increase in CRF release from the amygdala (Cook, 2004). These observations support the possibility that CRF in the amygdala contributes to the link between stress and alcohol withdrawal. Microinjection of a CRF1R antagonist (CRF1RA) into the central amygdala (CeA) prior to each stress application given prior to the final alcohol diet exposure prevented withdrawal anxiety (Fig. 3; Huang et al., 2010). This inhibitory effect of the CRF1RA antagonist in the CeA suggested that this site was a primary contributor to the stress induction of adaptation associated with the negative affect in alcohol abuse (Gilpin, Herman, & Roberto, 2015). CRF1RA microinjection into the dorsal raphe nucleus (DRN) or the bed nucleus of the stria terminalis (BNST) prior to each weekly stress exposure also prevented the sensitization of anxiety observed during alcohol withdrawal (Fig. 3; Huang et al., 2010; Knapp et al., 2011). Recently, Daniel and Rainnie (2015) reviewed evidence that the BNST has an opposing circuitry that can minimize stress modulation of anxiety-like behavior. From these findings, differing brain sites in an integrated CRF (stress) circuit appear to contribute to the repeated stress-induced cumulative

adaptation responsible for the anxiety induced during the single withdrawal from a CA exposure.

To further test the hypothesis that CRF acts on CRF1Rs in differing brain sites to contribute to the stress facilitation of alcohol-withdrawal anxiety, CRF was microinjected prior to a single 5-day cycle of alcohol exposure at bi-weekly intervals into the CeA, DRN, or BNST to substitute for weekly stress applications (Huang et al., 2010; Knapp et al., 2011). This repeated administration of CRF into these brain sites prior to the single CA exposure also sensitized anxiety during alcohol withdrawal (Fig. 4A). On the other hand, repeated microinjections of CRF into the hypothalamic PVN, the nucleus accumbens, and the CA1 region of the hippocampus prior to the withdrawal from alcohol did not support sensitization of anxiety during alcohol withdrawal (Huang et al., 2010). Furthermore, peripheral injection of the CRF1RA prior to each CRF injection into the CeA, DRN, or BNST prevented the anxiety arising from the subsequent withdrawal from the single 5-day alcohol exposure (Fig. 4B). These observations with CRF1RA injection constituted additional evidence that an integration of CRF action within specific brain sites is likely involved in the stress sensitization of anxiety during withdrawal from the subsequent single CA exposure (Huang et al., 2010). Importantly, microinjection of a specific CRF2R agonist, urocortin-3, into the positive CRF sites produced no sensitization of anxiety during alcohol withdrawal (Deussing et al., 2010). Collectively, this work that demonstrated brain site selectivity for CRF on CRF1Rs (Huang et al., 2010) supported involvement of an integrated neural circuit in the stress sensitization of anxiety during withdrawal from CA (Breese et al., 2011; Huang et al., 2010).

Neurocircuitry related to stress supports the negative affect (anxiety) induced by withdrawal from CIA exposure

Sensitization of anxiety is a major outcome of the repeated withdrawals from CIA exposure (Baldwin et al., 1991; Breese, Overstreet, Knapp, et al., 2005; Rassnick et al., 1993). An early review by Davis (2002) provided evidence for specific brain site involvement in anxiety and stress. More recent reviews have focused on defining specific neurocircuitry involvement in the chronic alcohol withdrawal associated with anxiety and stress (Herman & Roberto, 2015; Koob, 2014, 2015; Tovote, Fadok, & Lüthi, 2015). The evidence reviewed herein concerning the specific neurocircuitry associated with CIA withdrawal anxiety seems to indicate that circuitry associated with CRF involvement in stress and alcohol withdrawal is critical for negative affect (Breese et al., 2004, 2008; Huang et al., 2010). In accord with this concept, data from several sources have clearly implicated the CeA as a critical site for supporting CRF involvement in this withdrawal anxiety (Huang et al., 2010; Knapp, Overstreet, Angel, et al., 2007). Namburi et al. (2015) and Silberman and Winder (2015) have also suggested the involvement of a circuit involving the amygdala. Specific brain sites that must interact within a circuit involving CRF include the CeA, BNST, and BLA because CRF placed within these sites substituted for stress enhancement of withdrawal anxiety (Huang et al., 2010; Knapp et al., 2011; Koob, 2014). Supporting the association of CRF in the BNST with anxiety, Daniel and Rainnie's review (2015) documents the importance of CRF involvement in the BNST in stress-induced anxiety-like behavior. This latter review

also provides evidence that norepinephrine as well as serotonin action in the BNST can contribute to stress-induced anxiety – a clue that brain sites linked to these transmitter systems are associated with stress (Daniel & Rainnie, 2015).

The negative emotional state associated with the BNST following chronic stress and CA exposure was antagonized by a CRF1RA in support of CRF involvement (Daniel & Rainnie, 2015; Francesconi et al., 2009). A recent overview documents a role for the BNST in the anxiety and addiction associated with chronic stress in humans (Avery, Clauss, & Blackford, 2015). However, an issue to be clarified is whether the circuitry that is related to the CRF association with anxiety is identical to that activated by stress and CIA exposure (Breese et al., 2011; Shin & Liberzon, 2010; Sinha, 2001; Sinha et al., 2011; Tovote et al., 2015). Findings to date suggest that a neural circuit associated with CRF involvement in stress and alcohol withdrawal is critical for the negative affect noted during withdrawal (Silberman & Winder, 2015). In accord with this concept, Namburi et al. (2015) described a circuit mechanism that may be responsible for functional involvement of the amygdala. As these reports emphasize, the neurocircuitry associated with the role of stress and alcohol with alcohol and other abused drugs remains an active area of interest (Mantsch, Baker, Funk, Lê, & Shaham, 2015; Tovote et al., 2015).

One can conclude from the various reports on stress and CA consequences that the associated neurocircuitry supporting these changes related to alcohol withdrawal is complex. Even though documentation of the involvement of differing brain sites in the CRF and stress contribution to CA adaptation is informative, the means by which CRF action on CRF1Rs in these differing brain regions individually support the cumulative adaptation that sensitizes alcohol withdrawal to induce anxiety has not been defined. Consequently, efforts are warranted to define how CRF released by stress in these differing brain sites interacts within a neural circuit to sensitize anxiety during the withdrawal from CA intake (Breese et al., 2008). One focus could be on determining whether these brain sites form a circuit that supports the regional effects of CRF on sensitization of withdrawal anxiety. Future work should define how CRF released by stress in differing brain sites interacts within a proposed neural circuit to sensitize anxiety during alcohol withdrawal (Breese et al., 2008). A key experiment could test whether induction of anxiety by CRF placement into one of these sensitive sites would be prevented by simultaneous placement of a CRF1RA into the CeA or one of the other CRF sites supporting ethanol withdrawal-induced anxiety (Huang et al., 2010). Such research would also shed light on the degree to which these specific brain sites involved in CRF sensitization of withdrawal anxiety form an interactive circuit that is the basis of the regional effects of CRF action observed during stress and withdrawal.

The Roth laboratory has recently published several reviews related to Designer Receptors Exclusively Activated by Designer Drugs (DREADD) technology (Sternson & Roth, 2014; Urban & Roth, 2015). Utilizing a DREADD protocol, our laboratory demonstrated that inhibition of glutamate output from the BLA prevented the anxiety that follows withdrawal from CA exposure (unpublished observation) – a finding confirming that the BLA is involved in the adaptation that induces anxiety during withdrawal from CIA exposure. This latter observation is consistent with the finding that chronic early stress that activates the BLA facilitates anxiety (Rau, Chappell, Butler, Ariwodola, & Weiner, 2015). Whether this

aspect of BLA involvement in the withdrawal anxiety is associated with a selective BLA link to the CeA has yet to be confirmed. The degree to which GABA neural output from the medial portion of the CeA to distant sites is involved in the CA withdrawal and stress-induced anxiety also warrants further elucidation. Approaches to further identify the specific circuitry involved will be essential for fully understanding the neural basis of the persistent adaptation that supports alcohol-induced withdrawal anxiety. Future strategies may require new approaches to characterize fully the specific circuitry and sites involved in the withdrawal anxiety and sensitization of stress following withdrawal from CIA exposure. This new effort must also document how specific brain sites identified within a given circuit interact. The approach by Richiardi et al. (2015), who have explored how gene expression correlates with brain networks, could possibly be a means to explore interactions of differing brain regions associated with molecular and behavioral outcomes during withdrawal from chronic alcohol. Fig. 5 provides a summary of the possible brain sites that may interact to form circuits that support involvement of CRF and stress in withdrawal-induced anxiety associated with CA exposure, and illustrates how these sites may be linked to involvement of neuroimmune function (details to follow).

Withdrawal from chronic alcohol (CA) exposure facilitates an increase in cytokine mRNA in brain – relationship to stress

During the period when investigations implicated CRF involvement in the contribution of stress in alcohol withdrawal-induced anxiety, literature was accumulating that stress alone increased cytokines, cytokine mRNAs, and other neuroimmune factors in brain (Blandino, Barnum, & Deak, 2006; Blandino et al., 2009; Deak et al., 2005; Girotti, Donegan, & Morilak, 2011; Hueston et al., 2011; Johnson, O'Connor, Watkins, & Maier, 2004; Johnson et al., 2005; Minami et al., 1991; Nguyen et al., 1998, 2000; O'Connor et al., 2003; Porterfield et al., 2011; Shintani, Nakaki, Kanba, Kato, & Asai, 1995; Shintani, Nakaki, Kanba, Sato, et al., 1995; Shizuya et al., 1997; Suzuki, Shintani, Kanba, Asai, & Nakaki, 1997). The latter findings considered in the context of the increased susceptibility of alcoholics to stress (Gilman & Hommer, 2008; Gilman et al., 2008; Sinha, 2001) suggested the possibility that the adaptation induced by CIA exposure and stress might be associated with a facilitation of neuroimmune (cytokine) function (Breese et al., 2004; 2008, 2011). Blednov et al. (2005) had earlier provided evidence for this possibility by finding that deleting genes of chemokines altered alcohol reinforcement – a conclusion later confirmed (Blednov et al., 2012). Measurement of cortical cytokine mRNA levels prior to withdrawal from CA exposure revealed no increase in these mRNAs (Whitman, 2012; Whitman, Knapp, Werner, Crews, & Breese, 2013), even though stress alone activated neuroimmune function (Blandino et al., 2009; Deak et al., 2005; Johnson et al., 2004, 2005; Minami et al., 1991). Nonetheless, cytokine levels have been found to be elevated in plasma of alcoholics (Achur, Freeman, & Vrana, 2010; Kiefer, Jahn, Schick, & Wiedemann, 2002) as well as in their brains at autopsy (Crews, Qin, Sheedy, Vetreno, & Zou, 2013; He & Crews, 2008). Additionally, a cytokine-gene polymorphism was found to be associated with alcohol dependence (Liu, Hutchinson, White, Somogyi, & Coller, 2009; Saiz et al., 2009). The implications of these findings concerning neuroimmune function in alcoholism have not as yet been fully explored.

As noted earlier (Overstreet et al., 2002), three cycles of withdrawal from a 4.5% CIA exposure facilitated anxiety-like behavior whereas continuous alcohol exposure did not. A subsequent investigation assessed whether an increase in neuroimmune function would be altered in a similar fashion by these CA exposures (Whitman, 2012; Whitman et al., 2013). However, the cycled and continuous introduction of the lower 4.5% alcohol diet, which had previously been demonstrated to sensitize anxiety during withdrawal (Overstreet et al., 2002), did not increase cortical cytokine mRNAs or cytokines 24 h after withdrawal (Harper, Knapp, & Breese, 2015; Whitman, 2012; Whitman et al., 2013). In response to a 24-h alcohol withdrawal from 7% alcohol CIA diet, CCL2 (MCP-1), IL1β, and TNFα mRNAs were increased in cortex (Whitman, 2012; Whitman et al., 2013; Fig. 6). Unexpectedly, a comparable increase in neuroimmune mRNAs was also found in cortex following withdrawal from the 7% continuous alcohol diet exposure (Fig. 6), just as seen with withdrawal from the CIA 7% diet exposure (Whitman, 2012; Whitman et al., 2013). These comparable cytokine mRNA increases obtained during the CIA and continuous 7% alcohol exposures implicate a requirement for higher alcohol doses to increase cytokine mRNAs during alcohol withdrawal. Doremus-Fitzwater et al. (2014) have since reported that withdrawal from CA exposure also increases expression of both central and peripheral cytokine mRNAs. Additional investigations have found similar brain cytokine mRNA effects in adolescents exposed to a 5.3% alcohol diet compared with adults that received the 7% alcohol diet (Harper et al., 2015; Kane et al., 2014). These latter age-dependent findings could be relevant to reports that adolescent exposure to alcohol is associated with the appearance of alcoholism in adulthood (Hingson, Heeren, & Winter, 2006). Whether the increase in neuroimmune function associated with stress and withdrawal from chronic alcohol is associated with proliferation of microglia (Zou, Vetreno, & Crews, 2012) is not known. Likewise unknown is the degree to which lipopolysaccharide- (LPS) associated activation of neuroimmune function after CA exposure (Qin et al., 2007; 2008) is related to the increase in cytokine mRNAs associated with withdrawal from CA exposure. Additionally, given the extensive literature that females respond differently to chronic stress than males (see review by McEwen, Nasca, & Gray, 2015), further investigation of sex differences to withdrawal from CA exposure should be considered.

To follow up on the possibility that the stress increase in brain neuroimmune function (Johnson et al., 2004, 2005) could relate to CA withdrawal anxiety, Breese et al. (2008) found that individual cytokines (i.e., TNFα, IL1β, and CCL2 [MCP-1]) injected into brain prior to a single exposure to CA sensitized withdrawal anxiety (Fig. 7), just as multiple stresses and microinjections of CRF did when given prior to the single alcohol exposure (Breese et al., 2004, 2008; Huang et al., 2010). In accord with this latter work, Arakawa, Blandino, and Deak (2009) found that central infusion of an IL1β antagonist prevented the social interaction change produced by a subsequent stress exposure. Additionally, Knapp et al. (2011) determined that repeated administration of TNFα into the CeA prior to 5 days of alcohol exposure sensitized anxiety during withdrawal from a single alcohol exposure – a finding further implicating the CeA in the adaptation associated with sensitization of withdrawal-induced anxiety. Knapp, Whitman, Harper, and Breese (2015) subsequently demonstrated that withdrawal from exposure to continuous CA selectively induced a different pattern of CCL2-, IL1β-, and TNFα-mRNA levels in amygdala, hippocampus, and

hypothalamus than observed in cortex, a finding consistent with the idea that not all brain sites contribute equally to the neuroimmune response that occurs during withdrawal from CA exposure.

The prevention of brain cytokine mRNAs by a CRF1RA prior to initial withdrawals from CA in rats suggests a specific CRF mechanism for the withdrawal increase in cytokine mRNAs (Whitman et al., 2013). The observation that cytokine microinjection into the CeA substituted for CRF sensitization of withdrawal-induced anxiety supported the conclusion that cytokine mRNAs increase in sites where CRF acts to support adaptive changes associated with stress (Knapp et al., 2011). Given that CRF and cytokines in the CeA likely mediate stress influences on chronic alcohol-associated neuroimmune changes, a future investigation should determine whether microinjection of CCL2 and IL1β into the DRN or the BNST will sensitize anxiety during a withdrawal from a subsequent single alcohol exposure (Breese et al., 2008; Huang et al., 2010). This future investigation would determine whether all brain sites previously related to CRF sensitization of withdrawal anxiety also support cytokine sensitization of alcohol withdrawal-induced anxiety. Given the recent observation by Gray et al. (2015) that CRF drives anandamide hydrolysis to promote stress-induced anxiety, it may be warranted to test whether antagonism of anandamide action in the CeA, the DRN, or the BNST (Huang et al., 2010; Knapp et al., 2011) will prevent the anxiety and the accompanying increase in cytokine mRNAs that follow withdrawal from chronic alcohol (Knapp et al., 2011; Overstreet et al., 2002; Whitman et al., 2013). Such new information could be critical for understanding the persistent adaptation associated with the neuroimmune activation that follows CIA exposure.

Brain cytokine mRNAs after stress alone in controls and after CA withdrawal may be supported by distinct mechanisms

In a recent study from our laboratory (Knapp et al., 2015), restraint stress was confirmed to increase cytokine mRNAs in the cortex of control rats (Deak et al., 2005; Girotti et al., 2011; Johnson et al., 2004, 2005; Porterfield et al., 2011) to approximately the same degree observed following withdrawal from the CA treatment (Whitman, 2012; Whitman et al., 2013). Given that a CRF1RA prevents the increase in CCL2, IL1β, and TNFα mRNAs induced by withdrawal from continuous alcohol exposure (Whitman et al., 2013), it was presumed that the CRF1RA would prevent the stress increase in neuroimmune function in controls. However, the CRF1RA did not block the restraint stress increase in cytokine mRNAs in control rats (Knapp et al., 2015, & unpublished). These findings suggest that the mechanism of stress induction of cytokine mRNAs in cortex of controls is distinct from that responsible for the cortical increase in cytokine mRNAs induced during withdrawal from CA exposure (Knapp et al., 2015; Whitman et al., 2013).

A possible clue to the distinct mechanisms induced by these challenges comes from finding that a β-adrenergic receptor antagonist blocks the increase in neuroimmune function by stress in controls (Johnson et al., 2005; Porterfield et al., 2012) – an outcome that may be related to stress priming of microglia (Johnson, Zimomra, & Stewart, 2013). β-adrenergic input has also been shown to activate an inflammatory response in controls (Madrigal et al.,

2010), just as stress increases IL1β and other cytokine mRNAs (Porterfield et al., 2012). Accordingly, β-adrenergic antagonism also prevents microglial activation and anxiety-like behavior induced by stress (Wohleb et al., 2011). Whether β-adrenergic antagonism will prevent the increase in cortical cytokine mRNAs induced by withdrawal from CA exposure has yet to be defined. The later experiment should include an assessment of whether a β-adrenergic antagonist will alter brain cytokine mRNA increases in the CeA, the DRN, or the BNST following withdrawal from CA exposure. Another future experiment should also define whether a β-adrenergic antagonist during CIA exposure will influence sensitization of anxiety induced during withdrawal (Huang et al., 2010).

When stress is applied 24 h after withdrawal, the anxiety induced is enhanced (Breese et al., 2004; 2008, 2011); therefore, it was presumed that stress 24 h after the withdrawal from CA exposure would cause a further increase in selected cytokine mRNAs in brain. The extended absence from CA exposure was believed to be necessary for the stress to cause a greater response, because previous work had demonstrated that stress during withdrawal from an acute alcohol exposure did not increase neuroimmune function (Buck, Hueston, Bishop, & Deak, 2011). In an initial test of this hypothesis, stress increased CCL2 and IL1β mRNAs above that induced by withdrawal from CA intake alone. As this initial elevation of neuroimmune function by stress after CA exposure was not confirmed in a subsequent investigation, future studies will be required to resolve whether stress after CA exposure indeed causes a further increase in cytokine mRNAs after withdrawal from CA exposure. If an elevated level in cytokine mRNAs is confirmed to occur with stress after withdrawal from chronic alcohol, we will seek to define if a CRF1RA or a β-adrenergic receptor antagonist given prior to this stress challenge after CA withdrawal will affect the degree to which cytokine mRNAs are increased.

Cytokine influences on neural function

The close relationship of cytokine function to stress and CRF led many to posit that cytokines may act as neuromodulators (Adler, Geller, Chen, & Rogers, 2006; Bauer, Kerr, & Patterson, 2007; Lukàts, Egyed, & Karádi, 2005; Rostène, Kitabgi, & Parsadaniantz, 2007; Shintani, Nakaki, Kanba, Kato, et al., 1995). Given that a CRF1RA prevented the effect of cytokine microinjection into brain from substituting for stress (Knapp et al., 2011), a question that arose was whether this CRF1R antagonism provided indirect evidence for an association of cytokine actions in the CeA with neural function. Such efforts to understand cytokine influences on neural function have been proceeding on a number of fronts. A subsequent electrophysiological study demonstrated that TNFα decreased the threshold for triggering an action potential from CeA neurons without altering membrane properties during current-clamp recording (Ming, Criswell, & Breese, 2013). Further, TNFα increased amplitude but not frequency of mEPSCs from some but not all CeA neurons (Ming et al., 2013). Similarly, CCL2 was found to increase firing rates in the striatum (Guyon et al., 2009). In the hippocampus, CCL2 increased synaptic transmission via presynaptic influences on transmitter release (Zhou, Tang, Liu, Dong, & Xiong, 2011).

Electrophysiological studies also demonstrated that TNFα influences GABA function in the CeA (Ming et al., 2013). TNFα increased the frequency but not the amplitude of mIPSCs

from some but not all CeA neurons – an outcome consistent with a selective neural influence of TNFα on GABA function in this brain site. Bajo et al. (Bajo et al., 2014; Bajo, Herman, et al., 2015; Bajo, Varodayan, et al, 2015) have also reported that immune factors modulated alcohol influences on GABAergic transmission in the CeA. In unpublished work, our laboratory also found that CCL2 can enhance GABA release from some CeA neurons. In accord with these latter findings, Bajo and colleagues (Bajo, Herman, et al., 2015) found that IL1β influences mIPSCs of some CeA neurons but not others, further evidence for a similar influence of this cytokine on GABAergic function in the CeA as observed with TNFα (Ming et al., 2013). Bajo, Varodayan, et al. (2015) reported that an IL1β receptor antagonist prevented the IL1β increase in mIPSC frequency. Consistent with a relationship between alcohol and cytokine action, alcohol-like cytokines significantly increased eIPSP amplitude and increased mIPSC frequencies (Bajo, Varodayan, et al., 2015). While co-application of IL1β and alcohol did not alter the increase in eIPSP amplitude by alcohol, the alcohol-induced increase in mIPSCs was prevented by IL1β co-application (Bajo, Varodayan, et al., 2015). Liu et al. (2011) reported that the influence of binge alcohol drinking on $GABA_A$ receptor function was related to TLR4 expression in the amygdala. It is not clear whether this latter result directly relates to cytokine involvement in neural function.

Based upon work showing that CRF1RA into the CeA prevented CIA withdrawal-induced anxiety (Huang et al., 2010) and that CRF release is induced by IL1β (Turnbull & Rivier, 1995), Ming et al. (2013) explored whether a CRF1RA would alter the TNFα-induced increase in mIPSC frequency. The CRF1RA prevented the TNFα-induced increase in mIPSC frequency without altering the TNFα-induced amplitude increase in mEPSCs or the TNFα-reduced threshold for action potentials. (Ming et al., 2013). This finding suggested the possibility that at least some cytokine actions are mediated via indirect actions through CRF receptor signaling – a conclusion consistent with the observation that the CRF1RA blocked the action of TNFα to support alcohol withdrawal anxiety (Breese et al., 2004; Knapp et al., 2004; Knapp, Overstreet, Angel, et al., 2007; Overstreet et al., 2003, 2004a, 2005). Bale & Vale (2004) hypothesized that stress release of CRF causes a release of cytokines that in turn further facilitates CRF release (del Cerro & Borrell, 1990). To clarify how TNFα might involve CRF release in the presence of tetrodotoxin, the possibility that preventing glial activation with minocycline (Giuliani, Hader, & Yong, 2005; Gong, Zou, Fuchs, & Lin, 2015) would alter the elevated mIPSC frequency by TNFα was tested (Ming et al., 2013). The minocycline action prevented the TNFα-induced increase in mIPSC frequency – a finding consistent with a glial contribution in this CRF neural involvement (Ming et al., 2013). Cytokine effects on calcium and sodium channels have also been documented. For example, actions of CCL2 were found to depend upon T-type Ca^{2+} channels or Na^{+} channels (Banisadr, Gosselin, Mechigel, Rostène et al., 2005; Belkouch et al., 2011; Gosselin et al., 2005; van Gassen, Netzeband, de Graan, & Gruol, 2005). In the striatum, CCL2 was found to increase firing rates via CCL2 effects on membrane leak channels (Guyon et al., 2009). In other neuronal preparations, actions of CCL2 via T-type Ca^{2+} channels or Na^{+} channels have been identified (Banisadr, Gosselin, Mechighel, Rostène, et al., 2005; Belkouch et al., 2011; Gosselin et al., 2005; van Gassen et al., 2005). The possible relationship of these channels to CRF and CA influences on cells has not been identified.

These basic electrophysiological observations of cytokine action provide renewed support for the view that neural and immune functions in brain may interact to support withdrawal anxiety and the increases in brain cytokine mRNAs associated with CA adaptation. However, the observation that not all neurons in the CeA responded to TNFα also needs further clarification (Bajo, Herman, et al., 2015; Bajo, Varodayan, et al., 2015; Ming et al., 2013). Likewise, as only TNFα and IL1β have been tested in the CeA, the preliminary observation that CCL2 enhances mIPSC frequency will need confirmation, and a possible linkage of this CCL2 action to CRF function will need to be explored. Whether all cytokines linked to stress and CRF induction of withdrawal anxiety (Huang et al., 2010; Whitman et al., 2013) will be associated with neural actions in all brain regions like those observed in the CeA (Ming et al., 2013) also remains to be assessed.

CRF and neuroimmune signaling links to CA adaptation

Phenotypes related to anxiety and CA drinking are responsive to manipulations by stress, CRF, and cytokines (e.g., Blednov et al., 2005; Breese et al., 2008; Knapp et al., 2011). Further, demonstration of cytokines within neurons and glia (Banisadr, Gosselin, Mechighel, Kitabgi, et al., 2005; June et al., 2015) and evidence for cytokine receptors on both neurons and glia (Banisadr et al., 2002; Banisadr, Gosselin, Mechighel, Rostène, et al., 2005) suggest the potential for cytokine signaling systems across cell types and circuits that will influence neural function. The underlying signaling mechanisms that drive actions of cytokines and CRF are not well understood, although physiological studies described above (“Cytokine Influences on Neuronal Function”) provide clues that can guide future efforts to isolate relevant mechanisms. Involvement of the amygdala in anxiety has been clearly outlined for CRF and cytokines. In addition, classic neurotransmitters like GABA, glutamate, norepinephrine, and serotonin are all well known to play a role in amygdala contribution to anxiety-related behavior. These neural systems have long been known to directly impact both conditioned and unconditioned negative emotional responding via the amygdala. Less well understood is how cytokine and CRF systems may interact across these behavioral domains via common or independent signaling mechanisms to regulate behaviors influenced by the amygdala, including alcohol consumption and alcohol withdrawal-related behaviors. It is also possible that individuals at risk for alcohol abuse have differential engagement of CRF or cytokines in brain. The recent observation that CRF and alcohol action on glutamate transmission in the CeA is selectively altered in Marchigian Sardinian alcohol-preferring rats but not in Wistar rats is an example of such differential involvement in animals (Herman, Varodayan, et al., 2015). Assuming that alcoholism has a genetic basis, perhaps such distinction in CRF involvement observed in differing rat strains occurs between non-alcoholics and alcoholics. Such differential vulnerability across species and strains could be a fruitful area for additional research to elucidate mechanisms of the persistent adaptation that supports alcohol abuse.

Several laboratories have proposed that TLR4 signaling is a key link between alcohol and activation of neuroimmune function (Akira & Takeda, 2004; Alfonso-Loeches & Guerri, 2011; Alfonso-Loeches, Pascual-Lucas, Blanco, Sanchez-Vera, & Guerri, 2010; Alfonso-Loeches et al., 2012; Blanco, Vallés, Pascual, & Guerri, 2005; Fernandez-Lizarbe, Pascual, Gascon, Blanco, & Guerri, 2008; Fernandez-Lizarbe, Pascual, & Guerri, 2009; Gárate et al.,

2013; Pandey, 2012; Pascual, Baliño, Alfonso-Loeches, Aragón, & Guerri, 2011; Pascual, Baliño, Aragón, & Guerri, 2015; Weber, Frank, Sobesky, Watkins, & Maier, 2013; Wu et al., 2012; Yamada & Maruyama, 2007; Yang, Wang, Czura, & Tracey, 2005; Yu et al., 2006; Zou & Crews, 2010). In support of this view, TLR4 was increased in mouse brain following CA intake as well as in cortex of *post mortem* alcoholics (Crews et al., 2013; He & Crews, 2008). Likewise, the increase in cytokine production after withdrawal from prolonged alcohol was prevented in TLR4 knockout mice (Pascual, Baliño, Aragón, & Guerri, 2015; Pla, Pascual, Renau-Piqueras, & Guerri, 2014). Further, TLR4 mRNA was increased in cortex after withdrawal from both the CIA and continuous alcohol protocols (Whitman, 2012; Whitman et al., 2013). The increase in TLR4 mRNA induced by the CA exposures could be an important factor in the reported involvement of TLR4 signaling in alcohol action (Crews et al., 2013; Pascual et al., 2015). Montesinos et al. (2015) found that TLR4 elimination prevented synaptic and cognitive dysfunctions in adolescent mice that received CIA exposure. Additionally, Weber et al. (2013) found that blockade of TLR4 during stress prevented a subsequent LPS-induced neuroimmune response.

Other factors that may contribute to the potential involvement of TLR4 in CA adaptation are MyD88 and TRIF – adaptor proteins that influence the transcription factor NF-kB (Cheng, Taylor, Ourthiague, & Hoffman, 2015). Another endogenous factor linked to TLR4 is the high-mobility group box 1 protein (HMGB1) (Andersson & Tracey, 2011), which initiates cytokine release (Frank, Weber, Watkins, & Maier, 2015). Like TLR4 mRNA, HMGB1 mRNA was elevated in cortex after both CIA and continuous CA exposure (Whitman, 2012; Whitman et al., 2013). Functional antagonists of HMGB1 block the cumulative increase in cytokine mRNAs that follows withdrawal from CA exposure (Davé et al., 2009; Kim et al., 2006; Lin et al., 2011; Park et al., 2004, 2006; Su, Wang, Zhao, Pan, & Mao, 2011; Ulloa et al., 2002; Whitman et al., 2013). Collectively, these findings support an interactive link between TLR4 activity and the increase in neuroimmune function that follows withdrawal from CA exposure. Further investigation of the effects of TLR4 and factors linked to repeated CA withdrawals and stress activation of cytokine mRNAs in various brain regions is warranted.

Relatedly, CRF-amplified neuronal TLR4/MCP-1 (CCL2) signaling was shown to regulate alcohol drinking (June et al., 2015); therefore, it is apparent that regions of brain known to regulate drinking (e.g., accumbens, amygdala, and others) are attractive brain sites for further investigation of these cytokine interactions with CRF. Recent reports from Herman, Roberto, and colleagues (Bajo, Herman, et al., 2015; Bajo, Varodayan, et al., 2015; Herman & Roberto, 2014, 2015) provide a classic neurotransmitter and local circuit framework through which cytokine effects may be manifested. Moreover, these investigators (Bajo, Herman, et al., 2015; Bajo, Varodayan, et al., 2015; Herman & Roberto, 2014, 2015) have shown that different populations of CeA neurons defined via physiological phenotyping respond differentially to alcohol. Consequently, interleukin-1β (IL1β) receptors on these CeA-GABA neurons may regulate their effects via signaling that ultimately regulates amygdala outputs that control emotion and other behaviors. Such effects are even more notable in the context of data showing that IL1β induces anxiety through interactions with the endocannabinoid system in the brain (Rossi et al., 2012) – a finding consistent with an association of dysregulation of endocannabinoid function with anxiety (Lutz, Marsicano,

Maldonado, & Hillard, 2015). Additionally, Hodge and colleagues (Agoglia, Holstein, Reid, & Hodge, 2015) have provided evidence that cytokines acting through glutamate could be involved. These investigators (Agoglia et al., 2015) showed that binge drinking linked to glutamate function decreased CaMKIIα T286- and CaMKIIα GluA1S831-dependent phosphorylation (pGluA1S831) in adolescent rat amygdala. This observation (Agoglia et al., 2015) and others (Ming et al., 2013) that relate phosphatidyl inositol-3 kinase antagonist action to glutamate suggest that glutamate signaling may be another avenue through which cytokines could act to influence the persistent outcome of CA exposure.

The fact that kinases generally are worthy of further assessment in connection with cytokine/CRF interaction in brain is suggested by work showing that p38-mediated protein kinase is induced by CCL2 in cultured hippocampal cells (Cho & Gruol, 2008). The second messenger protein kinase C (PKC) epsilon, which supports CRF action in the amygdala, is likely engaged within GABA neurons (Bajo, Cruz, Siggins, Messing, & Roberto, 2008), by CRF1R action linked to Gs- and Gq-mediated signaling (Hanoune & Defer, 2001; Papadopoulou et al., 2004). Another established signaling mechanism involving CRF1-receptor signaling is PKA activation (Papadopoulou et al., 2004) – an engagement confirmed in the amygdala (Cruz, Herman, Kallupi, & Roberto, 2012). The CRF1R activation of PKA via the Gs and Gq proteins (Hanoune & Defer, 2001; Papadopoulou et al., 2004) likely regulates amygdala-related anxiety-like behavior via this mechanism. Thus, one might speculate that the action of CRF on CRF1Rs in other behaviorally significant regions such as the BNST and dorsal raphe may also be linked to these same kinase signaling mechanisms. Additionally, the CCL2 cytokine action on CCR2 receptors activates MAPK (Jiménez-Sainz, Fast, Mayor, & Aragay, 2003). The fact that a phosphatidyl inositol-3 kinase antagonist prevents the TNFα-increased amplitude of mEPSCs suggests TNFα has an intracellular influence on this neural function (Ming et al., 2013). Such findings imply that the potential routes through which CRF and cytokines interact to influence function are diverse. More generally, in areas where classic neurotransmitter receptors engage signaling systems important to behavior, particularly those behaviors regulated by amygdala activities following chronic alcohol exposure, cytokine modulation via their respective receptors may alter the effects associated with classic transmitters such as CRF. It would seem essential that future studies focus on cells in behaviorally relevant brain regions that have cytokine/chemokine receptors and their specific signaling systems in order to fully characterize the effects cytokines and CRF have on behavior during and after CA exposure.

Defining the signaling links will provide support for a number of avenues and mechanisms that could be explored to further define CRF and cytokine actions. This information will contribute to our understanding of the functional basis of the persistent adaptation responsible for the behavioral effects associated with CIA withdrawal and sensitization of stress after CIA exposure. For the future, an area that could be of interest would be defining whether second messengers are common to the varied types of neural systems that appear to be involved in CIA-induced withdrawal anxiety. Likewise, further exploration of stress having a differing basis for increasing cytokine mRNAs in the cortex than withdrawal from CA exposure could assist in resolving other unexpected outcomes. Defining the potential involvement of cytokines and other peptides in neural systems implicated in circuits associated with stress and withdrawal from CA would also seem worthy of future

investigation. Chief among these future explorations may be additional delineation of cytokine action on classic neurotransmitter effects in the amygdala and associated brain regions that relate to the anxiety that follows withdrawal from CA and CIA exposure. An understanding of the functional significance of cytokines interacting with classic neural types may be important to understanding the complex involvement of cytokines in the adaptation induced by chronic alcohol exposure. Through such studies, novel targets of therapeutic intervention could emerge.

Do cytokines act through neurocircuits to support negative affect (anxiety) induced by stress and withdrawal from CIA exposure?

The demonstration that cytokines can be linked to stress supports the examination of specific brain sites and circuits that may contribute to the anxiety associated with cytokine influences on withdrawal-induced anxiety. The possible involvement of cytokines in neurocircuitry associated with stress and CA exposure has been raised by Banisadr, Gosselin, Mechighel, Kitabgi, et al. (2005), who showed that CCL2 was highly localized with neurotransmitters and neuropeptides systems in brain. Other data suggest that the previously described increase in cortical cytokine mRNAs induced by CA withdrawal (Whitman et al., 2013) may also occur in the amygdala (Knapp et al., 2015). Szcytkowski and Lysle (2010) implicated DA1Rs in the BLA in the mediation of heroin-induced conditioned immunomodulation – another example of a drug of abuse influencing cytokine changes within a specific brain site. Additionally, the role of inflammasomes in the cytokine mRNA increases in differing brain regions associated with stress and withdrawal from CA is yet another area worthy of future investigation (Fleshner, 2013; Iwata, Ota, & Duman, 2013).

The recent research of Marshall et al. (2015) implicating IL-1 receptor signaling in the BLA in alcohol binge drinking in mice complements the finding that CRF amplifies neuronal TLR4/CCL2 signaling by alcohol self-administration (June et al., 2015). Muscatell et al. (2015) have also reported that stress enhanced inflammatory activity by way of cortical-amygdala coupling. Recently, Knapp et al. (2015) demonstrated that withdrawal from continuous CA exposure differentially affected levels of CCL2, IL1β, and TNFα mRNAs across the cortex, amygdala, hippocampus, and hypothalamus. These distinct regional differences in cytokine mRNA expression are plausibly mediated by distinct neurocircuitry. Whether selected cytokines placed into those specific brain sites with differing cytokine mRNA responses would all sensitize anxiety during a single alcohol-withdrawal exposure, just as is found when TNFα is placed in the CeA prior to alcohol exposure (Huang et al., 2010), would be of interest. Investigations of differences between adolescents and adults in immune responses across brain regions following stress and CIA withdrawal are also warranted (Kane et al., 2014), given the association of adolescent alcohol exposure with later alcoholism (Hingson et al., 2006). These strategies for studying immune involvement in alcohol action across brain sites and neural circuits may help point the way to mechanisms that support the association of cytokines with the adaptation induced by stress and withdrawal from chronic alcohol. Such new information about cytokines and brain sites could advance an understanding of the association of neuroimmune influences with the withdrawal anxiety linked to alcohol abuse.

Is stress activation of CRF and neuroimmune function linked to the neurobiology of alcoholism?

As outlined earlier, alcoholics have an increased susceptibility to stress (Gilman & Hommer, 2008; Gilman et al., 2008; Sinha, 2001) and neuroimmune function is related to stress (Breese et al., 2004, 2008, 2011). Further, the stress link to CRF and neuroimmune activation is associated with a “kindling”-like neural adaptation that supports the symptoms associated with chronic alcohol exposure (Breese et al., 2011). CRF acting through the CRF1R has been implicated in both stress sensitization of the anxiety during CIA exposure (Breese et al, 2011; Huang et al., 2010; Knapp et al., 2011) and the sensitization induced by repeated cytokine injection into brain prior to a single alcohol withdrawal (Knapp et al., 2011). Mantsch et al. (2015) have recently reviewed the various brain sites and transmitters involved in stress involvement in alcohol and other drugs of abuse. The Crews laboratory, citing evidence that alcohol can influence measures of neuroimmune function in alcoholics (Crews et al., 2013; He & Crews, 2008), has contended that induction of immune-related genes in brain contribute integrally to the neurobiology of addiction (Crews & Vetreno, 2011; Crews, Zou, & Qin, 2011) – a view shared by others (Achur et al., 2010; Mayfield, Ferguson, & Harris, 2013). The finding that repeated microinjections of cytokines into brain prior to a single alcohol exposure sensitized withdrawal anxiety is consistent with this view (Breese et al., 2008).

A CRF1RA does not block a stress increase in cytokine mRNAs in controls, but does prevent the CA withdrawal-induced increase in neuroimmune function (Whitman, 2012; Whitman et al., 2013) – findings that suggest distinct mechanisms support these neuroimmune changes induced by the different “stressful” challenges. Thus, released cytokines might not always be involved in the sensitization of anxiety that follows alcohol withdrawal. Nonetheless, the stress-induced return to alcohol seeking was prevented by a CRF1RA in animals (reviewed by Heilig & Koob, 2007). Overall, it seems reasonable to suggest that the CIA exposures and stress engage CRF and neuroimmune function to contribute to the persistent adaptation and the symptomatic consequences observed in animals (Breese et al., 2011; Cui et al., 2015; Koob, 2015).

The present overview clearly associates stress and alcohol withdrawal with CRF and neuroimmune involvement (Breese et al., 2008; Huang et al., 2010; Knapp et al., 2011). It is not known whether altered CRF and neuroimmune function associated with the kindling-like adaptation associated with repeated stresses and withdrawals from CA exposure contributes to the varied symptoms observed in abstinent alcoholics. In this context, Kwako et al. (2015) recently reported that CRF1RA administration to alcohol-dependent individuals after withdrawal did not affect stress or alcohol cues on craving, anxiety, or neuroendocrine responses. Such apparent inconsistencies between the animal and human findings point to the need for further work to elucidate the neural basis of the stress and CIA consequences responsible for the persistent adaptation that supports alcohol abuse.

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Highlights

- Withdrawals from chronic intermittent alcohol exposure involve stress release of CRF.
- CRF involvement in stress substitution for chronic intermittent alcohol withdrawals involves multiple brain sites.
- Withdrawal from chronic alcohol exposure facilitates an increase in cytokine mRNAs that is dependent upon CRF release.
- Brain regions are capable of increasing common cytokine mRNAs by distinct mechanisms.
- Neurocircuitry related to stress is involved in the anxiety and induction of cytokine mRNAs that follow withdrawal from chronic alcohol exposure.

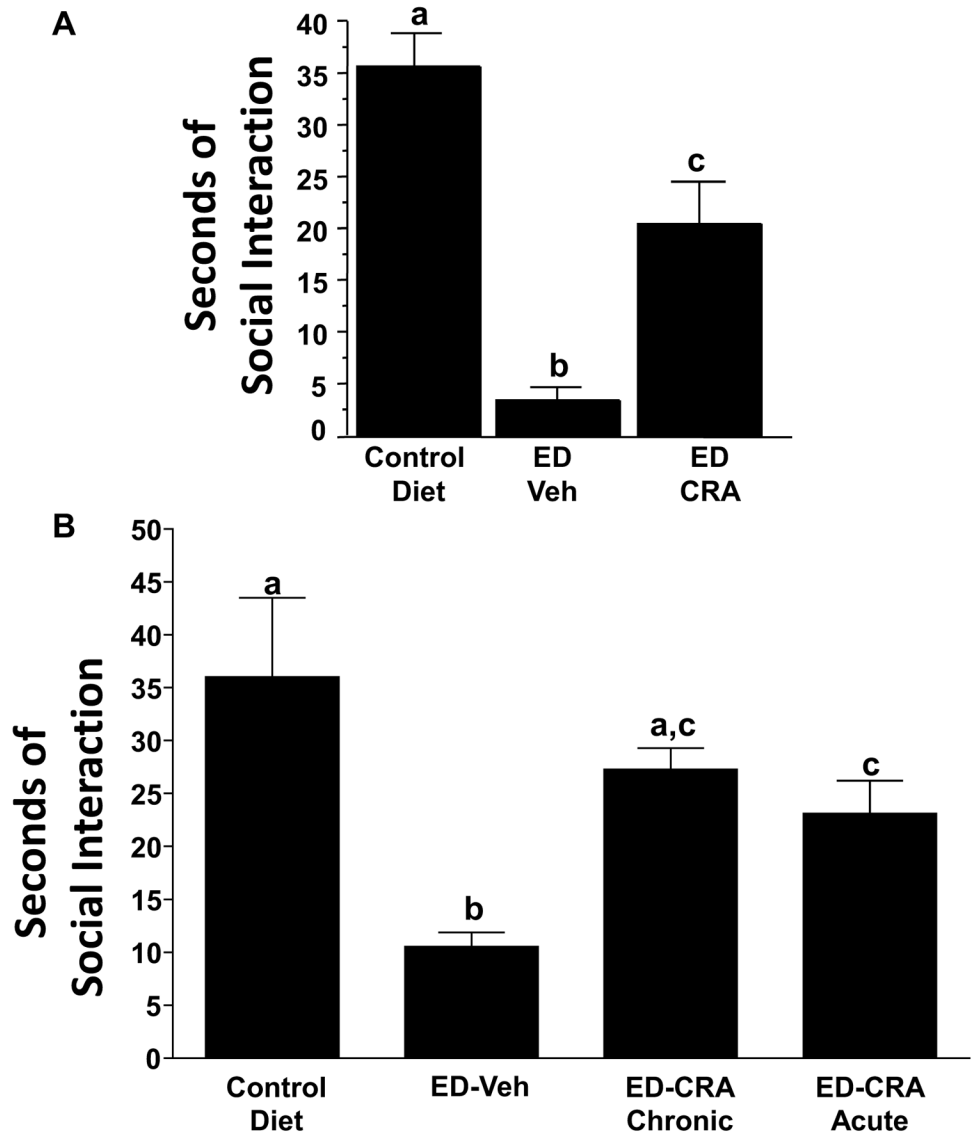

Fig. 1.

Fig. 1A. Mean social interaction time for control and alcohol-withdrawn (ED) rats treated with CRA1000. Rats were treated for 17 days with 7% (w/v) ethanol (alcohol) diet and then tested between 5 and 6 h into withdrawal. CRA1000 was given intra-peritoneally (i.p.) to one ED group 30 min prior to behavioral testing (1 mg/kg). Reduced SI behavior is interpreted as an elevated anxiety-like state. The data represent the mean seconds ± S.E.M. of time spent in SI. A one-way ANOVA showed significant group differences ($p < .01$). The letter(s) above each bar, if different from those appearing above other bars, reflect statistically significant *post hoc* comparisons between those respective bars according to Tukey's test ($p < .01$). CMC = 0.5% solution of carboxymethylcellulose vehicle. Adapted from Knapp et al., 2004.

Fig. 1B. Effects of the CRF1 (corticotrophin releasing factor-1) receptor antagonist CRA1000 (CRA) on social interaction (SI) behavior of rats subjected to repeated withdrawals from alcohol. Rats were exposed to control liquid diets throughout (n = 8) or

to three cycles of 5 days' exposure to an ethanol- (alcohol) containing liquid diet (ED, 7% w/v). The rats were maintained on control diet during the 2 days of withdrawal between the first and second, and the second and third cycles, and between alcohol withdrawal and behavior testing after the third cycle. One group was injected with CMC vehicle 4 h into the first and second withdrawal (ED–Veh), another was pretreated with 3 mg/kg CRA1000 at the same times (ED–CRA Chronic), and the final group was injected acutely with 1 mg/kg CRA1000 30 min before the SI test during the third withdrawal, 4.5 h after alcohol removal (ED–CRA Acute). The other groups exposed to ED were also tested in the SI arena 5 h after removal of alcohol. Error bars, letters above bars, and CMC are described in Fig. 1A above. Adapted from Overstreet et al., 2004.

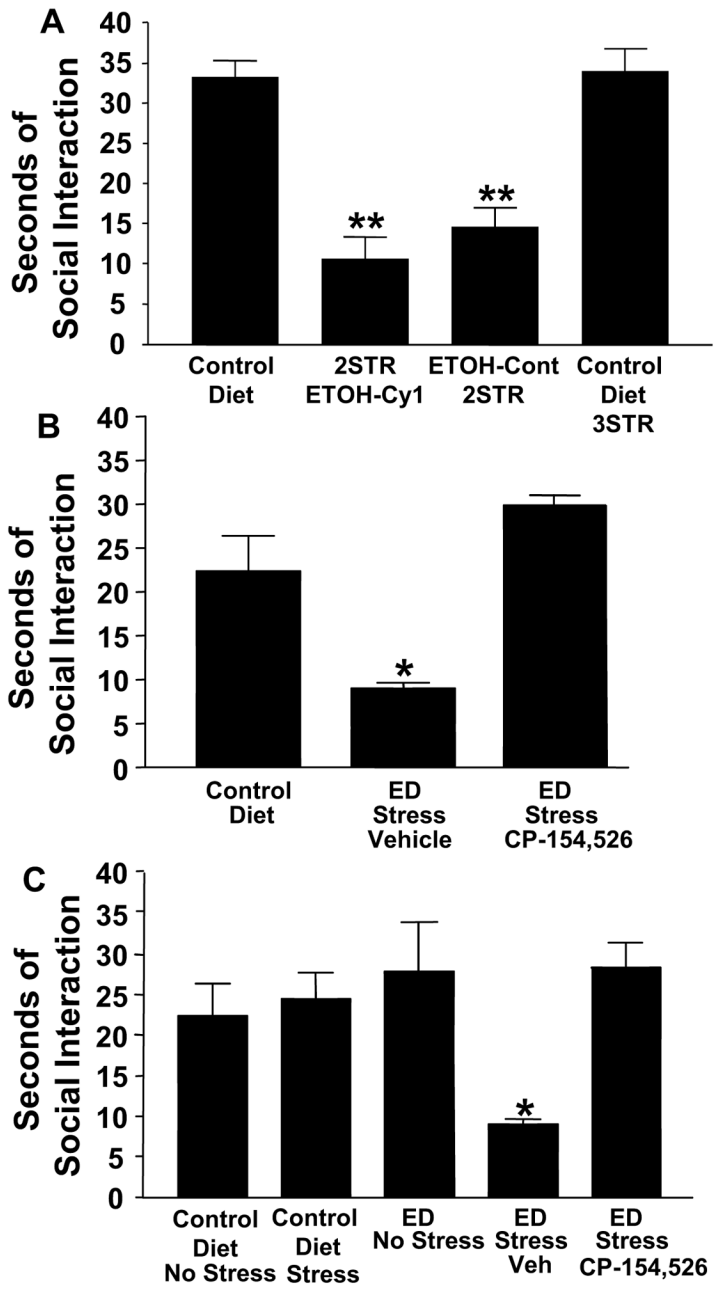

Fig. 2.

Fig. 2A (top). Sensitization of alcohol withdrawal-induced anxiety (reduced social interaction) with repeated stresses. Male rats were exposed to either continuous control diet or to stress applied at 6 and 11 days while on CD followed by a single 5-day cycle of 4.5% ethanol liquid diet (2STR-ETOH-Cy1). Social interaction testing commenced between 5 and 6-h after the ethanol exposure ended.

2STR-ETOH-Cy1 = two stresses + ethanol + withdrawal. Control Diet-3STR = stressed at 6, 11, and 16 days. ETOH-Cont-2STR = stress at 6 and 11 days during continuous (Cont)

ethanol diet + withdrawal. $^{**}p < 0.01$ compared to CD or CD-3STR. Adapted from Breese et al., 2004.

Fig. 2B (middle). Effect of a CRF-1R antagonist administered prior to testing for stress-induced reductions in social interaction behavior at three days into abstinence from repeated alcohol withdrawals. The CRF-1R antagonist CP-154,526 (10 mg/kg) was administered 30 min prior to restraining of rats for 45 min on day 3 after the last of three withdrawals. Vehicle (Veh) was given to rats exposed to ethanol (alcohol) diet and stress (ED Stress Veh). CP-154,526 significantly blocked the deficit induced by stress in repeatedly withdrawn rats ($^{*}p < 0.01$ compared to Control Diet group with no stress). Adapted from Breese et al., 2005.

Fig. 2C (bottom). Effect of a CRF-1R antagonist administered during repeated withdrawals on subsequent behavioral responses to stress three days into abstinence from repeated alcohol withdrawals. The CRF1-receptor antagonist, CP-154,526 (10 mg/kg) was administered during the first two withdrawal periods, but not the final withdrawal, of the multiple withdrawal protocol. Vehicle was given to the group that received alcohol diet and stress (ED Stress Veh). CP-154,526 significantly prevented the reduction in social interaction induced by stress applied three days following the repeated withdrawals. $^{*}p < 0.01$ compared to ED No Stress, Control Diet Stress, and Control Diet No Stress groups and CP-154,526 group. Adapted from Breese et al., 2005.

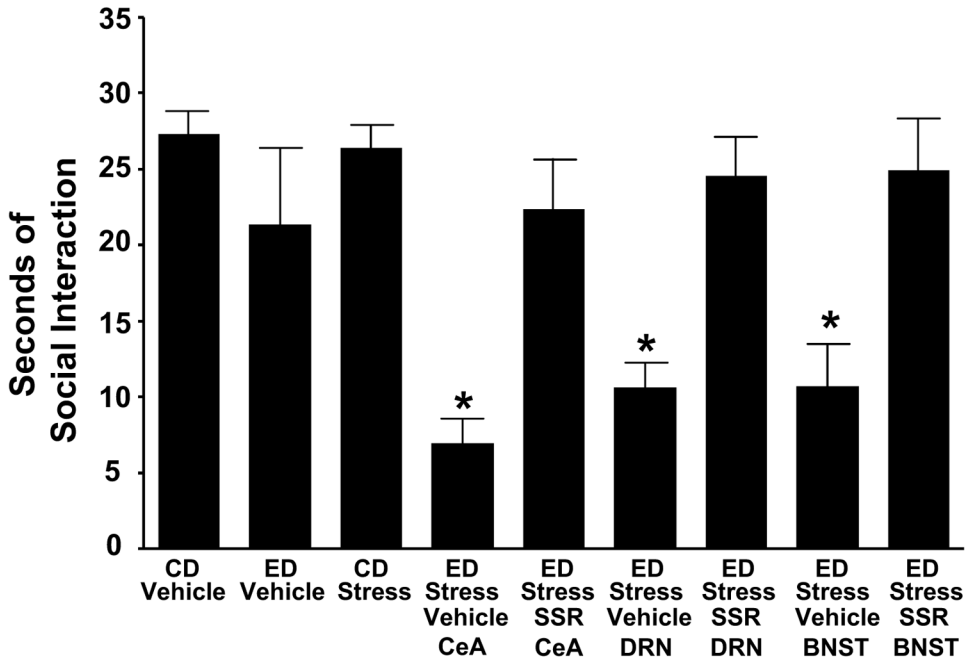

Fig. 3. Microinjection of corticotrophin releasing factor 1 receptor (CRF1R) SSR125543 (SSR) into the CeA, DRN, or d-BNST before restraint stress limits sensitization of alcohol withdrawal-induced anxiety-like behavior in rats

SSR125543 (SSR; 10 μg/0.5 μL) was microinjected into the CeA, DRN, or the d-BNST 15 min before the two weekly 60-min restraint stresses before exposure to 5 days of 4.5% alcohol (ethanol) liquid diet (ED). Social interaction (SI) was measured 5 to 6 h after the alcohol diet removal. The anxiogenic effect of stress on alcohol withdrawal-induced anxiety was blocked by the SSR microinjected in the selected brain sites. For the control diet- (CD) vehicle and ED-vehicle groups, vehicle was administered into each of the brain sites (n = 3–6 for each site). The vehicle data for each of these controls were combined because a significant change across sites was not observed. No significant effect on SI ($p > 0.05$) was observed during alcohol withdrawal when the CD-vehicle group was compared with the ED-vehicle group. *$p < 0.001$ compared with the CD-vehicle, ED-vehicle, and CD stress groups as well as the groups that received the CRF-1 receptor antagonist before the repeated stresses. [$F(8,75) = 7.385$, *$p < 0.001$]. CeA = central amygdala; DRN = dorsal raphe nucleus;

d-BNST = dorsolateral bed nucleus of the stria terminalis. Adapted from Huang et al., 2010.

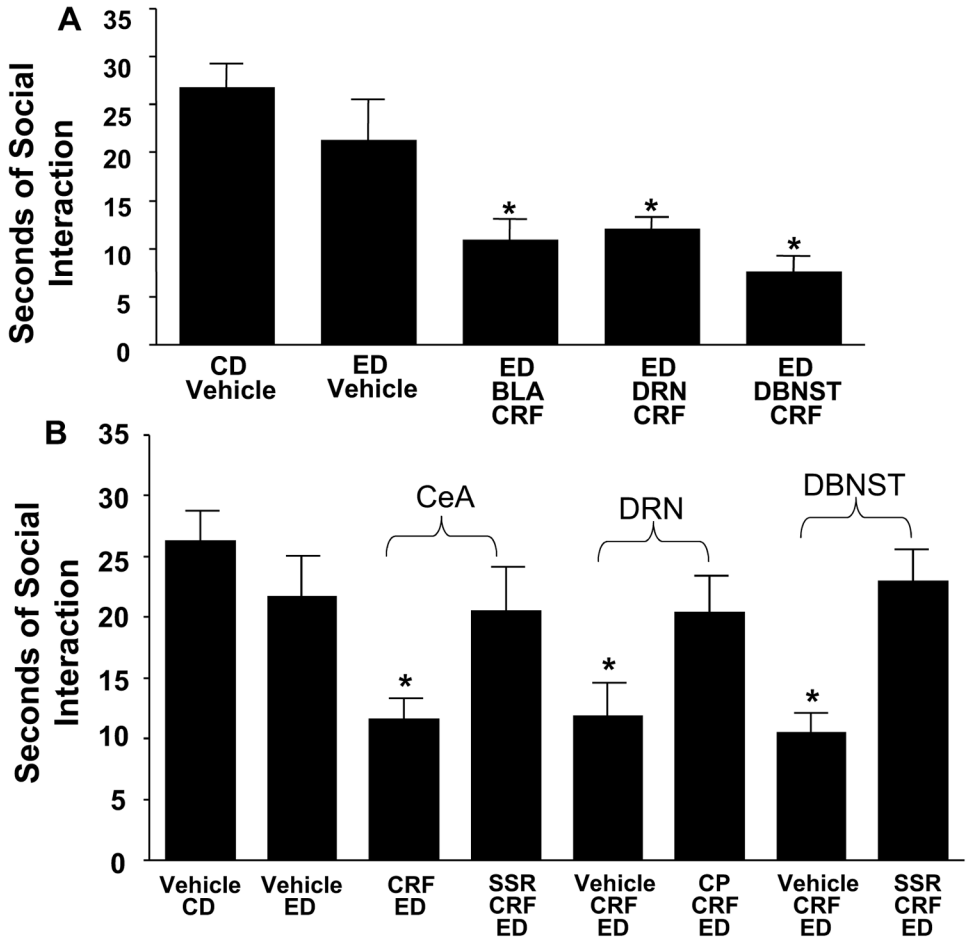

Fig. 4.

Fig. 4A. Effects of repeated corticotrophin releasing factor (CRF) into the basolateral amygdala (BLA), dorsal raphe nucleus (DRN), and dorsolateral bed nucleus of the stria terminalis (d-BNST) before exposure to chronic alcohol liquid diet reduces social interaction (SI) behaviors during withdrawal. CRF (0.5 μg/0.5 μL) was microinjected twice, once per week, into the BLA, DRN, or d-BNST before exposure to 5 days of 4.5% ethanol (alcohol) liquid diet (ED). In the Control Diet (CD)-vehicle and ED-vehicle groups, vehicle was administered into each of the brain sites (n = 3–4 for each site), and data for these vehicle injections were combined because a significant difference in SI across sites was not observed across these groups. No significant difference was found for the SI between the CD-vehicle and ED-vehicle groups ($p > 0.05$). A group that received CRF and was on CD only was not included for each of the present sites because previous data demonstrated that intracerebroventricular administration of CRF to rats that received CD sensitize SI deficits (Overstreet et al., 2004), and the repeated CRF in the CeA of control diet-treated animals likewise did not sensitize withdrawal-induced anxiety. SI was measured 5 to 6 h after the ED removal. *Significantly different from CD-vehicle and ED-vehicle [$F(4,42) = 9.227$, $p < 0.001$].

Fig. 4B. CRF-1 receptor antagonist blockade of CRF-sensitized alcohol withdrawal-induced anxiety-like behavior when CRF is injected into the central amygdala, dorsal

raphe, or dorsolateral bed nucleus of the stria terminalis. A 10 mg/kg dose of the CRF-1 receptor antagonist, SSR125543 (SSR), was administered i.p. 15 min before the microinjection of CRF (0.5 μg) into either the central amygdala or the d-BNST followed by the 5 days of ethanol (alcohol) diet. Another CRF-1 receptor antagonist CP154526 (CP; 10 mg/kg i.p.) was administered 15 min before each of the CRF (0.5 μg) microinjections into the dorsal raphe. CRF in these brain sites sensitized social interaction deficits (reduced SI), and this effect was prevented by the CRF-1 receptor antagonists. SI was measured 5 to 6 h after the removal of ED. In the CD-vehicle group and the ED-vehicle group, vehicle was administered into each of the brain sites (n = 4–6 for each site), and data were combined because a significant change across sites was not observed. No significant difference in SI ($p > 0.05$) was observed when the CD-vehicle group was compared with the ED-vehicle group. *$p < 0.01$ compared with vehicle CD- and vehicle ED-treated groups and the groups that received the SSR125543 systemically [$F(7,76) = 3.005$, $p < 0.01$]. Adapted from Huang et al., 2010.

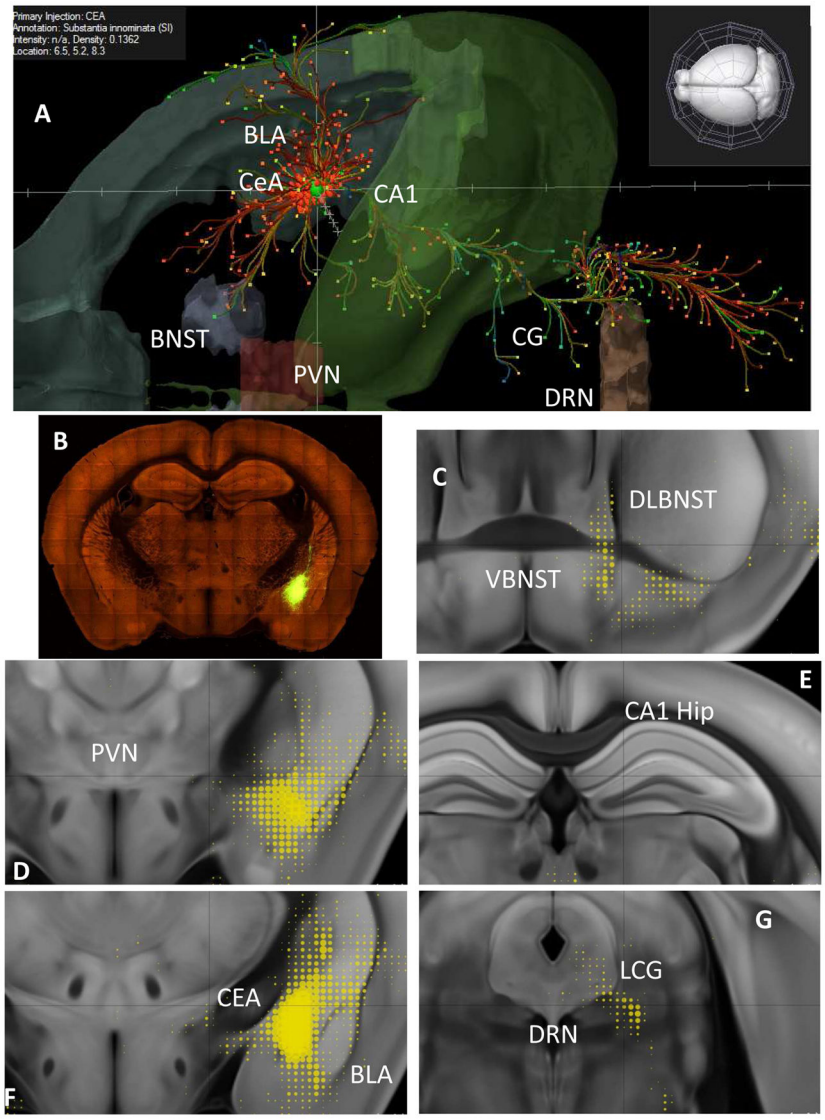

Fig. 5. Anatomical depictions of brain sites where CRF actions influence alcohol withdrawal-induced anxiety

Positive sites (central nucleus of the amygdala, CeA; basolateral nucleus of the amygdala, BLA; dorsolateral bed nucleus of the stria terminalis, DLBNST; and dorsal raphe nucleus, DRN) and negative sites (VBNST, PVN, and hippocampus) identified in Huang et al. (2010) are overlaid on mouse images derived from the Allen Mouse Brain Connectivity Atlas (2015, Allen Institute for Brain Science; http://connectivity.brain-map.org) and Oh et al., 2014. Above, the reference animal was an erbb4-2A-creERT2 mouse (Madisen et al., 2010; see also Bi et al., 2015) injected with virus (89-nanoliters) that led to expression in GABAergic neurons of the CeA (Bregma: −0.94, 2.43, 4.47) (Bi et al., 2015). **A.** CeA projection patterns customized from the Mouse Connectivity Atlas and extending anteriorly in the extended amygdala, laterally toward nearby cortex, and posteriorly into the brain stem. **B**. Serial two-photon tomography of the CeA injection site. **C.** Ventral and dorsal BNST projections (yellow; dot sizes reflect relative density of innervation of that region as per the Composite Projection Viewer). **D.** Absence of PVN projections. **E.** Absence of

hippocampal projections. **F.** CeA injection site and local/regional projections. **G.** DRN and lateral central gray (LCG) projections.

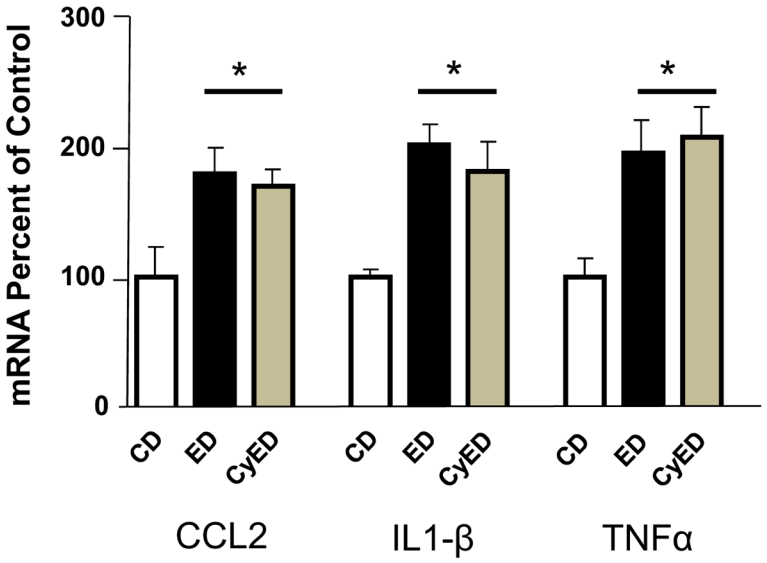

Fig. 6. Comparison of chronic 7% dietary ethanol (alcohol) (ED, 15 days continuous diet) and chronic intermittent/cycled alcohol diet (ED, three 5-day cycles with 2 days of control liquid diet (CD) between cycles) on cytokine mRNAs in brain cortex

Twenty-four hours into withdrawal from ED, both the chronic continuous ED and cycled ED treatment groups had elevated CCL2, IL1β, and TNFa mRNA, $F(2,18) = 5.211$, $p = 0.018$; $F(2,24) = 9.718$, $p = 0.0009$; $F(2,25) = 6.475$, $p = 0.006$, respectively. No significant difference was found between the cytokine mRNAs for the two chronic ethanol-treated groups relative to each other ($p > 0.05$). *Significantly different from CD ($p < 0.05$). CCL2, chemokine(C-Cmotif) ligand 2; IL1β, interleukin-1β, TNFα, tumor necrosis factor-α; CyED = chronic intermittent/cycled alcohol. Diet: ED = chronic continuous alcohol diet. Adapted from Whitman et al., 2013.

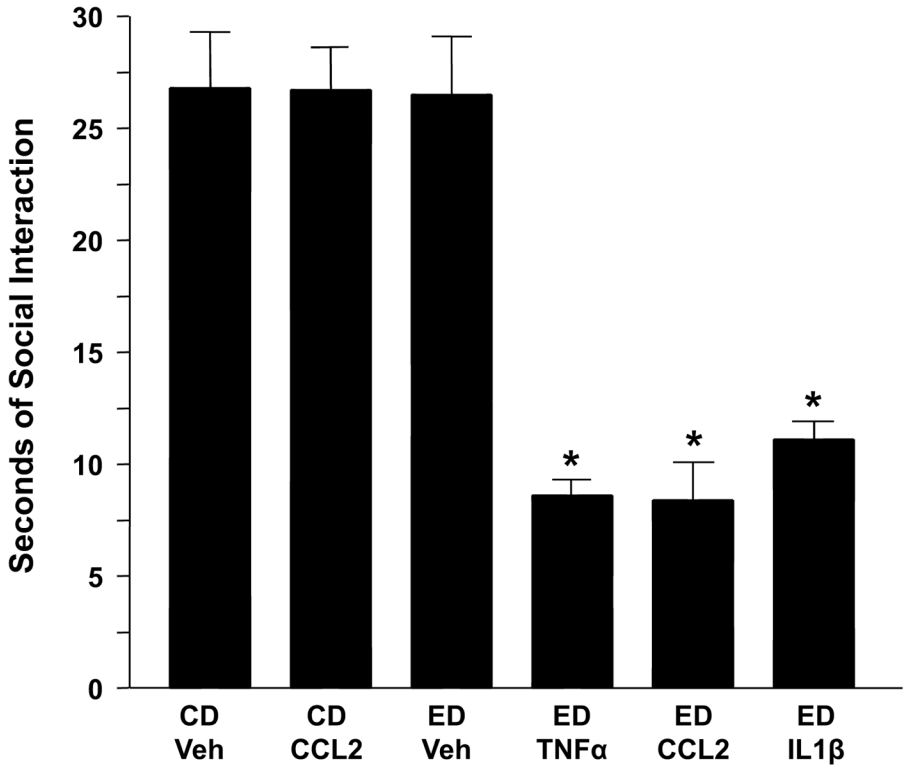

Fig. 7. Repeated IL1β, TNFα, or CCL2 sensitize alcohol withdrawal-induced anxiety-like behavior

Rats were injected twice at weekly intervals with either vehicle or cytokine (IL1β, TNFα, or CCL2; 100 ng/5 μL, intracerebroventricularly [i.c.v.]) while drinking control diet (CD) and then continued on CD or switched to a 4.5% alcohol (ethanol) diet (ED) for 5 days. MCP1 was also given twice in a group exposed only to CD to test for an effect on social interaction (SI) deficits (the operational definition of anxiety-like behavior) without prior ED exposure. SI for all groups was measured 5–6 h into withdrawal. CCL2 = CC chemokine ligand 2 (formerly MCP-1 = monocyte chemo-attractant protein-1); IL1β = interleukin-1β; TNFα = tumor necrosis factor-α;

CD = control diet; Veh = artificial cerebrospinal fluid. *$p < 0.001$ compared to CD-Veh or ED-Veh groups. Adapted from Breese et al., 2008.