Published in final edited form as:

Neuropeptides. 2013 August ; 47(4): 273–280. doi:10.1016/j.npep.2012.12.004.

DIFFERENTIAL ACTIVATION OF NEURONAL CELL TYPES IN THE BASOLATERAL AMYGDALA BY CORTICOTROPIN RELEASING FACTOR

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Abstract

Enhanced CRF release in the BLA is strongly associated with the generation of behavioral stress responses through activation of the CRF-R1 receptor subtype. Stress and anxiety-like behavior are modulated in part by the balance of peptide actions such as excitatory corticotropin releasing factor (CRF) and inhibitory neuropeptide Y (NPY) receptor activation in the basolateral nucleus of the amygdala (BLA). While the actions of CRF are clear, little is known about the cell type influenced by CRF receptor stimulation. These studies were designed to identify the cell types within the BLA activated by intra-BLA administration of CRF using multi-label immunohistochemistry for cFos and markers for pyramidal (CaMKII-immunopositive) and interneuronal [glutamic acid decarboxylase (GAD65)] cell populations. Administration of CRF into the BLA produced a dose-dependent increase in the expression of cFos-ir. Intra-BLA injection of CRF induced significant increases in cFos-ir in the CaMKII-ir population. Although increases in cFos-ir in GAD65-ir cells were observed, this did not reach statistical significance perhaps in part due to the decreased numbers of GAD65-ir cells within the BLA after CRF treatment. These findings demonstrate that CRF, when released into the BLA, activates projection neurons and that the activity of GABAergic interneurons is also altered by CRF treatment. Decreases in the number of GAD65-ir neurons could reflect either increased or decreased activity of these cells and future studies will more directly address these possibilities. The expression of increased cFos is associated with longer term regulation of gene expression which may be involved in the profound long term effects of neuropeptides, such as CRF, on the activity and plasticity of BLA pyramidal neurons.

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The authors do not have any financial or personal conflicts of interest.

Keywords

CaMKII; anxiety; interneuron; pyramidal neuron; GAD; GABA; CRF

Introduction

The generation of stress results from the integration of a number of environmental stimuli perceived by the limbic system which then produces the appropriate endocrine and behavioral responses. The amygdala is one brain region central to the acquisition and expression of emotional memory. Specifically, the basolateral nucleus of the amygdala (BLA) is integral to the regulation of fear and anxiety responses (Maren et al., 1996; Koo et al., 2004; Pare and Duvarci, 2012). The activity of the BLA is tightly regulated by the balance of excitatory (glutamatergic) and inhibitory (GABAergic) signals. Disruption of this balance results in the generation of fear and stress responses which can be reduced by administration of anxiolytic compounds (Zhang et al., 2000; Faria et al., 2012). In addition to the excitatory (glutamatergic) and inhibitory (GABAergic) transmitters, a number of other neurotransmitters, including neuropeptides, relay sensory information to the amygdala and contribute to the production of appropriate behavioral responses (Sajdyk et al., 2004; Alldredge, 2010).

Corticotropin-Releasing Factor (CRF) is a 41 amino acid peptide that is a member of a peptide family which also includes the urocortins (Ucns) I, II and III (Donaldson et al., 1996; Reyes et al., 2001). CRF was first characterized as a hypothalamic releasing factor, eliciting adrenocorticotropin hormone (ACTH) release from the pituitary (Vale et al., 1981). In addition to hypothalamic-pituitary-adrenal (HPA) axis activation, CRF also functions as a neurotransmitter in extra-hypothalamic brain regions (Merchenthaler, 1984; Rainnie et al., 2004). These peptides (CRF and Ucns) function through activation of two G-protein coupled receptors, CRF-R1 and CRF-R2. Binding of CRF, or the CRF-related peptide urocortin I (UCN), to these receptors stimulates adenylyl cyclase and increases intracellular levels of cAMP (Behan et al., 1996; Grammatopoulos, 2012). CRF expression has been identified in limbic brain regions including the BLA (Fischman and Moldow, 1982; Merchenthaler, 1984) where the CRF-R1 receptor is expressed and associated with glutamatergic neuronal activity (Justice et al., 2008; Refojo et al., 2011).

Central delivery of CRF or Ucn, either intracerebroventricularly (i.c.v.) or directly to the amygdala, increases anxiety-like behaviors as measured by social interaction (SI) (Dunn and File, 1987; Gehlert et al., 2005; Spiga et al., 2006), elevated plus maze and Geller-Seifter tests (Spina et al., 2002). Furthermore, CRF release, as assessed by microdialysis, is increased in the amygdala during acute restraint stress (Merlo et al., 1995; Merali et al., 1998). The CRF-R1 receptor mediates many of the anxiogenic effects of CRF (Heinrichs et al., 1997) and in support of this, CRF-R1 KO animals demonstrate impaired stress responses (Timpl et al., 1998). Conversely, CRF-overexpressing transgenic mice exhibit increased anxiety behaviors that can be attenuated by treatment with a CRF-R1/2 antagonist (Stenzel-Poore et al., 1994). Animals exposed to environmental enrichment exhibit decreases in stress-related behavior which is correlated with lower expression of CRF-R1 within the BLA (Sztainberg et al., 2010).

The present studies were designed to determine the cell type(s) in the BLA that mediates the actions of CRF. The BLA consists of two types of neurons, spiny pyramidal glutamatergic projection neurons, identified by the expression of Calcium Calmodulin Kinase II (CaMKII), and non-spiny GABA-ergic interneurons that do not express CaMKII (Hall, 1972; McDonald, 1982; McDonald and Pearson, 1989; Rostkowski et al., 2009). To

determine whether BLA pyramidal or interneuron populations are preferentially activated by CRF, we assessed immunoreactivity for the immediate early gene (IEG) cFos and the BLA pyramidal neuron marker, CaMKII, or the GABAergic cell marker, GAD (glutamic acid decarboxylase) following CRF delivery to the BLA. Determining the cellular substrates mediating the actions of CRF is an important step in elucidating the mechanisms underlying the control of anxiety and related disorders.

MATERIALS AND METHODS

Animals

Adult male (250–350g) Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were housed, unless otherwise noted, 3 to a conventional cage, with free access to standard lab chow and water, and maintained in a temperature (20–22°C), humidity (50–55%) and illumination (14:10h light:dark cycle) controlled, AAALAC-approved facility. Five days of acclimatization to our facilities were allowed before the animals were included in any experimental procedures. All procedures were approved by the Rosalind Franklin University of Medicine and Science (RFUMS) Institutional Animal Care and Use Committee (IACUC).

Implantation of BLA Cannulae

Animals were deeply anesthetized with ketamine/xylazine (90/20 mg/kg i.p. in 1 ml/kg volume; Wyeth, Madison, NJ). After confirming anesthesia by tail pinch, rats were placed in a stereotaxic apparatus (Stoelting, Wood Dale, IL) and the skull was exposed via longitudinal midline incision. After flushing the wound with lidocaine (2% in normal saline, Sigma-Aldrich, St. Louis, MO), a small hole was drilled in the skull. A 26-gauge stainless steel guide cannula (Plastics One, Roanoke, VA) was implanted through the hole in the right BLA at the following coordinates: anteroposterior: –2.6; mediolateral: –5.0; dorsoventral: –7.4; incisor bar: –3.3 mm according to a standard stereotaxic atlas of the rat brain (Paxinos and Watson, 1998). The cannula was secured to the skull with three stainless steel screws (2.8 mm; Plastics One) and acrylic dental cement (Flexacryl Hard, Lang Dental Mfg Co., Inc., Wheeling, IL). A stylet was placed in each cannula to maintain patency. After surgery, animals were given a non-steroidal anti-inflammatory drug (flunixin meglumine, 1.5mg/kg s.c., Sigma-Aldrich, St. Louis, MO) and were housed separately. During the five day recovery period, animals were handled daily and stylets were manipulated to acclimate the animals to the microinjection procedure.

Intracranial Injection Procedures

On the day of the experiment, the animals were brought to the experimental room and allowed to acclimate for 90 min before the stylets were replaced with injectors. Acute microinjections of all compounds were delivered via injectors (33 gauge; Plastics One) that fit into, and extended 1 mm beyond, the guide cannula. A cannula connector (Plastics One) was used to attach the injector to a 1cc Becton-Dickinson plastic syringe which was placed on an SP2201 infusion pump (WPI, Inc., Sarasota, FL) for delivery of equal volumes of vehicle [artificial cerebrospinal fluid (aCSF: 125 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 2.5 mM CaCl₂, 20 mM glucose, 1mM NaH₂PO₄, 25 mM NaHCO₃)] or appropriate concentration of peptide. Injector patency was confirmed after removal to ensure proper flow and that the injector had not become clogged during the injection procedure.

CRF was obtained courtesy of Dr. J. Rivier (The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, CA). Different doses of CRF (2, 20, 200 or 2000 fmol or 0.95, 9.5, 95, 950 pg, respectively) or vehicle (aCSF) were delivered in 250nL volume over 90 sec. These doses were chosen on the basis that 200 fmol of CRF has been

shown to exhibit a robust increase in anxiety behaviors (Sajdyk et al., 1999). The injection cannula remained in place for an additional 2 minutes to ensure complete delivery of the solution through the guide cannula. The injector was then removed, the stylet replaced and the animal returned to the home cage. Ninety minutes following CRF or vehicle delivery, animals were perfused for immunocytochemistry.

Verification of Probe Placement

Only animals with injectors inserted within the borders of the BLA were included in these studies. Probe placement was verified histologically. A schematic representation of typical injector placements for these studies and an example of a proper probe placement is shown in Figure 1. Five animals were excluded from these studies due to improper injector placement.

Tissue Preparation for Immunocytochemistry

Animals were deeply anesthetized with sodium pentobarbital (Sigma-Aldrich, St. Louis, MO, 100mg/kg i.p.) and transcardially perfused with 30mL of phosphate buffered saline (PBS: 10mM Na₂HPO₄, 150mM NaCl, pH 7.5) containing 0.1% procaine and 100U/mL heparin at 37°C followed by 60mL fixative solution consisting of 4% paraformaldehyde (PFA) in PBS at +4°C. Brains were rapidly dissected out, post-fixed overnight in 4% PFA solution, followed by an hour-long PBS wash at +4°C. Coronal brain sections were cut in a bath of ice-cold PBS at 40µm thickness using a vibratome (Vibratome 1000, Ted Pella, Inc., Redding, CA).

Multiple Label Immunocytochemistry

cFos & CaMKII or GAD—A biotinylated tyramide amplification immunofluorescence protocol adapted from Adams (1992) was used. Briefly, free-floating sections were rinsed through 3 changes of PBS over 10 minutes, followed by a 15 min wash in 1% H₂O₂ in PBS to diminish endogenous peroxidase activity. Next, tissues were blocked for 3 hours in immunocytochemistry (ICC) buffer (0.1 M PBS containing 0.2% gelatin, 0.01% thimerosal and 0.002% neomycin, pH 7.5) containing 0.1% TritonX-100 and 5% normal donkey serum (NDS; Equitech-Bio, Kerrville, TX) to block non-specific binding. Sections were then incubated at +4°C for 72 hours with anti-cFos antibody (1:12,000, rabbit; EMD, Madison, WI) in ICC with 2% NDS and 0.1% TritonX-100. Following incubation with primary antibody, sections were washed through 5 changes of ICC buffer over 50 minutes and then incubated with biotinylated, affinity purified donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; 1:2,000) for 1hr at room temperature. After ICC buffer rinses, sections were incubated in Vectastain Elite ABC (Vector Laboratories, Burlingame, CA; 2 µL/mL) for 30 minutes. Next, sections were rinsed with PBS and incubated in biotinylated tyramide solution (3 µg/mL biotinylated tyramide and 0.01% H₂O₂ in PBS) for 10 minutes. Tissues were then rinsed in ICC buffer and immersed in ICC buffer containing fluorescein isothiocyanate conjugated streptavidin (FITC-SA; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; 1:250) for 3 hours. Following washes in 4 changes of tris-buffered saline (TBS: 100mM Tris base, 150mM NaCl, pH 7.5) over 20 minutes, sections were incubated with antibody directed against CaMKII (1:5000, mouse monoclonal, clone 6G9, Millipore, Billerica, MA) or GAD65 (1:900, mouse monoclonal, Cat# MAB351, clone 6C9, Chemicon International, Temecula, CA) in ICC buffer with 2% NDS. Sections were subsequently washed through 5 changes of ICC buffer over 50 minutes followed by a 3 hour incubation in ICC buffer containing Cy3 donkey anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; 1:250) for visualization of CaMKII and GAD. Following 2 changes of TBS over 10 minutes, sections were briefly incubated with the nucleic acid stain ToPro-5 (1:5000,

Invitrogen, Corp., Carlsbad, CA) in TBS for 5 min. Following 2 rinses in TBS, sections were mounted onto Superfrost+ slides and coverslips were applied with 2.5% polyvinyl alcohol-1,4-diazabicyclo[2.2.2]octane (PVADABCO) anti-fade mounting medium.

Antibody Specificity

The CaMKII mouse monoclonal antibody used was raised against the α -subunit of CaMKII and recognizes both phosphorylated and nonphosphorylated CaMKII. Its specificity has been documented and produces a single band on Western blots at the expected weight of 50 kDa (Erondu and Kennedy, 1985). GAD antiserum was raised against purified GAD from rat brains and recognizes the lower molecular weight form of GAD (GAD65) as determined by western blot using rat brain tissue (Chang and Gottleib, 1988). Immunohistochemical labeling of BLA neurons with this antibody is consistent with that observed using other widely used GAD or GABA antibodies (Rostkowski et al., 2009). Additionally, omission of the primary antibody from the incubation mixture resulted in complete loss of staining and signal was only evident in the appropriate channels.

Confocal Microscopy and Stereological Procedures

All experiments required quantification of multiple antigens in cells that were labeled with multiple fluorescent markers. Accordingly, sections were analyzed using scanning laser confocal stereology. Images of the BLA were captured at 10 $\times$ magnification and the borders of the nucleus were manually outlined for each section using an Olympus Fluoview 300 microscope (Olympus, Melville, NY) equipped with a motorized x - y - z stage control. Starting at a random point, every 6th section (240 μ m apart) was taken for analysis for a total of 10 sections throughout the rostral-caudal extent of the nucleus from bregma -1.8 mm to bregma -4.16 mm according to Paxinos and Watson (1998). StereoInvestigator software (MBF Bioscience, Williston, VT) was used to implement the optical fractionator counting procedure (West et al., 1991; Peterson, 1999) and generate unbiased counting frames. The stereological parameters, which resulted in a sampling fraction of 1/500th of the BLA, included the following: sampling grid: $x = 500\mu\text{m}$; $y = 500\mu\text{m}$, optical dissector counting frame: $100\mu\text{m} \times 100\mu\text{m}$ (cFos/CaMKII study) or $240\mu\text{m} \times 180\mu\text{m}$ (cFos/GAD study), counting frame thickness: $10\mu\text{m}$ with a $2\mu\text{m}$ guard zone on either side. These parameters were employed for each experiment described. A mean of 56 ± 1 sites were analyzed for the cFos/CaMKII study and 57 ± 1 sites for the cFos/GAD study.

At each systematic randomly selected site, a serial confocal stack of each fluorophore was individually captured on the appropriate emission channel using a 60 $\times$ oil immersion objective (1.4 numerical aperture). The following excitation wavelengths were used: 488nm for the secondary fluorophore FITC, 568nm for Cy3 and 647nm for Cy5. Stacks were then merged and saved for counting offline. Colocalization was determined by overlapping signals observed at several focal planes through each cell by an experimenter blind to treatment groups. All cells whose nucleus came into focus within the inclusion limits of unbiased counting frames were counted. The section thickness was recorded at 3 sites per section and the average section thickness per animal was determined for stereological estimate calculations. Total neuron estimates were calculated by the software, using the numbers of counted neurons and the corresponding sampling probability. Brightness and contrast of the photomicrographs presented here were adjusted using Adobe Photoshop 6.0 to ensure the highest quality images for publication.

Statistical Analysis

Data are reported as mean $\pm$ SEM. Data were analyzed via Student's unpaired t-test or One-Way ANOVA followed by a Student-Newman-Keuls post-test where appropriate. Statistical significance was set at $p < 0.05$.

RESULTS

cFos-ir following CRF treatment in the BLA

Compared to vehicle-treated controls, CRF induced a robust increase in cFos-ir in the BLA on the injected side. No discernable cFos-ir, other than a few scattered cells, was observed on the non-injected side. Photomicrographs of representative fields of cFos-ir in the BLA after delivery of different doses of CRF are presented in Figure 2. Administration of CRF induced a dose dependent increase in cFos-ir that generated an inverted-U shape dose response curve [Figure 3; ($F=6.216$, $r^2=0.5305$, $df=4$, $p=0.0017$)]. cFos-ir (Figure 2, column 1, green) was colocalized with the nuclear marker ToPRO-5 (Figure 2, column 2, blue) indicating that cFos-ir was exclusively present in the nucleus as expected for a transcription factor. Negligible cFos immunoreactivity was present in the vehicle-treated (aCSF) group (Figure 2A1 and Figure 3). There was no significant difference in the number of cFos-ir cells between the 2 fmol ($12,469 \pm 4250$, $n=6$) and vehicle-treated groups (2168 ± 586 , $n=5$; Figure 3). Intra-BLA administration of 20 fmol ($24,436 \pm 5880$ cells, $n=4$) and 2000 fmol ($20,228 \pm 6033$, $n=5$) CRF significantly increased the number of cFos immunoreactive cells when compared to vehicle (aCSF)-treated controls (2168 ± 586 , $n=5$). The 200 fmol dose of CRF induced significantly more cFos-ir cells in the BLA ($30,942 \pm 4305$, $n=7$) compared to both vehicle- ($p<0.01$) and 2 fmol CRF-treated groups ($p<0.05$).

The 20 fmol CRF dose was chosen for post-injection cFos/CaMKII analysis since it elicited a submaximal response in cFos-ir. Choosing this dose was also important in that it avoided potential confounds of a dose on the inverted side of the dose response curve. Photomicrographs of cFos-ir in CaMKII-ir cells after vehicle or CRF treatment are depicted in Figure 4. In CRF-treated animals, many more cFos-ir/CaMKII-ir neurons are seen (Figure 4F, arrowheads) compared to aCSF-treated controls (Figure 4C). The results of the stereological analysis of cFos expression in CaMKII-ir cells are represented in Table 1. The number of double-labeled cells (cFos-ir/CaMKII-ir), i.e. the population representing activated pyramidal neurons, was significantly different [Student's t-test, unpaired ($t=3.363$, $df=5$, $p=0.0200$)] between vehicle control (1061 ± 601) and CRF-treated groups (9208 ± 2769). In the vehicle-treated control animals, $35.7 \pm 8.1\%$ of cFos-ir was expressed in CaMKII-ir (pyramidal neurons) compared with $64.2 \pm 8.1\%$ of cells single-labeled for cFos-ir. The degree of cFos expression in GABAergic (GAD-ir) interneurons is presented in Table 2. There are few GAD-ir neurons expressing cFos in the vehicle group (figure 4I), and while there appears to be an increase in this population after CRF administration (figure 4L), this is not statistically significant. Examination of the total cell counts for GAD-ir neurons indicates a significant decrease in the overall numbers of these cells after CRF injection. The number of CaMKII-ir cells remains unchanged 90 minutes after CRF injection (Figure 5; Tables 1 and 2).

DISCUSSION

The current studies demonstrate that intra-BLA administration of CRF, at anxiogenic doses, induces dose-dependent increases in cFos immunoreactivity in pyramidal cells indicating that these cells are a site of action of CRF. cFos immunoreactivity is also increased in GAD-ir interneurons of the BLA. While CRF treatment did not alter the total number of CaMKII-ir cells within the BLA, the overall number of GAD-ir interneurons was significantly decreased. In sum, these data suggest that CRF can stimulate BLA output (pyramidal) cells and that cFos-related signal transduction systems are part of the signaling pathways in the BLA.

The selection of cFos as a marker for cellular activation by CRF was based on a number of criteria. Firstly, the transcription of cFos in response to changes in neuronal and/or

metabolic activity is well documented (Sagar et al., 1988; Dragunow and Faull, 1989; Curran and Morgan, 1995). Secondly, cFos has been implicated in the formation of fear memories (Campeau et al., 1991; Beck and Fibiger, 2000; Reijmers et al., 2007; Lehner et al., 2008) and lastly, the second messenger systems to which CRF-R1 are coupled are known to be involved in cFos transcription. CRF receptors typically link to G_s proteins which activate adenylyl cyclase and increase intracellular cAMP (Chen et al., 1986; Battaglia et al., 1987) which leads to activation of cAMP response element binding protein (CREB) activation and cFos transcription (Ahn et al., 1998; Mayr and Montminy, 2001; Stanciu et al., 2001). Under basal conditions few cFosimmunopositive cells were identified in the BLA (Rostkowski and Urban, unpublished observations). The low cFos expression in aCSF vehicle-treated animals suggests that subsets of BLA neurons are tonically active. Injection of CRF into the BLA caused a reliable and dose-related increase in cFos-ir, and resulted in an inverted U-shaped curve with the highest doses of CRF eliciting smaller changes in cFos than moderate doses. Similar inverted U-shaped responses have been reported for anxiety-like behaviors following intra-amygdalar CRF delivery (Liang and Lee, 1988; Elkabir et al., 1990). This lower expression of cFos at higher doses of CRF might represent an increase in various compensatory mechanisms, such as increased GABA release or involvement of inhibitory neurotransmitters or neuropeptides, which are recruited to counteract the stressful stimuli and inhibit pyramidal cell activity.

CRF treatment produced a much higher proportion of cFos-ir expression in pyramidal neurons (CaMKII-ir cells) than in interneurons (GAD65-ir cells), a profile expected of an anxiogenic compound. This increase in cFos could result from a direct effect of CRF on the pyramidal cells (Justice et al., 2008; Giesbrecht et al., 2010). While these results support a direct role for CRF at the pyramidal cell body, there were also increases in cFos noted in interneurons. The relative percent of GAD65-ir cells expressing cFos-ir 90 minutes after CRF injection was about 10.3%. However, whether this accurately reflects the total number of GAD65/cFos-ir cells is obscured by the observation of a significant decrease in the overall number of GAD65-ir cell after acute CRF administration. The apparent activation of interneurons could result either from direct activation of CRF receptors on these cells or via feed-forward compensatory mechanisms from the pyramidal cells to activate subsets of interneurons. Previous studies indicate that stress-induced increases of CRF in the BLA (Lehner et al., 2008) produce GABAergic disinhibition (Rodriguez Manzanares et al., 2005; Isoardi et al., 2007), decreased GABA release (Stork et al., 2002) and GABA_A receptor-mediated neurotransmission (Martijena et al., 2002). While these previous studies examine the more immediate effects of CRF, these current data demonstrate a decrease in the expression of GAD65 immunoreactivity in the BLA 90 minutes after CRF treatment. The loss of GAD65 immunoreactivity could reflect a decrease in the synthesis of the protein or an increased turnover should these cells be stimulated by increased BLA glutamatergic neurotransmission. Further studies using *in situ* hybridization to assess changes in GAD65 gene expression would be useful to parse out these two options.

Additionally, while we used a general marker for GABAergic cells, it will be important to identify which subclass of interneuron, based on calcium binding protein profiles, was preferentially affected by this treatment. Hale et al., (2010) demonstrated that serotonin (5-HT) receptor 2A/parvalbumin-expressing interneurons are part of a dorsal raphe (DR)-BLA anxiety-related circuit whose numbers increase in response to a stressor. In the cortex, Mohila and Onn (2005) demonstrated CRF-ir contacts specifically on parvalbumin-ir interneurons. Together our data regarding the effects of CRF in the BLA provide an anatomical basis leading to the subsequently observed increase in pyramidal neuron activity and synchronicity seen in stressful or anxiety-provoking states (Pare and Collins, 2000). Our histological findings are consistent with both direct and indirect mechanisms of CRF on pyramidal neuron activity and subsequent BLA neuronal output.

While the present studies utilized acute delivery of CRF to the BLA, others have shown that repeated NPY or CRF receptor activation results in enduring changes in stress-coping, stress-resilience or stress-sensitivity (Sanders et al., 1995; Sajdyk et al., 1999; Rainnie et al., 2004; Shekhar et al., 2005; Truitt et al., 2007; Sajdyk et al., 2008). These long-lasting effects are likely mediated through the same receptor types as the acute effects but appear to additionally require additional mediators often associated with long-term potentiation (LTP; CaMKII) for CRF (Rainnie et al., 2004) or long-term depression (LTD; calcineurin) for NPY (Sajdyk et al., 2008). Comparison of the patterns of cFos expression in acute and repeated treatment with NPY and CRF will likely illuminate differences in activated neuronal populations in normal stress responses (acute) and pathological stress states (chronic). Future studies will focus on the downstream mechanisms that underlie the actions of CRF and contribute to the behavioral state of the individual.

Acknowledgments

The authors gratefully acknowledge Dr. Daniel A. Peterson for assistance with stereology and the Rosalind Franklin University of Medicine and Science Multiuser Microscopy and Imaging Facility. The authors also thank Ms. Gina DeJoseph for her excellent technical assistance.

This work was supported by NIH grants MH62621 and MH090927

References

- Adams JC. Biotin amplification of biotin and horseradish peroxidase signals in histochemical stains. *J Histochem Cytochem.* 1992; 40:1457–1463. [PubMed: 1527370]
- Ahn S, Olive M, Aggarwal S, Krylov D, Ginty DD, Vinson C. A dominant-negative inhibitor of CREB reveals that it is a general mediator of stimulus-dependent transcription of c-fos. *Mol Cell Biol.* 1998; 18:967–977. [PubMed: 9447994]
- Allredge B. Pathogenic involvement of neuropeptides in anxiety and depression. *Neuropeptides.* 2010; 44(3):215–224. [PubMed: 20096456]
- Battaglia G, Webster EL, De Souza EB. Characterization of corticotropin-releasing factor receptor-mediated adenylate cyclase activity in the rat central nervous system. *Synapse.* 1987; 1:572–581. [PubMed: 2843998]
- Beck CHM, Fibiger HC. Conditioned fear-induced changes in behavior and in the expression of the immediate early gene c-fos: with and without diazepam pretreatment. *J Neuroscience.* 2000; 15:709–720.
- Behan DP, Grigoriadis DE, Lovenberg T, Chalmers D, Heinrichs S, Liaw C, De Souza EB. Neurobiology of corticotropin releasing factor (CRF) receptors and CRF-binding protein: implications for the treatment of CNS disorders. *Mol Psychiatry.* 1996; 1:265–277. [PubMed: 9118350]
- Campeau S, Hayward MD, Hope BT, Rosen JB, Nestler EJ, Davis M. Induction of the c-fos proto-oncogene in rat amygdala during unconditioned and conditioned fear. *Brain Res.* 1991; 565:349–352. [PubMed: 1842702]
- Chang YC, Gottlieb DI. Characterization of the proteins purified with monoclonal antibodies to glutamic acid decarboxylase. *J Neurosci.* 1988 Jun; 8(6):2123–2130. 1988. [PubMed: 3385490]
- Chen FM, Bilezikjian LM, Perrin MH, Rivier J, Vale W. Corticotropin releasing factor receptor-mediated stimulation of adenylate cyclase activity in the rat brain. *Brain Res.* 1986; 381:49–57. [PubMed: 3019476]
- Chung L, Moore SD. Cholecystokinin enhances GABAergic inhibitory transmission in basolateral amygdala. *Neuropeptides.* 2007; 41:453–463. [PubMed: 17904218]
- Curran T, Morgan JI. Fos: an immediate-early transcription factor in neurons. *J Neurobiol.* 1995; 26:403–412. [PubMed: 7775973]
- Donaldson CJ, Sutton SW, Perrin MH, Corrigan AZ, Lewis KA, Rivier JE, Vaughan JM, Vale WW. Cloning and characterization of human urocortin. *Endocrinology.* 1996; 137:3896. [PubMed: 8756563]

- Dragunow M, Faull R. The use of c-fos as a metabolic marker in neuronal pathway tracing. *J Neurosci Methods*. 1989; 29:261–265. [PubMed: 2507830]
- Dunn AJ, File SE. Corticotropin-releasing factor has an anxiogenic action in the social interaction test. *Horm Behav*. 1987; 21:193–202. [PubMed: 2886414]
- Elkabir DR, Wyatt ME, Vellucci SV, Herbert J. The effects of separate or combined infusions of corticotrophin-releasing factor and vasopressin either intraventricularly or into the amygdala on aggressive and investigative behaviour in the rat. *Regul Pept*. 1990; 28:199–214. [PubMed: 2343163]
- Erondu NE, Kennedy MB. Regional distribution of type II Ca^{2+} /calmodulin-dependent protein kinase in rat brain. *J Neurosci*. 1985; 5:3270–3277. [PubMed: 4078628]
- Faria V, Appel L, Åhs F, Linnman C, Pissioti A, Frans Ö, Bani M, Bettica P, Pich EM, Jacobsson E, Wahlstedt K, Fredrikson M, Furmark T. Amygdala subregions tied to SSRI and placebo response in patients with social anxiety disorder. *Neuropsychopharmacology*. 2012; 37(10):2222–2232. [PubMed: 22617357]
- Fischman AJ, Moldow RL. Extrahypothalamic distribution of CRF-like immunoreactivity in the rat brain. *Peptides*. 1982; 3:149–153. [PubMed: 6980401]
- Gehlert DR, Shekhar A, Morin SM, Hipskind PA, Zink C, Gackenhaimer SL, Shaw J, Fitz SD, Sajdyk TJ. Stress and central Urocortin increase anxiety-like behavior in the social interaction test via the CRF1 receptor. *Eur J Pharmacol*. 2005; 509:145–153. [PubMed: 15733549]
- Giesbrecht CJ, Mackay JP, Silveira HB, Urban JH, Colmers WF. Countervailing modulation of Ih by neuropeptide Y corticotrophin-releasing factor in basolateral amygdala as a possible mechanism for their effects on stress-related behaviors. *J Neurosci*. 2010; 30:16970–16982. [PubMed: 21159967]
- Grammatopoulos DK. Insights into mechanisms of corticotropin-releasing hormone receptor signal transduction. *Br J Pharmacol*. 2012; 166(1):85–97. [PubMed: 21883143]
- Hale MW, Johnson PL, Westerman AM, Abrams JK, Shekhar A, Lowry CA. Multiple anxiogenic drugs recruit a parvalbumin-containing subpopulations of GABAergic interneurons in the basolateral amygdala. *Prog Neuropsychopharmacol Biol Psychiatry*. 2010; 34:1285–1293. [PubMed: 20647026]
- Hall E. The amygdala of the cat: a golgi study. *Z Zellforsch Mikrosk Anat*. 1972; 134:439–458. [PubMed: 4638299]
- Heinrichs SC, Lapsansky J, Lovenberg TW, De Souza EB, Chalmers DT. Corticotropin-releasing factor CRF_1 , but not CRF_2 , receptors mediate anxiogenic-like behavior. *Reg Peptides*. 1997; 71:15–21.
- Isoardi NA, Bertotto ME, Martijena ID, Molina VA, Carrer HF. Lack of feedback inhibition on rat basolateral amygdala following stress or withdrawal from sedative-hypnotic drugs. *Eur J Neurosci*. 2007; 26:1036–1044. [PubMed: 17666080]
- Justice NJ, Yuan ZF, Sawchenko PE, Vale W. Type 1 corticotropin-releasing factor receptor expression reported in BAC transgenic mice: implications for reconciling ligand-receptor mismatch in the central corticotropin-releasing factor system. *J Comp Neurol*. 2008; 511(4):479–496. [PubMed: 18853426]
- Koo JW, Han JS, Kim JJ. Selective neurotoxic lesions of basolateral and central nuclei of the amygdala produce differential effects on fear conditioning. *J Neuroscience*. 2004; 24:7654–7662.
- Lehner M, Taracha E, Skorzewska A, Turzynska D, Sobolewska A, Maciejak P, Szyndler J, Hamed A, Bidzinski A, Wislowska-Stanek A, Plaznik A. Expression of c-Fos and CRF in the brains of rats differing in the strength of a fear response. *Behav Brain Res*. 2008; 188:154–167. [PubMed: 18067977]
- Liang KC, Lee EH. Intra-amygdala injections of corticotropin releasing factor facilitate inhibitory avoidance learning and reduce exploratory behavior in rats. *Psychopharmacology (Berl)*. 1988; 96:232–236. [PubMed: 3148150]
- Maren S, Aharonov G, Fanselow MS. Retrograde abolition of conditional fear after excitotoxic lesions in the basolateral amygdala of rats: absence of a temporal gradient. *Behav Neurosci*. 1996; 110:718–726. [PubMed: 8864263]

- Martijena ID, Rodriguez Manzanares PA, Lacerra C, Molina VA. Gabaergic modulation of the stress response in frontal cortex and amygdala. *Synapse*. 2002; 45:86–94. [PubMed: 12112401]
- Mayr B, Montminy M. Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat Rev Mol Cell Biol*. 2001; 2:599–609. [PubMed: 11483993]
- McDonald AJ. Neurons of the lateral and basolateral amygdaloid nuclei: a Golgi study in the rat. *J Comp Neurol*. 1982; 212:293–312. [PubMed: 6185547]
- McDonald AJ, Pearson JC. Coexistence of GABA and peptide immunoreactivity in non-pyramidal neurons of the basolateral amygdala. *Neurosci Lett*. 1989; 100:53–58. [PubMed: 2569703]
- Merali Z, McIntosh J, Kent P, Michaud D, Anisman H. Aversive and appetitive events evoke the release of corticotropin-releasing hormone and bombesin-like peptides at the central nucleus of the amygdala. *J Neurosci*. 1998; 18:4758–4766. [PubMed: 9614249]
- Merchenthaler I. Corticotropin releasing factor (CRF)-like immunoreactivity in the rat central nervous system. Extrahypothalamic distribution. *Peptides*. 1984; 5(Suppl 1):53–69. [PubMed: 6384954]
- Merlo PE, Lorang M, Yeganeh M, Rodriguez dF, Raber J, Koob GF, Weiss F. Increase of extracellular corticotropin-releasing factor-like immunoreactivity levels in the amygdala of awake rats during restraint stress and ethanol withdrawal as measured by microdialysis. *J Neurosci*. 1995; 15:5439–5447. [PubMed: 7643193]
- Mohila CA, Onn SP. Increases in the density of parvalbumin-immunoreactive neurons in the anterior cingulate cortex of amphetamine-withdrawn rats: evidence for corticotropin-releasing factor in sustained elevation. *Cereb Cortex*. 2005; 15(3):262–274. [PubMed: 15238432]
- Pare D, Collins DR. Neuronal correlates of fear in the lateral amygdala: multiple extracellular recordings in conscious cats. *J Neurosci*. 2000; 20:2701–2710. [PubMed: 10729351]
- Pare D, Duvarci S. Amygdala microcircuits mediating fear expression and extinction. *Curr Opin Neurobiol*. 2012; 22:1–7. [PubMed: 22305967]
- Paxinos, G.; Watson, C. The rat brain in stereotaxic coordinates. Orlando, FL: Academic Press, Inc; 1998.
- Peterson DA. Quantitative histology using confocal microscopy: Implementation of unbiased stereology procedures. *Methods*. 1999; 18:493–507. [PubMed: 10491280]
- Rainnie DG, Bergeron R, Sajdyk TJ, Patil M, Gehlert DR, Shekhar A. Corticotrophin releasing factor-induced synaptic plasticity in the amygdala translates stress into emotional disorders. *J Neurosci*. 2004; 24:3471–3479. [PubMed: 15071094]
- Refojo D, Schweizer M, Kuehne C, Ehrenberg S, Thoeniger C, Vogl AM, Dedic N, Schumacher M, von Wolff G, Avrabos C, Touma C, Engblom D, Schütz G, Nave KA, Eder M, Wotjak CT, Sillaber I, Holsboer F, Wurst W, Deussing JM. Glutamatergic and dopaminergic neurons mediate anxiogenic and anxiolytic effects of CRHR1. *Science*. 2011; 333(6051):1903–1907. [PubMed: 21885734]
- Reijmers LG, Perkins BL, Matsuo N, Mayford M. Localization of a stable neural correlate of associative memory. *Science*. 2007; 317:1230–1233. [PubMed: 17761885]
- Reyes TM, Lewis K, Perrin MH, Kunitake KS, Vaughan JM, Arias C, Hogenesch JB, Gulyas J, Rivier J, Vale WW, Sawchenko PE. Urocortin II: a member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. *Proc Natl Acad Sci USA*. 2001; 98:2843–2848. [PubMed: 11226328]
- Rodriguez Manzanares PA, Isoardi NA, Carrer HF, Molina VA. Previous stress facilitates fear memory, attenuates GABAergic inhibition, and increases synaptic plasticity in the rat basolateral amygdala. *J Neurosci*. 2005; 25:8725–8734. [PubMed: 16177042]
- Rostkowski AB, Teppen TL, Peterson DA, Urban JH. Cell-specific expression of neuropeptide Y Y1 receptor immunoreactivity in the rat basolateral amygdala. *J Comp Neurol*. 2009; 517:166–176. [PubMed: 19731317]
- Sagar SM, Sharp FR, Curran T. Expression of c-fos protein in the brain: metabolic mapping at the cellular level. *Science*. 1988; 240:1328–1331. [PubMed: 3131879]
- Sajdyk TJ, Johnson PL, Leitermann RJ, Fitz SD, Dietrich A, Morin M, Gehlert DR, Urban JH, Shekhar A. Neuropeptide Y in the amygdala induces long-term resilience to stress-induced reductions in social responses but not hypothalamic-adrenal-pituitary axis activity or hyperthermia. *J Neurosci*. 2008; 28:893–903. [PubMed: 18216197]

- Sajdyk TJ, Schober DA, Gehlert DR, Shekhar A. Role of corticotropin-releasing factor and urocortin within the basolateral amygdala of rats in anxiety and panic responses. *Behav Brain Res.* 1999; 100:207–215. [PubMed: 10212068]
- Sajdyk TJ, Shekhar A, Gehlert DR. Interactions between NPY and CRF in the amygdala to regulate emotionality. *Neuropeptides.* 2004; 38:225–234. [PubMed: 15337374]
- Sanders SK, Morzorati SL, Shekhar A. Priming of experimental anxiety by repeated subthreshold GABA blockade in the rat amygdala. *Brain Res.* 1995; 699:250–259. [PubMed: 8616628]
- Shekhar A, Truitt W, Rainnie D, Sajdyk T. Role of stress, corticotrophin releasing factor (CRF) and amygdala plasticity in chronic anxiety. *Stress.* 2005; 8:209–219. [PubMed: 16423710]
- Spiga F, Lightman SL, Shekhar A, Lowry CA. Injections of urocortin 1 into the basolateral amygdala induce anxiety-like behavior and c-Fos expression in brainstem serotonergic neurons. *Neuroscience.* 2006; 138:1265–1276. [PubMed: 16488545]
- Spina MG, Merlo-Pich E, Akwa Y, Balducci C, Basso AM, Zorrilla EP, Britton KT, Rivier J, Vale WW, Koob GF. Time-dependent induction of anxiogenic-like effects after central infusion of urocortin or corticotropin-releasing factor in the rat. *Psychopharmacology (Berl).* 2002; 160:113–121. [PubMed: 11875628]
- Stanciu M, Radulovic J, Spiess J. Phosphorylated cAMP response element binding protein in the mouse brain after fear conditioning: relationship to Fos production. *Brain Res Mol Brain Res.* 2001; 94:15–24. [PubMed: 11597761]
- Stenzel-Poore MP, Heinrichs SC, Rivest S, Koob GF, Vale WW. Overproduction of corticotropin-releasing factor in transgenic mice: a genetic model of anxiogenic behavior. *J Neurosci.* 1994; 14:2579–2584. [PubMed: 8182429]
- Stork O, Ji FY, Obata K. Reduction of extracellular GABA in the mouse amygdala during and following confrontation with a conditioned fear stimulus. *Neurosci Lett.* 2002; 327:138–142. [PubMed: 12098654]
- Sztainberg Y, Kuperman Y, Tsoory M, Lebow M, Chen A. The anxiolytic effect of environmental enrichment is mediated via amygdalar CRF receptor type 1. *Mol Psychiatry.* 2010; 15:905–917. [PubMed: 20084060]
- Timpl P, Spanagel R, Sillaber I, Kresse A, Reul JM, Stalla GK, Blanquet V, Steckler T, Holsboer F, Wurst W. Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. *Nat Genet.* 1998; 19:162–166. [PubMed: 9620773]
- Truitt WA, Sajdyk TJ, Dietrich AD, Oberlin B, McDougale CJ, Shekhar A. From anxiety to autism: spectrum of abnormal social behaviors modeled by progressive disruption of inhibitory neuronal function in the basolateral amygdala in Wistar rats. *Psychopharmacology (Berl).* 2007; 191:107–118.
- Vale W, Speiss J, Rivier C, Rivier J. Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. *Science.* 1981; 213:1394–1397. [PubMed: 6267699]
- West MJ, Slomianka L, Gundersen HJ. Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. *Anat Rec.* 1991; 231:482–497. [PubMed: 1793176]
- Zhang Y, Raap DK, Garcia F, Serres F, Ma Q, Battaglia G, Van de Kar LD. Long-term fluoxetine produces behavioral anxiolytic effects without inhibiting neuroendocrine responses to conditioned stress in rats. *Brain Res.* 2000; 855:58–66. [PubMed: 10650130]

Figure 1. Histological verification of injector placement in the BLA

A) Schematic representation of injector placements as determined by histology. Injector tip placements are illustrated as symbols for each treatment; left side of the atlas section represents placements for CRF dose response experiment; right side illustrates placements for the coexpression study (cFos/CaMKII or cFos/GAD65). Illustrations of coronal brain sections are based on the rat brain atlas of Paxinos and Watson (1998). B) Representative section showing proper probe placement (between arrows) within the borders of the BLA. CeA, central amygdalar nucleus. Scale bar=200µm.

Figure 2. Photomicrographs of cFos-ir in the BLA following CRF delivery
 cFos-ir is seen in the first column (green), ToPro (nuclear marker) fluorescence is shown in the second column (blue) and merged images are presented in the third column. cFos-ir nuclei following intra-BLA delivery of A) vehicle (aCSF), B) 2 fmol CRF, C) 20 fmol CRF, D) 200 fmol CRF or E) 2000 fmol CRF are indicated with arrowheads. Scale bar= 20 μ m.

Figure 3. Total number of cFos-ir cells in the BLA following intra-BLA delivery of increasing concentrations of CRF

Intra-BLA delivery of 20, 200 or 2000 fmol of CRF resulted in a significantly higher number of cFos-ir cells in the BLA compared to vehicle (aCSF) treated control. Data are reported as mean + SEM. One-way ANOVA, followed by Student-Newman-Keuls post-hoc test: * $p < 0.05$, ** $p < 0.01$ compared to vehicle; + $p < 0.05$ compared to 2 fmol CRF, $n = 4-7$ /group.

Figure 4. Photomicrograph of cFos-ir in Pyramidal Cells and Interneurons in the BLA following CRF delivery

Compared to vehicle treated controls (A–C and G–I), intra-BLA delivery of 20 fmol CRF resulted in robust cFos-ir in CaMKII-ir cells (D–F) and GAD65-ir cells (J–L) in the BLA (arrowheads), respectively. CRF treatment also resulted in a reduction in the number of GAD65-ir cells. Single-labeled CaMKII-ir pyramidal neurons and GAD-65-ir interneurons are indicated with horizontal arrows and single-labeled cFos-ir cells with vertical arrows. Scale bar=20μm.

Figure 5. Effect of acute CRF treatment (20 fmol) on total number of CaMKII- and GAD65-ir cell numbers in the BLA

Total cell populations for CaMKII-ir and GAD65-ir cells were determined in the BLA 90 minutes after injection of either vehicle or CRF (20 fmol).

* $p < 0.05$ from corresponding vehicle-treated group, Student's *t* test.

Table 1

Distribution of cFos-ir in BLA pyramidal cells after 20 fmol CRF injection

	Total # cFos	Total # CaMKII	Total # Double Labeled Cells	% CaMKII-ir Cells Expressing cFos-ir
aCSF	2829 ± 1157	64820 ± 3600	1061 ± 601	1.7 ± 1.0
20 fmol CRF	14744 ± 516 ^{**}	70826 ± 6485	9208 ± 2768 [*]	13.1 ± 4.1 [*]

^{**}
p<0.001

^{*}
p<0.05 by Student's t-Test

Table 2

Distribution of cFos-ir in BLA interneurons after 20 fmol CRF injection

	Total # cFos	Total # GAD65	Total # Double Labeled Cells	% GAD65-ir Cells Expressing cFos-ir
aCSF	3473 ± 1241	5293 ± 506	91 ± 45	1.7 ± 0.9
20 fmol CRF	11385 ± 1627 *	3107 ± 662 *	319 ± 111	11.8 ± 4.3

*
p<0.05 by Student's t-Test