GABA and NMDA receptors in CRF neurons have opposing effects in fear acquisition and anxiety in central amygdala vs. bed nucleus of the stria terminalis

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Abstract

Beginning with Vale and Colleagues in 1981, corticotropin releasing factor (CRF) also called corticotropin releasing hormone (CRH) has repeatedly been identified as an important contributor to fear and anxiety behavior. These findings have proven useful to further our understanding of disorders that have significant fear-dysregulation, such as post-traumatic stress, as well as other stress- and anxiety-related disorders. Unfortunately, the data are not all in agreement. In particular the role of CRF in fear learning is controversial, with studies pointing to contradictory effects from CRF manipulation even within the same brain structure. Further, very few studies address the potentially promising role of CRF manipulation in fear extinction behavior. Here, we briefly review the role of CRF in anxiety, fear learning and extinction, focusing on recent cell-type and neurotransmitter-specific studies in the amygdala and bed nucleus of the stria terminalis (BNST) that may help to synthesize the available data on the role of CRF in fear and anxiety-related behaviors.

Keywords

CRF; GABA; NMDA; Fear; Extinction; Amygdala; BNST; Anxiety; PTSD; r121919

Introduction

CRF background

Corticotropin releasing factor CRH, also known as corticotropin releasing hormone (CRH), is a 41 amino acid peptide discovered by Vale and colleagues in 1981. CRF is largely expressed in stress responsive areas of the brain. In the brain, including the paraventricular nucleus of the hypothalamus (PVN), the central nucleus of amygdala (CeA) and the bed

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nucleus of stria terminalis (BNST) (Merchenthaler, 1984; Palkovits et al., 1985). CRF is widely studied due to its role in activating the hypothalamic–pituitary–adrenal (HPA) axis in response to a perceived threat and coordinating the autonomic and behavioral response to stress. While CRF is critical for responding to a threatening situation, hyper-activation of the CRF pathway is associated with severe emotional dysregulation including post-traumatic stress and other stress-related disorders (Bale, 2005; Nemeroff et al., 2006).

Our ability to study CRF is made more difficult because it is produced in many different types of cells (Kapcala and Dicke, 1992) and it is colocalized with numerous neurotransmitters and neuropeptides (Sawchenko and Swanson, 1985; Honkaniemi et al., 1992). One way to interrogate CRFergic cells directly is to utilize a transgenic mouse that allows CRF containing cells to be identified and manipulated apart from neighboring cells (Martin et al., 2010). After originally creating a mouse line in which cells expressing CRF also express Cre recombinase (CRF–Cre) (Martin et al., 2010), work in our lab utilized these CRF–Cre mice and crossed them with either "floxed" GABAAα1 (Gafford et al., 2012) or "floxed" NMDA (N-methyl-D-aspartate (NMDA) glutamate) receptor 1 (NR1) (Gafford et al., 2014) genes. The floxed gene of interest is surrounded (flanked) by LoxP sites which are recognized by Cre as the site of gene excision. The result of crossing CRF–Crewith floxed GABAAα1 or floxed NR1 mice is disruption of CRF producing-GABAAα1 or CRF producing-NR1 neurons throughout development. One concern particularly with manipulation of GABAergic neurons during development is that GABAergic neurons gradually shift from excitatory to inhibitory over the course of development, providing a basis for activity dependent neural circuit formation and for critical developmental periods (Hensch, 2005). Some work has shown that genetic disruption of GABAergic transmission early in postnatal life effects anxiety- and depression-related behaviors in adulthood (Shen et al., 2012). Therefore, it is possible that some of our behavioral effects were due to tuning of GABAergic neurons during these critical developmental periods even though we do see normal GABA receptor responses in electrophysiological recordings from these neurons (Gafford et al., 2012). These manipulations offer insight into inhibitory (GABAergic) and excitatory (glutamatergic) control over CRFergic cells in fear and anxiety behavior. This review will focus on these and other findings contributing to what is known regarding CRF in fear and anxiety in the BNST and CeA.

CRF and anxiety

The CeA and the BNST are highly interconnected (Alheid et al., 1998), receive information from and project to similar structures (Gray and Magnuson, 1987; Rosen et al., 1991; Gray and Magnuson, 1992; Dong et al., 2001) are both largely GABAergic (Sun and Cassell, 1993), receive glutamatergic projections from the lateral amygdala (LA) (Krettek and Price, 1978; Pitkanen et al., 1995; Dong et al., 2001) and express a wide variety of neuropeptides including CRF (Roberts et al., 1982;Woodhams et al., 1983). Even with the similarities between BNST and CeA, manipulation of CRF has vastly different effects on fear and anxiety behavior dependent on which structure is targeted as detailed below (Walker and Davis, 1997; Sullivan et al., 2004; Waddell et al., 2006; Sink et al., 2011).

CRF, anxiety and the BNST—CRF in the BNST has been implicated in mediation of anxiety-like responses (Sahuque et al., 2006; Lee et al., 2008). Within the BNST, work has shown that introduction of stressful stimuli increases CRF expression (Choi et al., 2006; Funk et al., 2006; Kim et al., 2006; Shepard et al., 2006) and overexpression of CRF1 or direct activation of CRF1 and CRF2 receptors with urocortin 1 enhances anxiety (Lee et al., 2008; Sink et al., 2013). Moreover, blockade of CRF with an antagonist infused into the BNST blocks sustained fear behavior that resembles anxiety (Davis et al., 2010). Recent work has implicated a specific subtype of CRF neuron in the BNST that may contribute strongly to the anxiety phenotype. Specifically, transgenic deletion of the GABAAα1 receptor only within CRF producing neurons was found to enhance anxiety as measured across a variety of different tasks (Figs. 1A, B), and this effect was reversed with systemic (Fig. 1C) or BNST (Fig. 1D) infusion of CRFR1 receptor antagonist R121919 (Gafford et al., 2012). These findings suggest that hyperactivation of CRFergic cells, through deletion of GABA receptors, is associated with increased anxiety in agreement with work showing increases in CRF enhance anxiety (Britton et al., 1982; Liang and Lee, 1988; Takahashi et al., 1989). Further, when CRFR1 activation is disrupted with administration of R121919 anxiety behavior is normalized.

CRF, anxiety and the CeA—In contrast to the effects of CRF manipulation in the BNST on anxiety, disruption of CRF in the CeA has not been shown to affect anxiety behavior (Lee and Davis, 1997; Callahan et al., 2013). One study took advantage of the finding that intracerebroventricular administration of CRF facilitates anxiety and directly tested whether this CRF mediated increase in anxiety can be blocked by NMDA lesions of the CeA or the BNST (Lee and Davis, 1997). The authors found that lesions of the BNST blocked CRF enhanced anxiety while CeA lesions had no effect, further demonstrating the important role of CRF in the BNST on anxiety behavior. Another recent study used RNA interference of CRF in the CeA to locally knock down CRF expression and found no effect on anxiety (Callahan et al., 2013). Interestingly, we have also tested the effect of disruption of CRF producing NR1 neurons on anxiety behavior. When we disrupted glutamatergic input onto CRF neurons we found no effect on anxiety measures (Gafford et al., 2014) (Figs. 1E, F). This may be due to the difference in expression of NR1 and GABAAα1 in the BNST since GABAAα1 is so heavily expressed in the BNST (Heldt and Ressler, 2007) compared to NR1 (Lein et al., 2007).

CRF and fear learning

CRF, BNST and fear learning—While the role of the BNST in anxiety behavior has been well established, there is little evidence to date that the BNST is engaged during fear learning (LeDoux et al., 1988; Hitchcock and Davis, 1991). However, a recent study showed that virally mediated overexpression of CRF in the BNST 2 weeks before fear conditioning resulted in attenuation of associative fear learning (Sink et al., 2013). In this same study when CRF was overexpressed after fear conditioning fear expression was significantly enhanced. Since intracranial administration of CRF has been shown to enhance stress responding (Cole and Koob, 1988; Sherman and Kalin, 1988) it is possible that CRF overexpression during fear consolidation enhanced anxiety resulting in enhanced fear memory consolidation, as shown previously (Cahill et al., 2003; Rau et al., 2005; Hui et al.,

2006). Our work using transgenic disruption of CRF in GABAAα1 neurons enhanced CRF expression in the BNST, PVN and CeA (Gafford et al., 2012) however, we show no effect on fear acquisition (Figure 3A, B). We do find significant disruption of fear extinction with disruption of CRF in GABAAα1 neurons. Since we did not specifically manipulate the BNST or amygdala during the fear conditioning experiments we cannot attribute the effects on extinction to a specific structure.

CRF, amygdala and fear learning—The amygdala is a structure critically engaged in fear learning (LeDoux, 2000). The major subnuclei of the amygdala include LA, basal (B), accessory basal (AB) and CeA. The term basolateral amygdala (BLA) is often used to refer to LA and B together (LeDoux, 2000). Evidence strongly supports the BLA as a structure critical for formation and storage of fear memory (LeDoux, 2000; Johansen et al., 2011). Studies have shown that the CeA is also required for the acquisition, consolidation, and expression of fear memories (Campeau and Davis, 1995; Goosens and Maren, 2003; Wilensky et al., 2006; Zimmerman and Maren, 2010) and potentially does so in parallel with the BLA (Pape and Pare, 2010). CRF has been shown to play a critical role within the amygdala in fear learning processes. The BLA contains a high density of CRF1 receptors (Baram and Hatalski, 1998; Chen et al., 2000) while the CeA has many CRF expressing neurons but lacks strong expression of CRF receptors (Sakanaka et al., 1986; Potter et al., 1994; Van Pett et al., 2000). Both the BLA and CeA are critical for fear memory (LeDoux et al., 1985; Wilensky et al., 2006). Infusion of a CRF receptor antagonist into the BLA disrupts contextual fear conditioning (Hubbard et al., 2007) and inhibitory avoidance learning (Roozendaal et al., 2002, 2008). Increases in CRF in the BLA facilitate performance in a variety of learning tasks (Liang and Lee, 1988; Roozendaal et al., 2008), but also see Isogawa et al. (2013) which found CRF infusion in LA *impairs* fear memory.

Interestingly, some investigators manipulated CRF around the time of auditory fear conditioning (Isogawa et al., 2013), after inhibitory avoidance training (Roozendaal et al., 2002) or at different times after contextual fear conditioning and found no disruption of learning tasks, even though other work has shown CRF expression is increased in the CeA after contextual fear conditioning (Thompson et al., 2004). In fact, other studies have found that CeA infusion of a CRF antagonist prior to contextual fear conditioning (Swiergiel et al., 1993) or CRF antisense at different time points after contextual fear conditioning (Pitts et al., 2009; Pitts and Takahashi, 2011) is effective at modulating fear memory. The lack of effectiveness of CRF receptor antagonists in the CeA in some studies may be partially explained by the relative expression of CRF receptors in the BLA compared to the CeA. The BLA strongly expresses CRF receptors (Baram and Hatalski, 1998; Chen et al., 2000) while the CeA has many CRF expressing neurons but fewer CRF receptors (Sakanaka et al., 1986; Potter et al., 1994; Van Pett et al., 2000) making the BLA a more target-rich environment for CRF receptor antagonists. A summary of these findings and further details from the infusion studies related to CRF regulation of fear behavior are detailed in Table 1.

Recent work from our lab has additionally manipulated specific subtypes of CRF containing neurons (CRF/NMDAR1 containing versus CRF/GABAAα1 containing, as described above) to examine whether cell-type and receptor specificity could help to explain some of the above discrepancies in fear-related behaviors. We find either enhancement of fear

conditioning with the transgenic (Fig. 2A) or lentiviral (Fig. 2D) mediated NMDAR1 deletion in CRF neurons compared to no effect on fear conditioning with the transgenic deletion of GABAAα1 in CRF neurons (Figs. 3A, D) (Gafford et al., 2012, 2014). The discrepancy in findings may again be due to the difference in the brain area affected by the manipulation as noted earlier in this review. The stronger expression of CRF containing NMDAR1 neurons within the CeA, a structure engaged in fear acquisition (Wilensky et al., 2006) may indicate a more critical role for these CRF containing neurons in fear conditioning, while CRF containing GABAAα1 neurons are more strongly expressed in the BNST which is less engaged in fear memory formation (Walker et al., 2009).

CRF and fear extinction

Fear extinction involves learning to no longer fear a previously fearful stimulus. Extinction is achieved by exposing a fear conditioned organism to multiple presentations of the feared stimulus without the previously paired aversive event (e.g., footshock) resulting in an eventual decrease of the fear behavior. This process is thought to be comprised of new inhibitory learning mechanisms (Myers and Davis, 2007). Fear extinction is a complex learning event that is reliant upon a broad network of structures including the amygdala (Pare and Duvarci, 2012; Furini et al., 2014), and multiple cellular networks within these structures mediate extinction behavior (Pare and Duvarci, 2012; Duvarci and Pare, 2014).

Methods to facilitate fear extinction has been proposed as a behavioral treatment for fear and anxiety disorders such as posttraumatic stress disorder (PTSD) (VanElzakker et al., 2014) since work has shown that those with PTSD have impairments in extinction (Orr et al., 2000; Peri et al., 2000; Rothbaum and Davis, 2003). Therapeutic outcome of those with PTSD who undergo fear extinction can be improved with administration of a partial agonist of the NMDA receptor (Walker et al., 2002; Difede et al., 2014; Rothbaum et al., 2014) and possibly impaired with administration of a benzodiazepine that potentiates GABAergic inhibition (Rothbaum et al., 2014). Work in humans has shown there is also a role for CRF in PTSD. Specifically, individuals with PTSD have been found to have enhanced CRF levels in their cerebrospinal fluid (CSF) (Bremner et al., 1997; Baker et al., 1999; Sautter et al., 2003; Risbrough and Stein, 2006) suggesting dysregulation of CRF may contribute to PTSD. It is unknown whether the increase in CRF is a predetermining factor in PTSD or a result of the development of PTSD.

Little research has directly manipulated CRF in fear extinction in the animal model. One recent study examined CRF in fear extinction by infusing either CRF, CRF binding protein which increases endogenous levels of free CRF or a CRF receptor antagonist into the BLA prior to fear extinction (Abiri et al., 2014). Abiri and colleagues found that endogenously increasing CRF in the BLA just prior to fear extinction with either infusion of CRF or CRF binding protein resulted in impaired fear extinction consolidation while application of a CRF receptor antagonist facilitated fear extinction (Abiri et al., 2014). These findings are in agreement with the work in humans with PTSD showing that increased CRF concentration resulted in disrupted fear extinction.

Work in our lab has also investigated the role of CRF in fear extinction learning. We assessed extinction with disruption of either GABAAα1 or NMDAR1 gene expression

within CRFergic neurons (Gafford et al., 2012, 2014). We found that transgenic disruption of GABAAα1 within CRFergic neurons did not affect fear conditioning (as noted previously) or retention behavior (Figs. 3A, B) but resulted in a significant and prolonged deficit in fear extinction (Gafford et al., 2012) (Fig. 3C). In a follow-up experiment we again show that transgenic disruption of GABAAα1 gene expression within CRF–containing neurons does not affect fear acquisition or retention of fear (Figs. 3D, E), but systemic infusion of the CRFR1 receptor antagonist R121919 could partially rescue the fear extinction deficit (Fig. 3F). In contrast, disruption of CRF NR1 neurons significantly facilitated fear acquisition (as noted previously) (Fig. 2A) and significantly enhanced fear retention tested the following day (Fig. 2B) without significantly effecting long term extinction retention (Fig. 2C). We replicated the finding of enhanced fear learning (Fig. 2D) and fear retention (Fig. 2E) with virus infusions that knocked down NR1 containing CRF neurons specifically within the CeA. However, with viral manipulation of CeA CRF NR1 neurons we show impaired fear extinction over 15 trials of extinction (Fig. 2F). The difference between disruption of CRFergic NR1 neurons in the transgenic mouse compared to the same disruption delivered via virus infusion directed at the CeA may be due to the targeted nature of the disruption. Altogether, our data may suggest that different CRF neuronal subpopulations selectively contribute to accelerated fear acquisition or disrupted fear extinction behavior. Specifically, transgenic disruption of inhibitory input onto CRFergic neurons strongly impairs fear extinction while transgenic of virus-mediated disruption of CRFergic NR1 neurons strongly enhances fear conditioning and fear retention. Since disorders such as PTSD have been linked to enhanced fear conditioning as well as disrupted fear extinction (Orr et al., 2000; Lissek et al., 2005; Blechert et al., 2007; Glover et al., 2011; VanElzakker et al., 2014), these data may highlighting a potential mechanism for further investigation of this dissociation.

Conclusion

In summary, CRF plays a critical, yet complex role in anxiety and fear memory. Both the BNST and CeA have similar connections to upstream and from downstream targets, receive highly processed sensory information and are rich with neuropeptides including CRF (Krettek and Price, 1978; Roberts et al., 1982; Woodhams et al., 1983; Gray and Magnuson, 1987; Rosen et al., 1991; Gray and Magnuson, 1992; Sun and Cassell, 1993; Pitkanen et al., 1995; Dong et al., 2001). However, a good deal of evidence shows that CRF in the BNST contributes to anxiety behavior while CRF in the amygdala contributes to fear memory processing. One reason for the difference in responsivity to fearful stimuli may lie in the different subtypes of CRF neurons in the BNST compared to the CeA. Recent technological innovations such as optogenetics and chemogenetic techniques may illuminate this question by allowing for specific manipulation of CRF neurons in the BNST or CeA. Further technological innovations such as FACS (Fluorescence Activated Cell Sorting) and TRAP (Translating Ribosome Affinity Purification) will allow genetic profiling of cell subtypes so that we may begin to determine differential genetic contributions responsible for normal and dysfunctional fear and anxiety behavior. These studies hold the potential to uncover novel, more directed and effective treatments for debilitating disorders of fear and anxiety such as PTSD.

Acknowledgments

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CRF GABAAα1
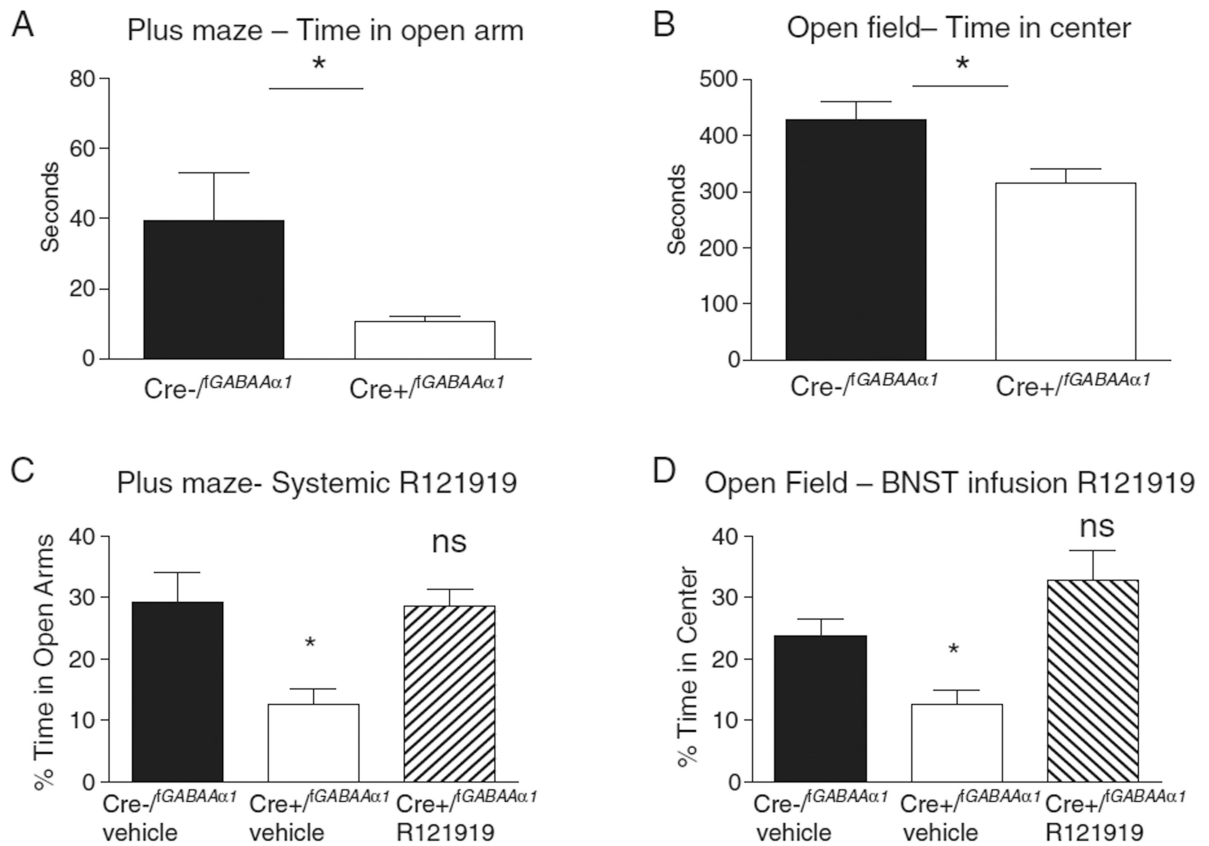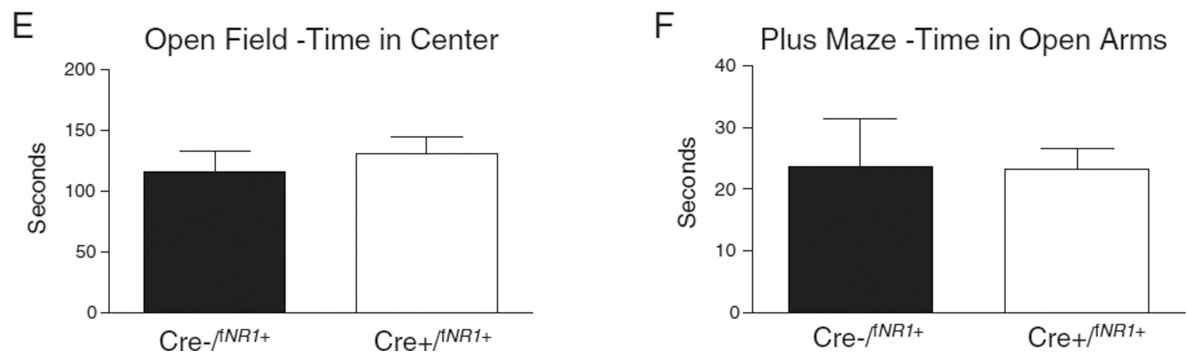
Fig. 1.
CRF GABAAα1 deficient mice show enhanced anxiety while CRF NR1 deficient mice show no difference from controls in anxiety. (A) CRF GABAAα1 deficient mice (Cre+/f*GABAAα1*) spent significantly less time in the open arm of the plus maze than Cre−/fGABAα1 mice. (B) Cre+/f*GABAAα1* mice also spent significantly less time in the center of an open field compared to Cre−/f*GABAAα1* mice. Both (C) systemic administration prior to the plus maze test and (D) BNST infusion prior to the open field of the CRF receptor 1 antagonist R121919 rescued the anxious phenotype in Cre+/f f*GABAAα1* mice. However,
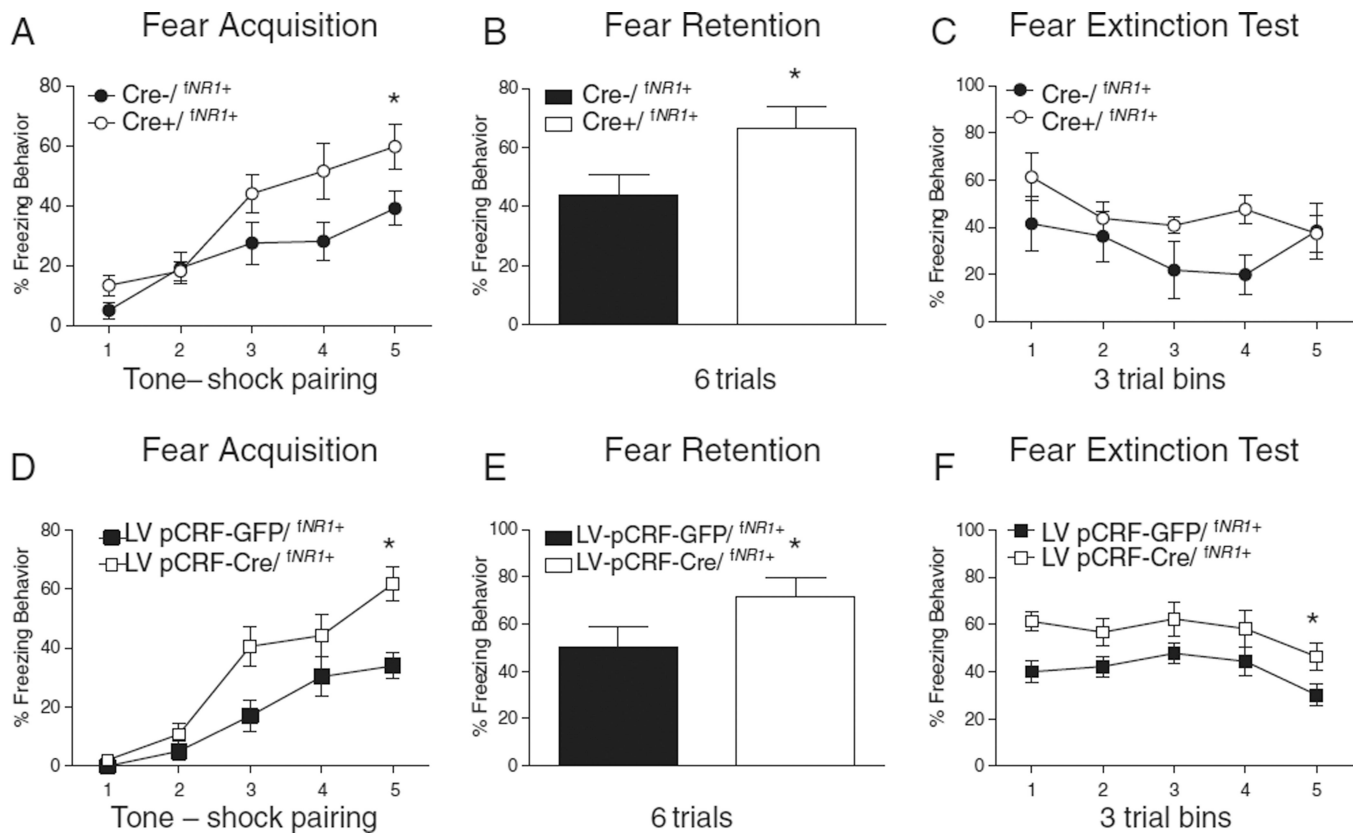
in CRF NMDAR1 deficient mice (Cre+/f*NR1*+) there were no differences in anxiety behavior in either (E) open field or (F) plus maze test compared to Cre−/f*NR1*+. Figures are adapted from recent work by Gafford et al. (2012, 2014).

Fig. 2.
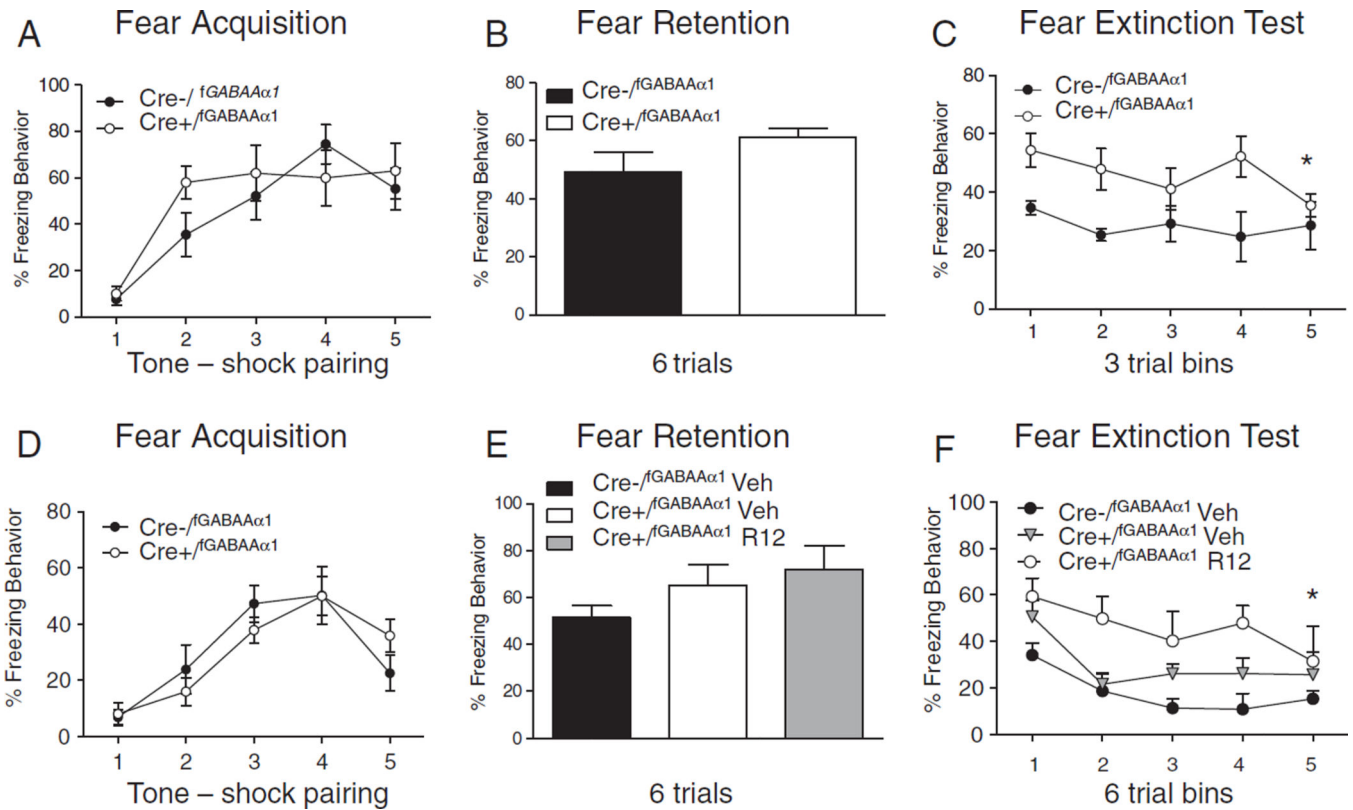Disruption of CRF NMDAR1 (NR1) neurons significantly disrupts fear acquisition without effects on fear extinction retention. (A) CRF NR1 deficient mice (Cre+/f*NR1*+) show significantly enhanced fear acquisition and (B) fear retention compared to Cre−/f*NR1*+ mice with no significant difference during the (C) fear extinction test. A follow-up experiment infused CRF promoter driven lentivirus into the CeA of f*NR1* mice resulting in disrupted CRF NR1 neurons only within the CeA. We again show significantly enhanced freezing behavior during (D) fear acquisition for mice with disruption of CRF NR1 neurons in the CeA (LV pCRF-Cre/f*NR1*+) compared to control virus infused mice (LV pGFP-Cre/f*NR1*+). LV pCRF-Cre/f*NR1*+ mice showed significantly enhanced (E) fear retention and (F) impaired fear extinction retention. Asterisk (*) indicates significant group difference. Figures are adapted from Gafford et al. (2014).

Fig. 3.
Disruption of CRF GABAAα1 neurons has no effect on fear acquisition but significantly disrupts fear extinction. (A) CRF GABAAα1 deficient mice (Cre+/f*GABAA*α*1*) show no difference from Cre−/f*GABAA*α*1* mice during fear conditioning or when tested for (B) fear retention. However, during the (C) fear extinction retention test they shown significant deficits. A follow-up experiment again shows no difference in freezing behavior during (D) fear acquisition or (E) fear retention for CRF GABAAα1 deficient mice (Cre+/f*GABAA*α*1*). However, Cre+/f *GABAA*α*1* mice given a systemic infusion of a CRF receptor 1 antagonist (R121919) prior to fear extinction show a (E) partial rescue of fear extinction behavior during the (F) fear extinction retention test compared to vehicle injected controls. Asterisk (*) indicates significant group difference.
Figures are adapted from Gafford et al. (2012).

Table 1

Findings from amygdala infusion studies related to CRF regulation of fear behavior.

Citation	Structure targeted	Drug infused	Drug action	Timing of infusion	Behavior tested	Results
Liang and Lee (1988)	Amygdala	CRH	Increases CRF	Immediately post training	Inhibitory avoidance	Improved retention
Roozendaal et al. (2002)	BLA	[9–41]-α-helical CRF	CRF receptor antagonist	Immediately post training	Inhibitory avoidance	Impairs retention.
				3 hour post training		No effect
	CeA			Immediately post training		No effect
Roozendaal et al. (2008)	BLA	CRF$_{6-33}$	CRF-binding protein ligand inhibitor	Immediately post training	Inhibitory avoidance	Enhancement of retention
		CRF$_{6-33}$ + α-helical CRF$_{9-41}$	CRF-binding protein ligand inhibitor + CRF receptor antagonist	Immediately post training		Blockade of retention enhancement
Swiergiel et al. (1993)	CeA	CRF$_{9-41}$,	CRF antagonist	Pre training	Contextual fear conditioning	Disrupted post shock freezing
				Pre testing		Disrupted freezing during test
Hubbard et al. (2007)	BLA	DMP696	CRF$_1$ receptor antagonist	5 min post training	Contextual fear conditioning	disrupted retention
				3 hour post training		disrupted retention
				9 hour post training		No effect
	CeA	DMP696	CRF$_1$ receptor antagonist	5 min post training		No effect
				3 hour post training		No effect
				9 hour post training		No effect
Pitts et al. (2009)	CeA	CRF antisense	Inhibits CRF	Pre training	Contextual fear conditioning	Disrupted retention
				Pre testing		No effect
Pitts and Takahashi (2011)	CeA	CRF antisense	Inhibits CRF	5 m post training	Contextual fear conditioning	Disrupted retention
				9 hour post training		Disrupted retention
				24 hour post training		Disrupted retention
				96 hour post training		No effect
Isogawa et al. (2013)	LA	CRH	Increases CRF	One hour pre training	Auditory fear conditioning	Disrupts retention

Citation	Structure targeted	Drug infused	Drug action	Timing of infusion	Behavior tested	Results
	CeA			Immediately post training		Disrupts retention
				1 hour pre testing		Disrupted retention
				One hour pre training		No effect
				Immediately post training		No effect
				1 hour pre testing		No effect