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The Center of the Emotional Universe: alcohol, stress, and CRF1 amygdala circuitry

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Alcohol is the most ubiquitous recreational drug in the world, causing more than three million global deaths in 2012 (World Health Organization, 2014). Anxiety and stress disorders are highly comorbid with alcohol abuse (Lai *et al*, 2015), suggesting the possibility of common underlying mechanisms or related circuitry dysfunction. The close relationship between substance use, which acutely elicits feelings of euphoria, and the more unpleasant effects of stress, anxiety, or withdrawal from drug exposure reflects an important aspect of emotional information processing in the brain: the same circuitry can underlie both rewarding and aversive states. Consistent with this idea, the general experience of stress can also be either rewarding or aversive, depending on the type and duration of stress experienced and the state of the organism. The transition from alcohol use to alcohol abuse is characterized by a change in motivational state from drug reward-dependent intake early in alcohol use to intake motivated by withdrawal-induced aversion as the organism becomes increasingly dependent upon alcohol (Koob and Le Moal, 2001). Investigating the common cellular mechanisms of alcohol and stress in the brain allows for the dissection of specific circuits that underlie seemingly oppositional hedonic states and may provide insight into inherent vulnerability and/or new treatment directions for alcohol use and stress-related disorders as well as unique strategies for understanding their comorbidity.

The amygdala has emerged as a key brain region in the ontogeny of both alcoholism and stress/anxiety disorders. The amygdala is activated by alcohol-related cues in human participants (Dager *et al*, 2013) and by acute alcohol in rodent models (McBride, 2002), and has been shown to functionally regulate alcohol self-administration (Koob, 2003). Withdrawal from alcohol is associated with both changes in amygdala activity and anxiety-related behaviors (McCool, 2011; Overstreet *et al*, 2004). The relationship between fear/anxiety and the amygdala is robust. In both naïve rodents and healthy human participants, amygdala activity is associated with fear-paired cues during fear conditioning (Duvarci and Pare, 2014). In the context of anxiety disorders, amygdala activity both at rest and in response to anxiety-associated cues is enhanced in patients with post-traumatic stress disorder (PTSD), and similar amygdala responsivity has been observed in other anxiety

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disorders in humans (see VanElzakker et al., 2014 for review). The amygdala is therefore a region where alcohol and anxiety-associated circuitry converge and is closely associated with the behavioral manifestations of both disorders.

Elucidating the specific cellular mechanisms that underlie the role of the amygdala in alcoholism, anxiety and their comorbidity has been complicated by the heterogeneous nature of the amygdala. Comprising multiple subnuclei, each with a diversity of cell types and projections to and from other brain regions, the specific role of the amygdala in emotional or disease-related states depends upon the region and cell population. With the advent of transgenic mice, chemo- and optogenetics, and more precise tools for molecular mapping of individual neurons, the role of discrete neural circuits and cell populations within the amygdala is coming into focus. These techniques have indicated a critical role for cell type-specific neuroadaptations within the amygdala in the effects of both acute and chronic alcohol and anxiety-related behaviors. Precise local and projection-based control of specific neuronal populations is emerging as a key regulator of intra-amygdala activity, amygdala output, and behavior.

The Amygdala Complex

The amygdala complex is composed of distinct nuclei and subdivisions including the lateral (LA), basolateral (BL) and basomedial (BM) amygdala, the medial (Imp), lateral paracapsular (lpc) and main intercalated cell cluster (IN) and the lateral and medial central amygdala (CeA) (Pitkanen and Amaral, 1994; Sah *et al*, 2003). The main flow of information follows a lateral-to-medial path, with sensory information from the thalamus and cortex entering the amygdala by afferents synapsing in the LA, which sends glutamatergic projections throughout the amygdala including to the BM, lateral CeA, and to the Imp. The BM then sends glutamatergic projections to the medial CeA, while the Imp sends GABAergic projections to the lateral CeA, which forms an inhibitory microcircuit with the medial CeA. Both the lateral and medial CeA function as major output nuclei, sending projections to downstream regions regulating rewarding and/or aversive behaviors. The BLA is primarily composed of glutamatergic neurons that are under precisely regulated inhibitory control by lpc neurons and local interneurons (Marowsky *et al*, 2004; McDonald, 1982; McDonald and Betette, 2001; Muller *et al*, 2003). Although interneurons make up a small percentage of BLA neurons, the multiple levels of inhibitory control of pyramidal neurons demonstrate that interneurons have significant effects on output of the BLA. In contrast, the CeA is predominately GABAergic cells, (McDonald, 1985; McDonald and Augustine, 1993), which comprise the vast majority of both projection neurons and interneurons.

The circuitry of the amygdala has been implicated in a number of important brain functions, but is best known for its role in emotional processing, specifically in the interpretation of emotionally-relevant stimuli or the attachment of emotional relevance to otherwise neutral stimuli (Pitkanen *et al*, 1994). In particular, the amygdala nuclei mediate the negative association processing behind the acquisition and expression of conditioned fear (for review see Ehrlich 2009) and recent evidence suggests that amygdala microcircuits are also critical for the coding of both positive and negative-valence cues (Beyeler *et al*, 2018; Beyeler *et al*,

2016). The high degree of interconnectivity in the amygdala underlies the complex information processing that takes place as multi-modal input is received, integrated and processed to produce relevant behavioral output.

CRF and Amygdala Microcircuitry

Corticotropin releasing factor (CRF) is a neuropeptide that has a well-established role in the stress response of the mammalian central nervous system. CRF is one of the principal activators of the hypothalamic-pituitary-adrenal (HPA) axis, which is responsible for a host of physiological and behavioral responses to stress, including alterations in heart rate, blood flow, reactivity, locomotion and motivated behavior (Keller *et al*, 2006). CRF activation produces behaviors similar to those seen following acute and chronic stress. Transgenic mice with constitutive overproduction of CRF exhibit reduced locomotor activity in a novel environment and decreased time spent in the open arm of the elevated plus maze, consistent with an anxiogenic phenotype, an effect which was reversed with the intra-ventricular infusion of a CRF antagonist (Stenzel-Poore *et al*, 1994). Similarly, intraventricular infusion of CRF produces a variety of pro-anxiety behavioral responses in rodent models, including novelty reactivity (Britton *et al*, 1982), social conflict (Britton *et al*, 1985), acoustic startle (Swerdlow *et al*, 1986), and reduced time in the open arms on an elevated plus maze (Adamec *et al*, 1991). In human participants, CRF levels in the cerebrospinal fluid are elevated in patients with anxiety disorders relative to healthy controls (Baker *et al*, 1999; Bremner *et al*, 1997). Two G-protein coupled receptors for CRF have been identified in the mammalian brain, CRF-1 and CRF-2. CRF and the CRF receptors are expressed throughout the amygdala (Van Pett *et al*, 2000) and have been implicated in neuroplastic changes related to fear (Hubbard *et al*, 2007), anxiety (Overstreet *et al*, 2004; Rainnie *et al*, 2004) and alcohol exposure (Herman *et al*, 2013a; Lovinger and Roberto, 2013; Nie *et al*, 2004; Roberto *et al*, 2010).

Within the BLA, both CRF and the CRF1 receptor have been shown to functionally regulate synaptic activity. *In vivo*, CRF release into the BLA originates from projections from the CeA (Roozendaal *et al*, 2002). Central administration of both CRF and CRF1 agonists have been shown to activate the BLA and produce anxiogenic behavioral responses (Dube *et al*, 2000; Rainnie *et al*, 2004). Exogenous application of CRF peptide in BLA slices likewise increased the excitability of neurons (Ugolini *et al*, 2008), an effect which was diminished following chronic unpredictable stress and shown to be mediated via the CRF1 receptor (Sandi *et al*, 2008). Microinjection of CRF specifically within the BLA activated CaMKII-containing projection neurons but not GAD-containing interneurons (Rostkowski *et al*, 2013). Chronic administration of the CRF1 and CRF2 agonist urocortin produced long-lasting anxiety responses that required NMDA receptor and CaMKII activation and produced hyperexcitability of the BLA network due to reduced local inhibition (Rainnie *et al*, 2004; Sajdyk *et al*, 1999). Together, these findings indicate a general enhancement of excitatory synaptic transmission in the BLA following acute *in vivo* and *ex vivo* application of CRF that is blunted by chronic stress and chronic CRF activation.

The CRF system also plays an important role in the regulation of synaptic transmission in the central amygdala. Although the CeA receives input from CRF+ projections originating

in the paraventricular hypothalamus (Hernandez *et al*, 2015) and the bed nucleus of the stria terminalis (Gungor *et al*, 2015), CRF function in the CeA is also driven by local microcircuitry (Sanford *et al*, 2017). CRF and GABA are colocalized within the CeA (Day *et al*, 1999), and CRF has been to shown to regulate GABAergic neurotransmission in this brain region. Exogenous application of CRF to CeA slices increases presynaptic GABA release in a CRF1, but not CRF2, -dependent manner (Kang-Park *et al*, 2015; Nie *et al*, 2004; Roberto *et al*, 2010). The effects of CRF on inhibitory neurotransmission in the CeA are regulated in part by protein kinase C-epsilon (PKCε); PKCε knockout mice exhibited increased baseline GABAergic tone in the CeA but were not responsive to the acute effects of exogenous CRF application, and PKCε inhibition occluded the effects of acute CRF in wild type mice (Bajo *et al*, 2008; Blasio *et al*, 2017). CRF has also been shown to alter excitatory transmission within the CeA. CRF application reduced evoked glutamate responses and enhanced glutamate release in the CeA of Sprague-Dawley rats (Liu *et al*, 2004; Varodayan *et al*, 2017) and C57BL/6 mice (Silberman *et al*, 2013a). In Wistar rats, CRF application had divergent effects on glutamatergic signaling, with a subset of CeA neurons exhibiting enhanced glutamate release following exogenous CRF application and a subset exhibiting reductions in glutamate release (Herman *et al*, 2016c).

Despite the expression of CRF and CRF1 in the amygdala and the relevance of the CRF system to behavioral conditions involving amygdala circuitry, how and where CRF1 fits into the known circuitry remains unclear. Previous technical limitations related to identifying CRF1 prevented the direct examination of CRF1-containing neurons in the amygdalar subnuclei and their place in the larger circuitry of the amygdala complex. This limitation had previously confounded the integration of cellular and behavioral data in determining the effects of CRF on specific circuitry within the amygdala and how alterations in the activity of that circuitry lead to adverse behavioral outcomes.

Previous limitations in identifying and targeting CRF1 neurons in the amygdala were overcome by the development of a BAC transgenic mouse line expressing green fluorescent protein (GFP) in CRF1-containing neurons (Justice *et al*, 2008). Immunohistochemical examination of GFP expression in the amygdala complex in this mouse indicates that CRF1 neurons makeup discrete cell populations with restrcied expression in discrete amygdala sub-nuclei (Figure 1A). Earlier work by our group utilized this transgenic mouse model approach to examine inhibitory transmission in CRF1-containing (CRF1+) and unlabeled (CRF1−) neurons in the CeA. We validated this model to establish that GFP expression was a reliable indicator of CRF1+ neurons in the amygdala and found that CRF1+ and CRF1− neurons exhibited distinct inhibitory characteristics. CRF1+ CeA neurons exhibited a significant ongoing tonic conductance mediated by $GABA_A$ receptors containing the α1 subunit. In contrast, unlabeled (CRF1−) CeA neurons possessed the potential for a tonic conductance mediated by δ subunit-containing $GABA_A$ receptors (Herman *et al*, 2013a). Paired recordings demonstrated that CRF1− neurons form local inhibitory synapses onto CRF1+ neurons, a portion of which project out of the CeA into the bed nucleus of the stria terminalis (Herman *et al*, 2016a). The specific role of CRF1+ and CRF1− cells in the CeA suggests that CRF1+ neurons may make up distinct neuronal populations in other amygdala nuclei, including the BLA, and that CRF1 neurons may display distinct patterns of inter- and intra-amygdalar connectivity (Figure 1B).

Alcohol and the Amygdala

Alcohol and GABAergic transmission

Although alcohol is known to engage many brain systems, GABAergic transmission is particularly sensitive to alcohol and is involved in the actions of acute alcohol as well as alcohol tolerance and dependence (Eckardt *et al*, 1998; Grobin *et al*, 1998). There are two main types of $GABA_A$ receptor transmission that function in a cell- and region-specific manner. Phasic signaling involves the generation of inhibitory postsynaptic currents (IPSCs) that are the result of 'point to point' transmission that occurs at synapses and results in 10 – 100 ms of inhibition. Tonic signaling is characterized by the presence of persistent inhibitory currents that are the result of low levels of ambient GABA acting at highly sensitized extrasynaptic $GABA_A$ receptors (for review, see Brickley and Mody, 2012; Belelli *et al*. 2009;Glykys and Mody, 2007). The functional characteristics of $GABA_A$ receptors are determined by the subunit composition. Previous research suggests that extrasynaptic $GABA_A$ receptors containing the α4, α6, and/or δ subunit have an increased sensitivity to alcohol (Wallner *et al*, 2003; Wei *et al*, 2004), prompting some researchers to suggest that tonic receptors are the primary target for alcohol in the brain (Mody *et al*, 2007; Wallner *et al*, 2003), although the direct action of alcohol on extrasynaptic $GABA_A$ receptors remains controversial (Baur *et al*, 2009; Borghese and Harris, 2007; Botta *et al*, 2007).

Alcohol and the Basolateral Amygdala

Considering the known anxiolytic properties of acute alcohol (Koob and Britton, 1996; Kushner *et al*, 2000) and the increased anxiety phenotype associated with alcohol withdrawal (Menzaghi *et al*, 1994), it is not surprising that the BLA has been implicated in both the behavioral and cellular effects of acute and chronic alcohol. Previous studies have shown that glucose metabolism is increased in the BLA following alcohol self-administration in rats (Porrino *et al*, 1998), and more recently it has been shown that inactivation of the BLA disrupts the renewal of conditioned Pavlovian-alcohol seeking (Chaudhri *et al*, 2013) and inhibition of BLA pyramidal neurons by a serotonin type-2 receptor agonist suppressed alcohol seeking behavior (McCool *et al*, 2014). In addition, cellular studies have demonstrated that synaptic activity in the BLA is altered by both acute and chronic alcohol. Acute alcohol enhances both basal and evoked inhibitory currents in BLA pyramidal neurons (Samson *et al*, 2003; Zhu and Lovinger, 2006) from two distinct inhibitory pathways, the feed-forward inhibition provided by lpc neurons and the feedback inhibition provided by local interneurons (Silberman *et al*, 2008), although the effects of alcohol on local inhibitory transmission display an acute tolerance involving the $GABA_B$ receptor (Silberman *et al*, 2009; Silberman *et al*, 2008; Zhu *et al*, 2006). In contrast, acute alcohol has relatively minor effects on AMPA and NMDA-receptor mediated glutamatergic transmission, although it does decrease kainate receptor-mediated excitatory transmission onto BLA pyramidal neurons (Lack *et al*, 2009). Consistent with the effects on inhibitory and, to a lesser extent, the effects on excitatory transmission, acute alcohol has also been shown to decrease the firing of BLA pyramidal neurons projecting to the nucleus accumbens (Perra *et al*, 2008), possibly through increased inhibitory input and/or decreased excitatory input.

In contrast to the minimal effects of acute alcohol on glutamatergic transmission in BLA pyramidal neurons, chronic intermittent alcohol (CIE) and withdrawal (WD) produced anxiety behavior as well as significant increases in excitatory transmission mediated by both AMPA and NMDA receptors (Lack *et al*, 2007) and CIE, but not WD, increased kainate receptor-mediated glutamatergic transmission (Lack *et al*, 2009). Notably, CIE only produced increases in *spontaneous* action potential-dependent excitatory transmission, suggesting that CIE alters the activity of local intra-BLA glutamatergic neurons (Lack *et al*, 2008). The specific effects of CIE on BLA function may also vary based on synaptic connections; at BLA cells receiving input from the external capsule, CIA enhanced glutamatergic activity in a postsynaptic fashion, whereas cells connecting to stria terminalis afferents exhibited presynaptic enhancement of glutamate release (Morales *et al*, 2018). CIE and WD have also been shown to produce long-lasting structural and functional changes in inhibitory BLA circuitry. CIE increased the functional expression of $GABA_A$ receptor currents in BLA neurons and no tolerance to the acute effects of alcohol was observed (Diaz *et al*, 2011; McCool *et al*, 2003). CIE and WD also significantly dampened evoked feed-forward inhibitory input from distal lpc neurons, but did not alter action potential-independent presynaptic GABA release from local interneurons onto BLA pyramidal neurons (Diaz *et al*, 2011). CIE and WD also decreased total and surface levels of the α1 subunit and increased surface α4 subunit expression at lpc synapses, which was accompanied by decreased zolpidem sensitivity (Diaz *et al*, 2011).

Tonic inhibition has been described in principal cells of the BLA (McCool *et al*, 2003; Olmos-Serrano *et al*, 2010) and principal cells as well local interneurons in the LA (Marowsky *et al*, 2012). The tonic inhibition in the BLA is mediated by α3-subunit containing $GABA_A$ receptors but the receptor composition of neurons in the LA is unknown (Marowsky *et al*, 2012). The α1 $GABA_A$ receptor subunit displays selective expression in the LA (Marowsky *et al*, 2004; Wiltgen *et al*, 2009) and plays a critical role in LA plasticity and auditory fear learning (Wiltgen *et al*, 2009). The α4 and δ subunits are expressed in the LA and BLA, although expression is sparse (Pirker *et al*, 2000). Chronic alcohol has been shown to produce changes in α1 and α4 $GABA_A$ receptor subunit trafficking and expression in BLA neurons (Diaz *et al*, 2011) and chronic alcohol and withdrawal have been shown to produce changes in the surface expression of multiple $GABA_A$ receptor subunits as well as functional changes in the sensitivity to acute alcohol exposure in BLA neurons (Lindemeyer *et al*, 2014). However, alterations in tonic inhibitory conductance and subsequent changes in the excitability of distinct neuronal populations (i.e., CRF1-containing BLA neurons) following chronic alcohol exposure remains to be studied. As tonic signaling is a critical determinant of overall network activity and tone (Semyanov *et al*, 2004), differential tonic conductance in discrete populations of amygdala neurons would allow for multiple levels of network regulation and multiple sites for potential dysregulation by alcohol.

Much of the previous work on the effects of alcohol has focused on synaptic transmission in BLA pyramidal neurons, as the major source of output from the BLA. These studies provide compelling evidence of the dysregulating effects of alcohol on pyramidal BLA neurons, however the effects of acute and chronic alcohol on other BLA subpopulations including interneurons and local intra-BLA projection neurons that connect distinct BLA subnuclei (e.g. LA and BM) remains unclear. The BLA is known to have a heterogeneous population

of interneurons that make synaptic connections with each other as well as purported pyramidal neurons (Muller *et al*, 2003) and provide important feed-forward and feedback inhibitory control of pyramidal neurons (Muller *et al*, 2003; Zhu *et al*, 2006). The contribution of alcohol-induced alterations in this inhibitory control on changes in pyramidal neuron activity in the BLA remains understudied. Recent work in the central amygdala highlights the importance of local inhibitory microcircuits in the processing of fear acquisition and expression (Ciocchi *et al*, 2010; Haubensak *et al*, 2010), but it is not clear if a similar system of local inhibitory microcircuitry is present in the BLA and if or how it contributes to the effects of acute and chronic alcohol.

Alcohol and the Central Amygdala

As the main output region of the amygdala, the CeA sends projections to brain regions known to mediate the rewarding/consummatory aspects of alcohol use (such as the nucleus accumbens) as well as structures that have been implicated in alcohol dependence and withdrawal (such as the bed nucleus of the stria terminalis). Connectivity with both of these critical limbic regions may underlie the role that the central amygdala plays in both hedonic and anhedonic aspects of alcohol abuse. In postmortem CeA tissue collected from human participants, AMPA, NMDA and $GABA_A$ receptor mRNA was altered in participants with a history of alcoholism (Jin *et al*, 2014). Similar alterations in AMPA (Cannady *et al*, 2017; Salling *et al*, 2016), NMDA (Roberto *et al*, 2006) and $GABA_A$ (Freeman *et al*, 2013) receptors have been observed in rodent models. Animal studies have also been used to establish a functional role for the CeA in alcohol self-administration behavior. Lesions to the CeA (Moller *et al*, 1997) and 5HT-3 antagonists microinjected into the CeA (Dyr and Kostowski, 1995) reduce voluntary alcohol consumption in non-dependent animals. Similarly, intra-CeA modulation of GABA signaling reduces operant alcohol self-administration in models of alcohol dependence (Hyytia and Koob, 1995; Roberts *et al*, 1996).

Like the BLA, the CeA has also been shown to respond to the presence of alcohol at the level of synaptic transmission. Acute alcohol enhances GABA signaling in CeA slices from alcohol-naïve rats via both presynaptic enhancement of GABA release and postsynaptic alterations in $GABA_A$ receptor function (Roberto *et al*, 2003). In terms of excitatory signaling, acute alcohol has been shown to increase spontaneous glutamate transmission in mice (Silberman *et al*, 2015; Silberman and Winder, 2013b), but suppress both action-potential dependent endogenous glutamatergic signaling and evoked glutamatergic transmission in rats (Roberto *et al*, 2004b; Zhu *et al*, 2007). These alterations in glutamatergic signaling are accompanied by mRNA and protein changes in subunits of the glutamatergic NMDA receptor (Roberto *et al*, 2006). However, alcohol-induced suppression of glutamatergic transmission is intact in the presence of NMDA receptor antagonists, indicating that non-NMDA glutamate receptors (presumably the AMPA receptor) also contribute to this effect (Zhu *et al*, 2007).

Models of chronic alcohol exposure have also identified significant adaptations in the CeA. Chronic alcohol exposure increases concentrations of glutamate in the CeA at baseline, and causes enhanced glutamate release into the CeA following acute alcohol infusion as

compared with naïve rats (Roberto *et al*, 2004b), in contrast to the general suppression of glutamatergic signaling seen following acute alcohol exposure. These changes are accompanied by alterations in the NMDA receptor GluN2Bsubunit (Roberto *et al*, 2006). Interestingly, the effects of chronic alcohol exposure on inhibitory neurotransmission in the CeA appear to be similar to those of acute alcohol. Chronic alcohol consumption enhances presynaptic GABA release in the rat CeA and may also enhance GABAergic activity via postsynaptic mechanisms (Roberto *et al*, 2004a). The similarity of this effect following both acute and chronic alcohol exposure suggests a lack of tolerance to alcohol-induced enhancement of GABAergic activity (Roberto *et al*, 2012). These changes in GABAergic transmission are accompanied by alterations in $GABA_A$ receptor subunit expression in the amygdala as a whole (Papadeas *et al*, 2001), although cell type-specific alterations within the CeA remain to be explored. Alterations in $GABA_A$ mRNA have been reported in the CeA following withdrawal from chronic alcohol (Freeman *et al*, 2013). Tonic GABA conductance has also been reported in the CeA, with alcohol inducing a tonic current in CeA slices in a cell-type specific manner in both rats and mice (Herman *et al*, 2013a; Herman and Roberto, 2016b). Together, these findings suggest a general enhancement of GABAergic signaling in the CeA following both acute and chronic alcohol exposure.

Alcohol and the CRF1 System

Given the marked effects of alcohol on inhibitory neurotransmission in the amygdala and the regulation of amygdalar GABAergic activity by CRF and activity at the CRF1 receptor, the CRF system is a logical site for the actions of alcohol in this region. Human studies have indicated a potential link between *crhr1*, the human gene for the CRF1 receptor, polymorphisms and risk for developing AUD (Glaser *et al*, 2014; Ribbe *et al*, 2011; Treutlein *et al*, 2006). Indeed, withdrawal from alcohol is associated with both increases in anxiety-like behavior as well as increases in extracellular CRF concentration in the amygdala (Funk *et al*, 2006; Merlo Pich *et al*, 1995; Zorrilla *et al*, 2001). Chronic models of alcohol exposure have been shown to increase amygdalar CRF and CRF1 receptor expression (Roberto *et al*, 2010), and sub-chronic alcohol drinking has been shown to alter CRF immunoreactivity and mRNA, respectively, in the CeA of both adult (Lowery-Gionta *et al*, 2012; Zhou *et al*, 2013) and adolescent rats (Allen *et al*, 2011; Karanikas *et al*, 2013). Recently, the development of more precise pharmacological tools and transgenic mouse lines have facilitated the investigation of the specific role of the CRF1 receptor in the acute and chronic actions of alcohol in the amygdala.

Acute Alcohol and the CRF1 System

Consistent with the observed alterations in amygdalar CRF following acute alcohol exposure, CRF1 receptor signaling has been shown to mediate some of the effects of acute alcohol on amygdalar GABAergic activity. The alcohol-induced increase in GABA neurotransmission in the CeA is absent in CRF1 receptor knockout mice, and CRF1 receptor antagonists block this effect of alcohol in wild-type mice (Nie *et al*, 2004). These effects appear to be driven by presynaptic CRF1 receptors (Nie *et al*, 2009). Previous work by our group using CRF1-GFP transgenic reporter mice assessed the effects of acute alcohol on CRF1+ cells within the CeA (Herman *et al*, 2016a). Acute alcohol increased the firing

discharge of CRF1+ neurons and decreased the firing discharge of CRF1− neurons. Further, CRF1+ cells exhibited basal tonic GABA conductance mediated by the α1 $GABA_A$ receptor subunit that was insensitive to acute alcohol application, whereas alcohol induced a δ subunit-dependent tonic conductance in CRF1− cells that lacked $GABA_A$ tone at baseline. As CRF1− cells were shown to form inhibitory synapses onto CRF1+ cells, a subset of which projected to the BNST, these findings revealed an inhibitory microcircuit within the CeA that is sensitive to the presence of alcohol. Increased inhibitory tone in CRF1− cells following alcohol exposure disinhibits CRF1+ cells, increasing their firing and enhancing CeA output into the BNST (Herman *et al*, 2016a). These findings add complexity to our understanding of the circuit-specific effects of ethanol and CRF in the CeA. Although our work thus far has investigated the effects of ethanol on CRF1-containing neurons, not on the direct actions of CRF on CRF1 receptors, it raises the possibility that CRF, like ethanol, has specific actions at distinct sites in CeA microcircuitry. The CRF1:GFP mice used in these studies only labels neurons containing CRF1, it does not offer information on the localization of these receptors. Additional studies investigating the functional effects of CRF and CRF1 activation in CeA microcircuitry are needed.

The majority of studies on the regulation of alcohol-induced alterations in GABAergic signaling by CRF1 have focused on the CeA. Like the CeA, the BLA does exhibit basal tonic inhibition and alcohol has been shown to potentiate phasic inhibition in the rat BLA (Silberman *et al*, 2008). The ability of CRF1 to modulate alcohol effects on tonic and phasic inhibition in the BLA has yet to be assessed.

The CRF1 receptor also plays a role in the effects of acute alcohol on excitatory synaptic transmission in the CeA. Several studies (Herman *et al*, 2016c; Silberman *et al*, 2015; Silberman *et al*, 2013b; Varodayan *et al*, 2017) have shown that acute alcohol can potentiate spontaneous glutamatergic EPSCs in the CeA. This potentiation was blocked by pretreatment with a CRF1 receptor antagonist, but persisted despite the ablation of CRF-containing neurons in the CeA and whole organism (Silberman *et al*, 2015). This finding suggests that the CRF1 receptor, but not CRF peptide, is required for the effects of alcohol on spontaneous excitatory transmission in the CeA. Other endogenous CRF1 agonists, such as the Urocortins, may be responsible for the role of CRF1 in these alcohol-induced effects. Prior work also indicates that acute alcohol decreases evoked EPSCs in the CeA (Herman *et al*, 2016c; Roberto *et al*, 2004b; Varodayan *et al*, 2017), which points to differences in spontaneous versus evoked glutamate activity in the context of alcohol. The ability of either CRF peptide or the CRF1 receptor to regulate the effects of alcohol on evoked EPSCs in CeA has not yet been established.

Evidence from behavioral models of acute alcohol drinking indicate a role for CRF1 within the extended amygdala and other limbic brain regions in non-dependent alcohol consumption. Microinjection of the CRF1 antagonist antalarmin into the CeA reduced binge alcohol consumption in adult rats with a history of early life stress (Gondre-Lewis *et al*, 2016) as well as C57BL/6J mice with no history of stress (Lowery-Gionta *et al*, 2012). Recently, CRF1 receptor inhibition specifically within the VTA reduced binge alcohol drinking, and chemogenetic inhibition of BNST CRF1+ neurons that project to VTA produced similar reductions in alcohol intake (Rinker *et al*, 2017).

Chronic Alcohol and the CRF1 System

Chronic alcohol exposure also impacts the CRF1 system within the CeA. A four-week history of voluntary alcohol drinking increased GABA release and blunted sensitivity to the CRF1 antagonist R121919 in Marchigian Sardinian (msP) rats (Herman *et al*, 2013b). These alcohol-preferring rats also exhibit elevated basal GABA release relative to outbred strains. In Sprague-Dawley rats, 2–4 weeks of chronic alcohol vapor enhanced sensitivity to the effects of CRF and CRF1 antagonists to alter CeA GABA release (Roberto *et al*, 2010). However, the degree to which chronic alcohol-induced increases in extracellular GABA depend upon CRF1 signaling mechanisms has yet to be conclusively established.

Behavioral pharmacology experiments have also indicated a role for the CRF1 receptor in discrete brain regions in chronic alcohol drinking. Chronic treatment with a CRF1 antagonist prevented the withdrawal-induced increase in alcohol drinking observed in dependent rats (Roberto *et al*, 2010). Similar reductions in alcohol drinking following CRF1 antagonism were reported during alcohol deprivation in mice with a history of heavy alcohol drinking, accompanied by alterations in CRF1 mRNA in the amygdala (Correia *et al*, 2015). Microinjection of the CRF1/2 antagonist D-Phe-CRF(12-41) into the CeA reduces alcohol intake in alcohol-dependent, but not non-dependent, rats (Funk *et al*, 2006). CRF1 activation has also been implicated in the anxiety behavior induced by alcohol withdrawal (Overstreet *et al*, 2004) but the mechanisms behind this effect are not known.

Due to the substantial preclinical literature suggesting a role for CRF1 signaling in alcoholism, CRF1 has become an attractive target for the treatment of AUD. However, two studies in human participants have failed to demonstrate an effect of centrally-active CRF1 antagonists on alcohol craving and stress-related behavior during abstinence (Kwako *et al*, 2015; Schwandt *et al*, 2016). The lack of translation of systemic CRF1 antagonism from rodent models to humans may speak to the greater complexity of these systems in the primate brain, the specific outcomes measured (craving, as opposed to relapse and alcohol intake), a relatively greater degree of individual differences in native CRF/CRF1 activity in human populations versus rodent studies (many of which use inbred strains with reduced intra-individual variation), or differences between the chronic stress models typically employed in preclinical studies versus the often dynamic role of stress in human conditions such as alcoholism (Koob and Zorrilla, 2012; Spierling and Zorrilla, 2017).

Together, a wealth of studies indicate that the amygdala plays a central role in the acute and chronic effects of alcohol and can functionally regulate alcohol drinking behavior in binge-like and dependent models of alcohol drinking. Further, CRF1 signaling in the CeA has emerged as an important mediator of the effects of acute and chronic alcohol in this brain region.

Fear and the Amygdala

The amygdala has been recognized as a major regulator of anxiety and fear-related behaviors for many years. Human participants with PTSD have been found to have reductions in amygdala volume compared with healthy controls (Starcevic *et al*, 2014), and PTSD has been shown to alter functional connectivity both within amygdalar subnuclei and between

these regions and the cortex (Brown *et al*, 2014). Similar evidence for dysregulation of cortical connectivity with the amygdala has been demonstrated in generalized anxiety disorder patients (Etkin *et al*, 2009). Amygdala hyperexcitability is a particularly consistent feature of anxiety disorders in human functional neuroimaging studies (Shin and Liberzon, 2010). Increases in amygdala activity have also been observed after the presentation of stress or fear-related visual stimuli in social phobia (Stein *et al*, 2002), arachnophobia (Schienle *et al*, 2005) and PTSD (Liberzon and Sripada, 2008), although results from patients with generalized anxiety disorder have been mixed (Mochcovitch *et al*, 2014).

Animal models of anxiety-like behavior have revealed some of the underlying neurological mechanisms behind the alterations in amygdalar activity seen in human studies. Dysregulation of GABAergic inhibition of the BLA results in hyperexcitability (Muller *et al*, 2015; Truitt *et al*, 2009), which has been associated with anxiety in rodent models and humans (Nuss, 2015; Prager *et al*, 2016; Terburg *et al*, 2012). Lesions to the CeA prevent the expression of anxiety-related behavior in the elevated plus maze in response to acute stressors (Ventura-Silva *et al*, 2013). Optogenetic activation of cells projecting from BLA to CeA promotes anxiolysis and inhibition of these same cells produces anxiogenesis in the open-field and elevated plus maze tasks (Tye et al 2011). These studies suggest that changes in activity within the CeA contribute to the expression of anxiogenic behaviors.

The amygdala also plays a significant role in fear learning. Generally speaking, the LA has been recognized as a site for the acquisition of fear conditioning, whereas the CeA is involved in the expression of fear-related conditioned responses (Maren and Quirk, 2004). Inputs to the LA are thought to strengthen with repeated pairings of the unconditioned stimulus (US) with the conditioned stimulus (CS) and promote recruitment of downstream targets in the CeA_M (Blair *et al*, 2001; Sigurdsson *et al*, 2007). CeA_L neurons exhibit increased firing in response to fear conditioned stimuli, and activation of these cells triggers freezing behavior in mice (Ciocchi *et al*, 2010). Information relating to fear conditioning can relay from the LA to the CeA_M via two pathways: an excitatory direct tract from the BLA, or an inhibitory indirect projection from the CeA_L to the CeA_M (Duvarci *et al*, 2014).

Amygdala Microcircuitry and Fear Learning

Recent work has begun to dissect functional amygdala circuits that are responsible for the acquisition and expression of fear learning. Ciocchi et al. (2010) identified separable populations of cells within the CeA_L that have opposite responses to CS presentation, those that increase firing in response to the CS (CEl_{on} cells) and those that decrease firing in response to the CS (CEl_{off} cells). In a related paper, Haubensak and colleagues (2010) demonstrated that the CEl_{off} population are likely PKCδ-containing neurons that send a GABAergic inhibitory projection to the CeA_M, regulating CeA_M activity and thereby altering the expression of conditioned fear. Both studies also provided evidence of reciprocal inhibition of CEl_{on} and CEl_{off} cells within the CeA_L. Consistent with these findings, genetic silencing of PKCδ-containing cells increased freezing behavior in a subset of conditioned animals. Together, these results identified an inhibitory microcircuit within the CeA_L that functionally regulates CeA_M activity and may in turn alter the expression of conditioned fear.

Subsequently, Li et al. (2013) demonstrated plasticity of glutamatergic signaling onto the SOM+ GABAergic cells of the CeA_L. This population is distinct from the PKCδ+ neurons characterized by Ciocchi and Haubensak and does not project to the CeA_M; rather, these cells form local inhibitory microcircuits that gate activity of the CeA_L. Stimulation of this SOM+ cell population triggered freezing behavior in naïve mice, and inhibition of this population reduced the expression of conditioned fear. Additional populations of SOM+ cells in the CeA_L project to the periacqueductal gray and paraventricular nucleus of the hypothalamus, where they regulate freezing behavior (Penzo *et al*, 2014). More recent work from this group has demonstrated that SOM+ CeA_L cells activate in real-time in response to external threats; activation of this population is associated with more passive defensive strategies such as freezing, whereas inhibition of this population promotes active avoidance behaviors (Yu *et al*, 2016). As a whole, these studies suggest that following fear conditioning, the LA can relay information to the CeA_L, where activation of PKCδ+ cells that project to the CeA_M triggers the expression of conditioned fear responses, and conversely activation of the local SOM+ CeA_L cells inhibits PKCδ+ cells, reducing the expression of conditioned fear responses. However, the added complexity of local versus projection SOM+ neurons exerting opposite effects on freezing behavior, and recent reports of significant overlap at the level of mRNA between cell markers in the CeA_L (McCullough *et al*, 2018), highlight the need for further characterization of specific cell populations recruited during fear-related behavioral tasks and the functional role of these circuits in the cellular and behavioral output of the amygdala as a whole.

CRF Involvement in Fear/Anxiety

Given the ability of CRF and CRF1+ cells to regulate BLA and CeA neuronal activity, it is unsurprising that the CRF system has also been shown to regulate fear and anxiety-like behaviors. Acute stress enhances CRF release in the amygdala (Koob and Heinrichs, 1999), and the systemic administration of CRF increases anxiety-like behavior in rodent models, including acoustic startle, elevated plus maze, forced swim test, and defensive withdrawal in an open field (see Dunn & Berridge, 1990 for review.) Restraint stress has also been shown to increase CRF1 receptor mRNA in the CeA and BLA of Wistar rats (Ciccocioppo *et al*, 2014). CRF1 agonists generally produce pro-stress behavioral effects, whereas CRF1 antagonists generally produce anxiolysis in behavioral tests of stress responsivity (for review, see Koob 2010). The Marchigian Sardinian P (msP) rat strain exhibits increased anxiety-like behaviors accompanied by constitutive increases in CRF1 expression within the CeA (Cippitelli *et al*, 2015; Natividad *et al*, 2017). CRF activity within the CeA has also been shown to functionally regulate anxiety-like behavior (Rassnick *et al*, 1993). In the laterocapsular division of the CeA, CRF peptide and the CRF1 receptor have also been shown to play a role in nonciception (Ji *et al*, 2013; Ji and Neugebauer, 2008), cellular adaptations to chronic pain (Fu and Neugebauer, 2008; Ji and Neugebauer, 2007b), and the expression of pain-related anxiety behaviors (Ji *et al*, 2007a).

To date, the role of CRF and CRF1+ neurons within the amygdala in the expression of anxiety-like behavior and conditioned fear, and where these cell populations fit into the fear-related microcircuitry already identified in the CeA, has not been fully characterized. Recent evidence supports a key role for the CRF1+ cell populations of the CeA in conditioned fear.

One study has identified CRF1+ neurons in the rostral CeA_L as a distinct population that does not overlap with PKCδ+ or SOM+ cells. These CRF1+ cells exhibit long-term potentiation following fear conditioning, and inactivation of these cells inhibits the acquisition, but not expression, of fear learning about weak threats (Sanford *et al*, 2017). Additional evidence suggests that the CRF1+ cells of the CeA regulate conditioned freezing behavior whereas the SOM+ cells of the CeA mediate conditioned escape behaviors, with reciprocal inhibitory connections between these populations gating the expression of passive or active defensive behavior (Fadok *et al*, 2017). However, the role of CRF1+ cells in the CeA_M in the expression of fear conditioning to weak threats, and the alterations in CRF1+ cells in the CeA_L following chronic intense fear conditioning, are unknown.

The CRF System, Fear and Alcohol in the Amygdala

Current studies in the BLA circuitry underlying specific fear behaviors provide a framework for an improved understanding of the neurobiological underpinnings of alcohol use disorders and dependence. Notably, work on the specific alterations in amygdala microcircuitry produced by fear conditioning can act as a model and as a point of comparison with the effects of alcohol on amygdala circuitry. As fear conditioning and alcohol dependence can both be conceptualized as examples of aversive conditioning and can be measured in terms of learned behavior, they may engage the circuitry in the same way and produce similar effects at the cellular and whole animal level. Conversely, if conditioned fear is viewed as a useful physiological function and alcohol dependence is viewed as a maladaptive pathological process, then while they may engage the same microcircuitry they will produce different (and possibly opposing) effects. This hypothesis is supported by studies demonstrating increased GABA release following acute alcohol (Zhu *et al*, 2006) and decreased GABA levels following fear conditioning (Stork *et al*, 2002). In addition, experimental evidence also indicates that binge-like alcohol exposure impairs fear conditioning in humans (Stephens *et al*, 2005) and that repeated alcohol withdrawal impairs the acquisition of fear conditioning in rats (Ripley *et al*, 2003). However, it has also been shown that the retrieval of fear memory increased alcohol consumption in alcohol-withdrawn rats (Bertotto *et al*, 2010), suggesting that there may be some mechanisms for behavioral overlap. One potential point of overlap is the CRF and CRF1 system, which has been implicated in both fear conditioning and alcohol dependence. Fear conditioned rats display increased CRF dialysate levels in the BLA that are positively correlated with the expression of fear behavior (Mountney *et al*, 2011), the CRF1 gene *Crhr1* is upregulated in the BLA of dependent rats (Sommer *et al*, 2008) and selective deletion of the α1 $GABA_A$ receptor subunit in CRF+ neurons enhanced anxiety and impaired fear extinction (Gafford *et al*, 2012). Additionally, polymorphisms in the human *crhr1* gene have been repeatedly associated with increased risk for alcoholism following stressful life events (Blomeyer *et al*, 2008; Ray *et al*, 2013; Schmid *et al*, 2010). The possible interference by alcohol on normal functioning of BLA microcircuitry adds a potential new layer to the existing knowledge on how alcohol alters normal brain function to contribute to the pathological phenotype of dependence. Impaired learning circuitry could explain why alcoholics continue to drink despite negative consequences, as well as why extinction therapy often fails in treating alcoholics. If chronic alcohol exposure alters amygdala circuitry that is critical for learned

associations, then it could result in a compromised ability to process physiologically-relevant negative associations and/or generate appropriate behavioral responses to aversive conditions.

Although the findings detailed above provide ample evidence that the activity of the CRF1 receptor in the amygdala plays a role in the acute and chronic effects of alcohol exposure, several important areas of investigation remain understudied. The majority of the work on CRF1-alcohol interactions have been studied in the CeA, but the BLA clearly plays an important role in alcohol-related behaviors and undergoes significant molecular adaptations following alcohol exposure. Future work should examine the effects of alcohol on CRF1+ and CRF1− cells within the BLA to complement the existing work from the CeA. At present, work on the CRF system and alcohol in the amygdala has focused on three discrete facets of this system: CRF peptide, the CRF1/CRF2 receptors, and CRF1-containing cell populations. It will be critical for future work to establish clear roles for these various component of the CRF system in alcohol-related behaviors, and to understand how they interact with one another to produce alterations in amygdala activity and behavior. Additionally, a functional role for CRF1 activity on alcohol-induced alterations in CeA glutamatergic activity has not yet been established. Lastly, two studies (Herman *et al*, 2013a; Roberto *et al*, 2010) provide initial evidence of the impact of chronic alcohol exposure on the CRF system of the CeA, but specific circuit dissection and the identification of mechanisms behind this effect have not been performed.

Recently, PKCε has emerged as an important regulator of CeA GABAergic transmission, CRF1 activity, and alcohol effects in acute alcohol models. Studies utilizing systemic knockout and intra-amygdala knockdown of PKCε have resulted in reduced alcohol intake, reduced alcohol intoxication, and altered GABAergic transmission both at baseline and in response to alcohol (Choi *et al*, 2008; Lesscher *et al*, 2009). Binge-like alcohol drinking, in which non-dependent subjects achieve a high blood-alcohol concentration during a limited window of alcohol access, has been shown to increase expression of PKCε in the CeA, and inhibition of PKCε specifically within the CeA reduced alcohol consumption in the binge model (Cozzoli *et al*, 2016). Given that PKCε has been shown to mediate some of the effects of CRF and alcohol on CeA GABAergic activity (Bajo *et al*, 2008; Blasio *et al*, 2017), the relationship between PKCε, CRF1 signaling and alcohol consumption warrants further investigation. If indeed native PKCε serves to inhibit GABAergic activity in the CeA, the acute increase in PKCε expression following sub-chronic alcohol exposure may represent a compensatory mechanism to offset the acute enhancement of GABAergic signaling induced by alcohol. However, the effect of chronic alcohol on PKCε expression or activity, and the potential functions of PKCε in models of alcohol dependence, remain to be explored. PKC has also been implicated in the stress-inducing effects of intra-ventricular CRF infusion (Toth *et al*, 2013) and has a well-established role in learning and memory (Sun and Alkon, 2014). These features make PKCε a particularly attractive target for investigation of the overlap, or lack thereof, of amygdalar signaling mechanisms in anxiety and alcoholism. Inhibitors that are selective for PKCε may also represent a more fine-tuned approach to pharmacological intervention in AUD, given the failure of systemic CRF1 antagonists to reduce alcohol craving in human participants (Kwako *et al*, 2015; Schwandt *et al*, 2016).

Another important consideration in the ongoing quest to clarify the role of CRF1 signaling in alcoholism and anxiety is the role of developmental stage. Adolescence is a time of heightened emotionality and stress responsivity (Casey *et al*, 2010), accompanied by alterations in the HPA axis generally and the cortisol system specifically (Apter *et al*, 1979). Findings from studies utilizing models of adolescent social isolation stress have demonstrated alterations in alcohol intake, amygdala function and anxiety-like behaviors (Butler *et al*, 2016; Karkhanis *et al*, 2015; Rau *et al*, 2015; Skelly *et al*, 2015), suggesting the potential for overlapping circuitry for these behaviors during adolescent brain development. Exposure to alcohol (Grant and Dawson, 1997) and stress (Burke and Miczek, 2014) during adolescence has also been shown to substantially enhance the lifetime risk for alcoholism in human participants and increase adult alcohol consumption in rodent models. However, the specific molecular and cellular mechanisms responsible for this increased risk remain unclear. CRF represents a system where stress and alcoholism converge, and therefore may play a role in mediating the long-lasting effects of adolescent alcohol exposure. To date, this possibility has received relatively little investigation. Adolescent alcohol exposure has been shown to both increase (Karanikas *et al*, 2013) and decrease (Allen *et al*, 2011) CRF immunoreactivity in the CeA. Additionally, age and sex differences in the ontogeny of the CRF1 receptor have been catalogued in many limbic brain regions, including the hypothalamus, ventral tegmental area, nucleus accumbens and hippocampus (Lukkes *et al*, 2016; Weathington *et al*, 2014), but the development of this receptor in the amygdala has not been studied. Age differences in sensitivity to CRF1 antagonist effects on alcohol consumption and GABAergic neurotransmission in brain regions relevant to addiction and anxiety have also not been explored. Such studies would provide important insight into the progression of alcohol use disorders over the lifespan and may reveal potential molecular mechanisms underlying age differences in alcohol intake and lifetime risk of AUD.

Finally, the recent work to define the microcircuitry of the central amygdala and the role of specific cell populations in fear conditioning can stand as a useful model for advances in preclinical alcohol research. To date, the role of the amygdala in alcohol-related behaviors, and reward-related behaviors in general, has focused on projections between the amygdala and other limbic circuitry. For instance, several studies have identified a role for an excitatory projection from the BLA to the NAc in behavior related to both natural and drug reward. Stuber et al. (2011) characterized this cell population and demonstrated that activation of this projection was sufficient to maintain self-administration of optical stimulation. Further, inhibiting this pathway reduced cued sucrose intake. Two recent papers have established a role for this pathway in alcohol-related behaviors. Keistler et al. (2017) found that selective ablation of cells in the BLA to NAc projection reduces reinstatement of alcohol seeking in rats. Millan et al. (2017) demonstrated that activation of this pathway reduced alcohol drinking and cue-induced alcohol seeking. These findings provide compelling evidence of the central role the amygdala plays in the regulation of downstream brain regions and the expression of alcohol-related behaviors. However, the role of intra-amygdala microcircuits on the overall activity of amygdalar subnuclei and the ways in which this circuitry modulates alcohol seeking, consumption and withdrawal behaviors has not been assessed. The recent work to characterize local microcircuits within the CeA in the context of fear conditioning has identified several important cell populations, including the

CRF1+, SOM+, and PKCδ+ cells of the CeA_M and CeA_L whose sensitivity to acute and chronic alcohol has received limited investigation. Differences in the functional role of these populations on alcohol-related behaviors remain to be studied. Engagement of this circuitry by alcohol may drive changes in amygdala activity associated with anxiety and stress, and conversely activation of some or all of these populations by stress may alter their sensitivity to the effects of alcohol. With the identification of these discrete cell populations within the amygdala, both in the context of fear conditioning and alcohol exposure, the stage is set for the thorough mechanistic evaluation of the functional role of the intra-amygdala architecture in the cellular, regional and behavioral effects of alcohol.

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HIGHLIGHTS

- The amygdala is a brain region where alcohol and anxiety-associated circuitry converge and is closely associated with the behavioral manifestations of both disorders.
- Precise local and projection-based control of specific neuronal populations is emerging as a key regulator of intra-amygdala activity, amygdala output, and behavior.
- The corticotropin releasing factor (CRF) system is implicated in the effects of alcohol, stress, and anxiety in distinct amygdala nuclei, but the specific circuitry involved in these effects and the role of these circuits in stress and anxiety are only beginning to be understood.

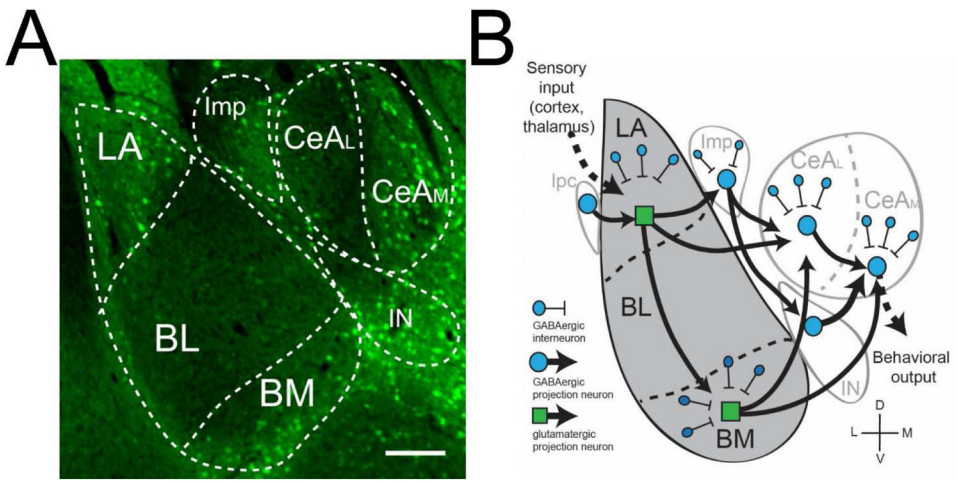

Figure 1.

CRF1 subpopulations in the amygdala complex. **A.** GFP expression in CRF1-containing neurons in the amygdala, including discrete populations in the LA. BM, Imp, IN, and CeA_M. Scale bar = 400 μm. **B.** Schematic diagram of proposed CRF1 circuitry showing inter- and intranuclear connections. BL: basolateral; BM: basomedial; CeA_L: lateral central amygdala; CeA_M: medial central amygdala; Imp: medial paracapsular intercalated; IN main intercalated; LA lateral amygdala.