Supplemental Methods

Immunohistochemical Localization of CRF Receptors in the DR

All histochemical analyses (immunofluorescence and immunoelectron microscopy) were carried out at the approximate rostral-caudal level indicated within the region delineated by the trapezoid (Fig. S1,A). Immunofluorescent detection of CRF receptors alone and in conjunction with 5-HT was carried out in rats that underwent transcardial perfusion with ice cold 4% paraformaldehyde in 0.1M phosphate buffer (PB; pH 7.4). Coronal sections (40 μm) through the DR were made using a vibratome and subsequently processed. Sections were rinsed in 0.1M PB, followed by 0.1M tris-saline buffer (TS; pH 7.6) and incubated in a blocking solution of bovine serum albumin (BSA; 0.5%) in TS containing 0.25% Triton X-100. Sections were incubated overnight in one of the following primary antibodies/combinations prepared in 0.1%BSA/0.25% triton: CRF_1, CRF_2, 5-HT and CRF_1, or 5-HT and CRF_2. Immunoreactivity for CRF receptors (CRF_1 or CRF_2) was detected using a TRITC conjugated secondary antibody (anti-rabbit; Jackson ImmunoResearch Laboratories, West Grove, PA). Sections processed for dual immunolabeling of CRF receptors and 5-HT were incubated in a cocktail of TRITC- (CRF receptors) and FITC-conjugated (anti-rat; 5-HT) secondary antibodies. Sections were mounted on slides, dehydrated, and coverslipped from xylenes using DPX mounting medium. Images were captured using a Zeiss LSM 510 Meta (Carl Zeiss, Inc., Thornwood, NY) confocal microscope and imported using the LSM 5 image browser. Image stacks through the z-plane (7-24 optical sections of 0.38μm) were collapsed to yield a projection image that was optimized with respect to brightness/contrast prior to the addition of text and labels in Adobe Photoshop 7.

For electron microscopic studies, rats were perfused with 3.8% acrolein and 2% paraformaldehyde in 0.1M PB. Immunohistochemical detection of CRF receptors using

immunogold was identical to methods described previously (1, 2). At the ultrastructural level, CRF_2 and 5-HT were detected using immunogold and immunoperoxidase, respectively. Immunodetection of 5-HT with immunoperoxidase was completed in a 5-6 minute empirically derived reaction using 22mg of 3,3'-diaminobenzidine tetrahydrochloride dehydrate (DAB; Sigma) with 10 µl of 30% H_2O_2 in 100ml 0.1M TS. DR sections used in the ultrastructural analysis of stress effects on CRF receptors were incubated in antibodies directed against either CRF_1 or CRF_2 and visualized using a silver-enhanced gold conjugated secondary antibody. Alternate sections were processed in parallel for detection of either CRF_1 or CRF_2 in the DR.

Antibodies and Immunohistochemical Controls

A rabbit polyclonal antibody directed to amino acids 230-444 of human CRF_1 was used in electron microscopy studies (sc-5543; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). A goat polyclonal antibody detecting CRF_1 (sc-1757; Santa Cruz Biotechnology, Inc.) raised toward an epitope at C-terminal end of the human CRF_1 receptor was used in immunofluorescence studies in conjunction with 5-HT. The CRF_2 antibody (NLS3570; Novus Biologicals, Inc., Littleton, CO) is a rabbit polyclonal antibody raised against a synthetic peptide (~15 amino acids) near amino acid 60 within the N-terminal extracellular region of CRF_2. CRF_1 and CRF_2 were used at concentrations of 1:1000 for immunofluorescence and electron microscopy studies. 5-HT was detected using an antibody raised in rat (1:500; Harlan-Sera-Lab, Belton, England). Fluorophore-conjugated (1:200) and biotinylated (1:400) secondary antibodies were raised in donkey (minimal cross-reactivity; Jackson ImmunoResearch Laboratories) and 1-nm gold secondary antibody (1:50) was raised in goat (Amersham Biosciences, Piscataway, NJ).

To evaluate the specificity of the CRF receptor primary antibodies, sections from CRF_1 deficient mice, CRF_2 deficient mice and wildtype control littermates were processed for immunohistochemical detection of the receptors. Brains from CRF_1 deficient mice and wildtype littermates were kindly provided by Dr. Stephen Gammie (University of Wisconsin, Madison, WI). Brains were obtained after perfusion with 5% acrolein and cryoprotection in 30% sucrose. CRF_2 deficient mice and their wildtype littermates were kindly provided by Dr. Tracy Bale (University of Pennsylvania, Philadelphia, PA). These mice were perfused with 3.8% acrolein and 2% paraformaldehyde and cryoprotected in 30% sucrose. Additional controls including the processing of tissue sections in the absence of primary antiserum for CRF_r or 5-HT or incubation in preabsorbed antisera (CRF_1 and CRF_2) did not produced detectable immunoreactivity in these sections.

Receptor immunoreactivity was examined in DR sections from CRF_1 knockout mice, CRF_2 knockout mice and wildtype littermates to evaluate CRF receptor antibody specificity. CRF_1 immunoreactivity was detected in wildtype (Supplementary Data, Fig. S1,B-C), but not CRF_1 knockout mice (Fig. S1,E-F). CRF_2 immunoreactivity was visible in the DR of wildtype mice (Fig. S1,D) but absent in CRF_2 knockout mice (Fig. S1,G). Further, immunolabeling for CRF_1 was detected in DR sections from CRF_2 knockout mice and was not noticeably different from wildtype counterparts (not shown) suggesting that there is not significant cross-reactivity of the antibodies used to detect the CRF_1 receptor with the CRF_2 receptor peptide.

Swim Stress

Rats were administered vehicle (aCSF) or the CRF_1 antagonist, antalarmin (20 mg/kg, i.p.), 30 min prior to swim stress. The procedures used for swim stress were identical to those

described previously (2). Rats were placed in a cylindrical glass tank (46 cm high x 20 cm diameter) filled with water (25 ± 1°C) to a depth of 30 cm for 15 min allowing active behavior without contact with the bottom of the tank. They were removed from the tank at the conclusion of the 15-minute session and immediately towel dried and placed into a warming cage (37°C) that contained a heating pad covered with towels for 15 min. Rats were subsequently returned to their home cage where they remained until perfusion (24 h later). Non-swim control animals were handled briefly and returned to their home cage until perfusion, which occurred 24 hours later.

DR Neuronal Recording

Rats were exposed to a 15 min swim stress or were handled as described above. Twenty-four hours after the stress or handling, rats were anesthetized with isofluorane (1-2% in air) and positioned in a stereotaxic instrument with the head in the horizontal plane for recording extracellular single unit activity from DR neurons as previously described (3). Double barrel glass micropipettes were used to record DR neuronal activity and simultaneously microinfuse CRF adjacent to the recording electrode, as previously described (3). Spontaneous discharge rate was recorded for 3-5 min prior to CRF (30 ng in 30 nl) administration. Injection of the entire volume at this rate usually required 60-90 sec. Initially a dose of 10 ng was administered, as this was previously shown to inhibit DR neuronal activity in unstressed halothane-anesthetized rats (3, 4). However, pilot studies determined that isofluorane anesthesia renders DR neurons somewhat less sensitive to CRF. Neuronal activity was recorded for a period of 5-15 min after CRF administration. CRF was administered only one time to an individual rat. Some rats received the CRF_2 antagonist, antisauvagine-30 (3 μg in 3 μl), administered through an

intracerebroventricular cannula (i.c.v.) 10 min prior to CRF. At the end of the experiment, pontamine sky blue dye was iontophoresed from the recording pipette to mark the recording site and neutral red dye was injected (3 μl, i.c.v.) to verify i.c.v. cannula placement. Rats were killed with an anesthetic overdose and the brains removed. The brains were first cut to visualize neutral red in the ventricular system. Frozen 40 μm sections were cut and mounted on slides and stained with neutral red for visualization of the PSB spot. Only data from recordings within the DR and with dye in the ventricular system (in applicable experiments) were used in the analysis.

Supplemental References

1. Reyes BA, Fox K, Valentino RJ, Van Bockstaele EJ (2006): Agonist-induced internalization of corticotropin-releasing factor receptors in noradrenergic neurons of the rat locus coeruleus. *Eur J Neurosci*. 23:2991-2998.

2. Reyes BA, Valentino RJ, Van Bockstaele EJ (2008): Stress-induced intracellular trafficking of corticotropin-releasing factor receptors in rat locus coeruleus neurons. *Endocrinology*. 149:122-130.

3. Kirby LG, Rice K, Valentino RJ (2000): Effects of corticotropin-releasing factor on neuronal activity in the serotonergic dorsal raphe nucleus. *Neuropsychopharmacology*. 22:148-162.

4. Price ML, Kirby LG, Valentino RJ, Lucki I (2002): Evidence for corticotropin-releasing factor regulation of serotonin in the lateral septum during acute swim stress: adaptation produced by repeated swim. *Psychopharmacology*. 162:406-414.

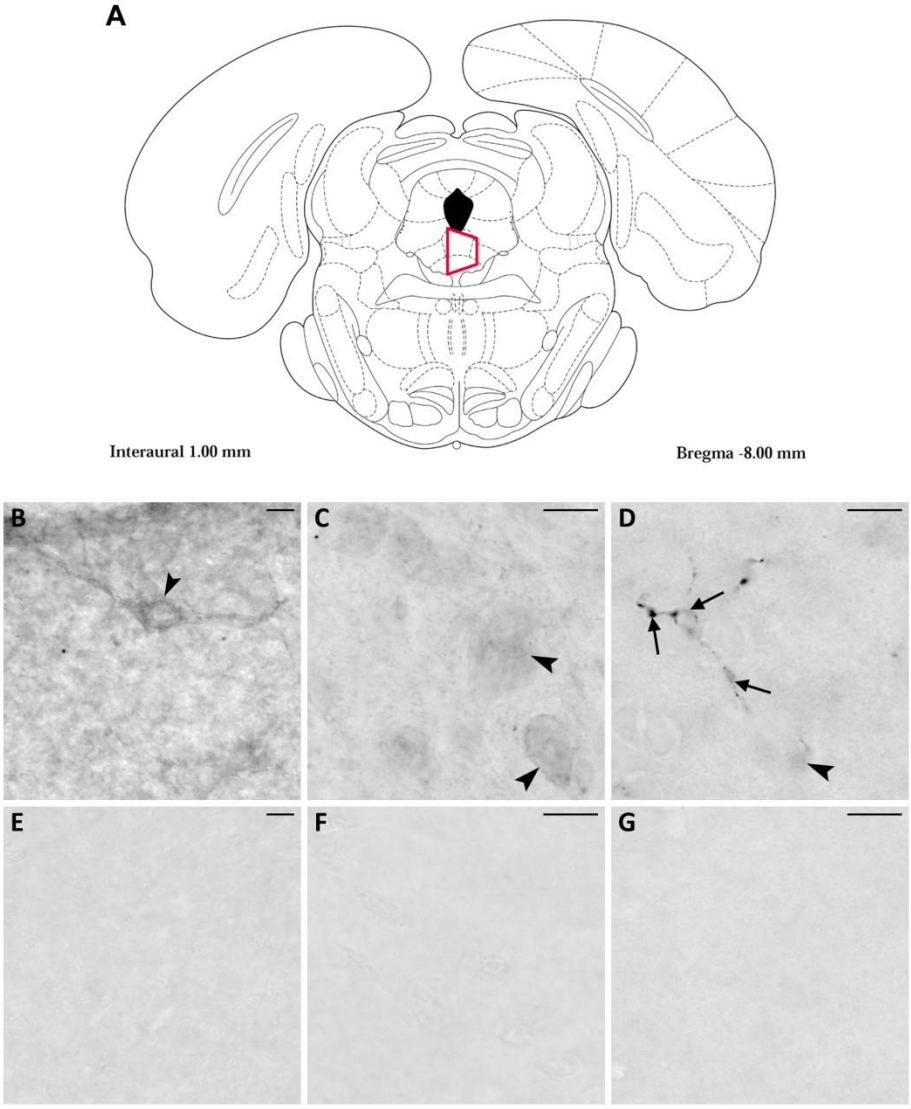
5. Paxinos G, Watson, C. (1998): The Rat Brain in Stereotaxic Coordinates. North Ryde: Academic Press.

Table S1: Ratio of cytoplasmic to total immunogold particles representing the cellular distribution of CRF_1 and CRF_2 in the DR: individual subject data.

Ratio of cytoplasmic to total immunogold label per animal

	Unstressed	***Stress***	***Stress/Antagonist***
CRF_1			
*A**	0.58 n_d=163	0.62 n_d=164	nd
B	0.45 n_d=152	0.71 n_d=108	nd
C	0.58 n_d=50	0.65 n_d=95	nd
CRF_2			
A	0.85 n_d=135	0.55 n_d=195	0.69 n_d=50
B	0.85 n_d=110	0.51 n_d=115	0.81 n_d=56
C	0.81 n_d=112	0.63 n_d=178	0.80 n_d=55

*A, B, and C represent the three subjects used for quantification. nd: not determined; n_d: number of dendrites sampled


A
Interaural 1.00 mm
Bregma -8.00 mm
B
C
D
E
F
G

Supplementary Figure S1: Immunodetection of CRF receptors in the DR of wildtype and CRF receptor mutant mice. A: Schematic representation of the approximate region sampled for both immunofluorescence and immunoelectron microscopy studies according to the rat brain atlas of Paxinos and Watson (5). **B:** CRF_1 immunoreactivity detected using the goat anti $CRF_{1/2}$ antibody (Santa Cruz sc-1757; 1:500) in the DR of wildtype mice was absent in the DR of CRF_1 deficient mice processed in parallel (E). **C:** CRF_1 immunolabeling in the DR was also evident when using a $CRF_{1/2}$ antibody raised in rabbit (Santa Cruz sc-5543; 1:500) which produced no detectable immunolabeling in the DR of CRF_1 deficient mice (F). **D:** CRF_2 immunoreactive cells and processes were detected using a polyclonal antibody raised in rabbit (Novus, NLS 3570; 1:500) in the DR of wildtype mice but immunoreactivity was indiscernible in the DR of CRF_2 knockout mice (G). Arrowheads indicate cell bodies; arrows indicate processes. Scale bars = 10μm.