Chronic Administration of the Triazolobenzodiazepine Alprazolam Produces Opposite Effects on Corticotropin-Releasing Factor and Urocortin Neuronal Systems

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In view of the substantial preclinical evidence that supports a seminal role of central corticotropin-releasing factor (CRF) neuronal systems in the physiology and pathophysiology of stress and anxiety, it is reasonable to suggest that the anxiolytic properties of benzodiazepines are mediated, at least in part, via regulation of CRFergic function. To begin to test this complex hypothesis, we examined the effects of acute and chronic administration of the triazolobenzodiazepine agonist alprazolam on CRF peptide concentrations, receptor-binding density, and mRNA expression in the CNS. Additionally, we measured mRNA expression for urocortin, a recently discovered neuropeptide that is generally considered to be a second endogenous ligand for CRF receptors. Both acute and chronic alprazolam administration was found to decrease CRF concentrations within the locus coeruleus. Furthermore, chronic alprazolam decreased

basal activity of the hypothalamic–pituitary–adrenal axis, CRF mRNA expression in the central nucleus of the amygdala, and CRF₁ mRNA expression and receptor binding in the basolateral amygdala. In marked contrast, urocortin mRNA expression in the Edinger-Westphal nucleus and CRF_{2A} receptor binding in the lateral septum and ventromedial hypothalamus were increased. Similar findings of an inverse relationship between the CRF₁ and CRF_{2A} receptor systems have been reported in an anxiety model based on adverse early-life experience, suggesting the intriguing possibility that CRF neuronal systems may be comprised of two separate, but interrelated, subdivisions that can be coordinately and inversely regulated by stress, anxiety, or anxiolytic drugs.

Key words: amygdala; anxiety; benzodiazepines; corticotropin-releasing factor; locus coeruleus; stress; urocortin

The neuropeptide corticotropin-releasing factor (CRF) coordinates the endocrine response to stress via its action as the major physiological regulator of the hypothalamic–pituitary–adrenal (HPA) axis (Owens and Nemeroff, 1991). In addition to the paraventricular nucleus of the hypothalamus (PVN), CRF-containing neurons have also been localized to extrahypothalamic limbic structures and brainstem nuclei that subserve behavioral and autonomic regulatory functions, such as the bed nucleus of the stria terminalis (BNST), the central nucleus of the amygdala (CeA), and the locus coeruleus (LC) (Swanson et al., 1983). There is overwhelming evidence (Valentino et al., 1993; Gray and Bingaman, 1996; Lee and Davis, 1997) that suggests that CRF functions as a neurotransmitter in these regions. In this manner, it is hypothesized that CRF neuronal systems are strategically located to integrate not only the endocrine but also the behavioral, immune, and autonomic responses to stress (Dunn and Berridge, 1990; Owens and Nemeroff, 1991).

Recently, the existence of two distinct CRF receptor subtypes with contrasting neuroanatomical distributions has been demonstrated: CRF₁ and CRF₂ (Chalmers et al., 1996). In rats, CRF₁ is the predominant receptor within the pituitary, cerebellum, and neocortex. However, in nonhuman primates, there is considerable

CRF₂ receptor expression and binding in the neocortex and anterior pituitary (Sanchez et al., 1999). The CRF₂ receptor consists of at least two splice variants, CRF_{2A} and CRF_{2B}. The former is more prevalent in subcortical regions, particularly the lateral septum (LS), ventromedial hypothalamus (VMH), and dorsal raphe, whereas the latter is more abundant in the periphery.

Another relatively recent and important discovery is that of urocortin, a second endogenous mammalian ligand for the CRF receptors (Vaughan et al., 1995; Donaldson et al., 1996; Zhao et al., 1998). Urocortin binds with equal affinity to both CRF receptor subtypes but demonstrates ~10-fold higher affinity for CRF₂ receptors than does CRF itself (Chalmers et al., 1996). In the rat, urocortin-containing perikarya and urocortin mRNA expression are most prominent in the Edinger-Westphal nucleus (EWN) and the lateral superior olive (Vaughan et al., 1995), regions that do not contain CRF mRNA. The highest density of urocortin innervation is observed in the LS and dorsal raphe (Vaughan et al., 1995; Wong et al., 1996; Kozicz et al., 1998). It is of interest to note that these two regions nearly exclusively express CRF_{2A} mRNA. This overlapping distribution, along with the higher affinity of urocortin for the CRF₂ receptor as compared with CRF, provide evidence that urocortin may be the endogenous CRF₂ ligand.

Although little is known about the role of urocortin and the CRF₂ receptor system in stress and the pathophysiology of psychiatric disorders, there is considerable data supporting a role for CRF in the pathogenesis of anxiety disorders. Central administration of CRF produces behavioral evidence of anxiety in laboratory animals (Dunn and Berridge, 1990), and transgenic mice

Received July 12, 1999; revised Nov. 9, 1999; accepted Nov. 11, 1999.

This work was supported by National Institutes of Health Grants DA08705 and MH42088, as well as the National Institutes of Health Medical Scientist Training Program Grant 5 T32 GM08169.

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that overexpress CRF also demonstrate heightened anxiety (Heinrichs et al., 1997a). In humans, elevated levels of CRF have been found in the CSF of post-traumatic stress disorder patients (Bremner et al., 1997; Baker et al., 1999) and during alcohol withdrawal, both of which are characterized by increased anxiety symptom severity (Hawley et al., 1994; Adinoff et al., 1996). Conversely, CRF antagonists and antisense oligonucleotides directed against either CRF or CRF₁ receptor mRNA are effective anxiolytics in laboratory animal studies (Heinrichs et al., 1992; Rassnick et al., 1993; Skutella et al., 1994; Liebsch et al., 1995). The recent demonstration that CRF₁ receptor knock-out mice exhibit decreased basal and stress-induced anxiety further supports a role for CRF in the mediation of anxiogenic behaviors (Timpl et al., 1998).

Benzodiazepines, a class of drugs that potentiate GABA-mediated Cl[−] current and have been clinically used as anxiolytics since the 1960s, appear to produce many effects opposite to those of centrally administered CRF. These drugs have been demonstrated to suppress HPA axis activation (Krulik and Cerny, 1971; Kalogeras et al., 1990; Rorher et al., 1994; Cowley et al., 1995), to attenuate stress-induced CRF neuronal activation (*c-fos* mRNA), as well as the subsequent increase in CRF production (CRF mRNA) and release (Imaki et al., 1995; Pich et al., 1995), and to block or diminish the anxiogenic effects of CRF as measured behaviorally (Britton et al., 1985; Swerdlow et al., 1986; Dunn and File, 1987).

In view of the substantial evidence relating CRF to stress and anxiety, it seems plausible that the anxiolytic actions of benzodiazepines may be at least partially attributable to their demonstrated ability to antagonize the production, release, and behavioral effects of CRF. To test this hypothesis, we sought to examine whether acute or chronic administration of the benzodiazepine alprazolam altered measurable parameters of central CRF and/or urocortin activity, including peptide and receptor concentrations or binding and mRNA expression.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats (225–275 gm on arrival; Charles River Laboratories, Raleigh, NC) were housed three per cage (acute-dosing experiment) with food and water available *ad libitum* in an environmentally controlled animal facility with a 12 hr light/dark cycle (lights on at 0730 hr). Rats receiving alprazolam incorporated into a liquid diet for the chronic-dosing experiments were housed individually and without access to a separate food supply. Animals were weighed and handled daily starting 7 d before the experiment (acute-dosing experiment) or throughout the course of the experiment (chronic-dosing experiment). Rats were killed by decapitation between 0900 and 1030 hr. After decapitation, brains were rapidly removed, frozen on dry ice, and stored at −80°C until cryostat sectioning or dissection.

Drug treatment

Acute-dosing experiment. Rats ($n = 12$ per group) received a single subcutaneous injection of either (1) alprazolam [0.00 mg/kg (vehicle) or 0.10, 0.33, 1.0, or 3.3 mg/kg in 50% polyethylene glycol (PEG) 400 (Sigma, St. Louis, MO) in water], (2) flumazenil [0.00 mg/kg (vehicle) or 0.33, 1.0, 3.3, or 10.0 mg/kg in 50% PEG 400], or (3) FG7142 [0.00 mg/kg (vehicle) or 0.33, 1.0, 3.3, or 10.0 mg/kg in 45% hydroxypropyl- β -cyclodextrin (Research Biochemicals, Natick, MA)]. The injection volume was 1 ml/kg, and injections were made in staggered order, between 0730 and 0900 hr. Rats were killed exactly 90 min after drug or vehicle injection.

Chronic-dosing experiment. Based on our previous studies (Owens et al., 1991), an oral alprazolam dose of 90 mg·kg^{−1}·d^{−1} was administered, with the alprazolam content of the diet adjusted daily for the rats' weight. Rats ($n = 16$ per group) received a liquid, fat emulsion-based diet (Bio-Serv, Frenchtown, NJ) supplemented with 0.01% (w/v) saccharin

and varying amounts of alprazolam dissolved in PEG 400 or PEG 400 vehicle alone (control diet). The diet was available *ad libitum* and was the only source of food available to the rats. Intake was recorded daily for individual rats throughout the course of the experiment. The rats were killed after 14 d of feeding.

RIAs

ACTH RIA. Trunk blood was collected on ice in EDTA-containing glass tubes and centrifuged for 10 min at 2500 rpm. ACTH was measured in duplicate samples of rat plasma by a two-site immunoradiometric assay (Nichols Diagnostics, San Juan Capistrano, CA) with a coefficient of variation of 5% and sensitivity (blank $\pm$ 2 SD) of 1 pg/ml.

Corticosterone RIA. Trunk blood was collected on ice in polycarbonate tubes and centrifuged for 10 min at 2500 rpm. Corticosterone was assayed in duplicate samples of rat serum by double antibody RIA (ICN Biochemicals, Costa Mesa, CA) with a coefficient of variation of 6% and sensitivity (blank $\pm$ 2 SD) of 1.2 ng/ml.

CRF RIA. Regional brain tissue samples from the acute alprazolam experiment, as well as the amygdala samples from the chronic alprazolam experiment, were dissected as described in Owens et al. (1989). Briefly, the frozen brains were sliced with a razor blade into sections of varying thickness (~1 mm for the LC and 2–3 mm for the amygdala) that were then dissected on ice with a scalpel to remove the region of interest, based on the atlas of Paxinos and Watson (1986). Because of concerns about the proximity of the LC to the parabrachial nucleus, the LC samples from the chronic alprazolam experiment were micropunched (0.8 mm) from 300- μ m-thick coronal frozen brain slices thaw-mounted onto glass slides. The CRF concentrations in these brain tissue samples were determined using a modified version of the RIA originally described by Vale, as modified by Ladd et al. (1996). The primary antibody was either oC30 rabbit anti-CRF (acute experiments; donated by W. Vale, The Salk Institute for Biological Studies, La Jolla, CA) or Peninsula (Belmont, CA) rabbit anti-CRF (chronic experiments) at a final dilution of 1:16,000 or 1:70,000, respectively. A standard curve was prepared with rat, human CRF (Bachem, Torrance, CA) comprising the concentration range from 0.625 to 5120 pg/tube. The sensitivity of the assay was 1.25 pg/tube.

In situ hybridization

Serial coronal brain sections (20 μ m) were prepared on a cryostat at −18°C, thaw-mounted onto SuperFrost Plus slides (Fisher, Pittsburgh, PA) under RNase-free conditions, and stored with Humi-Cap desiccant capsules (Life Technologies, Gaithersburg, MD) at −80°C until the assay. In situ hybridization was performed according to the procedures described by Simmons et al. (1989) with minor modifications. Briefly, slides were warmed in a stepwise manner to room temperature, post-fixed in 4% paraformaldehyde, pH 7.4, for 20 min, and rinsed twice in 10 mmol/l PBS, pH 7.4, for 2 min. Next, the slides were treated for 15 min with proteinase K (Promega, Madison, WI; 10 μ g/ml in 0.1 mol/l Tris with 50 mmol/l EDTA) at room temperature, followed by 10 min of acetylation (0.5% acetic anhydride in 0.1 mol/l triethanolamine, pH 8.0), two rinses in 2 $\times$ SSC, and dehydration through a graded ethanol series. The sections were then air-dried for at least 1 hr before hybridization.

The CRF riboprobe was constructed from a 1.2 kb *EcoRI* fragment of a full-length rat CRF cDNA (K. Mayo, Northwestern University, Evanston, IL) subcloned into a pBluescript II-SK⁺ plasmid. The urocortin riboprobe was constructed from an ~0.6 kb *EcoRI*–*EcoRI* fragment ligated into a pBluescript II-SK⁺ plasmid (W. Vale, The Salk Institute for Biological Studies). The insert includes ~70 bp of 5'-untranslated cDNA and the full-length coding region for rat urocortin, followed by ~130 bp of 3'-untranslated cDNA. The CRF₁ riboprobe was constructed from a 1.3 kb *PstI*–*PstI* fragment, containing the full-length coding region for the rat CRF₁ receptor, ligated into a pBluescript II-SK⁺ plasmid (W. Vale, The Salk Institute for Biological Studies). The CRF_{2A} riboprobe was constructed from a 275 bp fragment, encoding the N-terminal region of the CRF_{2A} receptor, ligated into a pBluescript II-SK⁺ plasmid (T. Lovenberg, Neurocrine Biosciences, San Diego, CA). Radiolabeled antisense cRNAs were synthesized by incorporating either α -³⁵S-UTP (DuPont NEN, Boston, MA) into the CRF and urocortin probes or α -³⁵S-CTP plus α -³⁵S-UTP into the CRF₁ and CRF_{2A} receptor probes. The transcription reactions were performed using the Ambion MAXIScript kit (Austin, TX) with SP6 (CRF), T7 (urocortin and CRF₁), or T3 (CRF_{2A}) RNA polymerases according to the instructions provided. After transcription and removal of the cDNA template with 2 U of DNase (Ambion), the cRNA probes were recovered via ethanol precipitation

and/or gel filtration using a G-50 Sephadex Quick Spin column (Boehringer Mannheim, Indianapolis, IN).

The brain sections were hybridized overnight at 58–60°C with 2×10^6 cpm of ^{35}S -labeled cRNA probe diluted into hybridization buffer (50% formamide, 10% dextran sulfate, 0.3 mol/l NaCl, 1× Denhardt's solution, 10 mmol/l Tris, 1 mmol/l EDTA, 2 mg/ml yeast tRNA, and 10 mmol/l DTT) in humidified Nunc trays (Nunc, Naperville, IL). The next day, slides were allowed to cool to room temperature before being washed four times in 4× SSC for 5 min on a rotating platform at 60 rpm. The sections were then treated with 250 $\mu\text{g/ml}$ RNase A (Promega) for 30 min at 37°C. Subsequently, the slides underwent a series of SSC washes (supplemented with 1 mmol/l DTT) with salt concentrations decreasing from 2× to 0.5×, followed by a 60 min high-stringency wash with 0.1× SSC + 1 mmol/l DTT at 60°C and then dehydration through a graded ethanol series. The slides were air-dried for at least 1 hr and then apposed to Kodak Biomax MR film (Eastman Kodak, Rochester, NY) for 12 hr to 21 d (depending on the probe and brain region examined) with ^{14}C standards (Amersham, Piscataway, NJ). Controls performed to establish specificity of the signal included hybridization with sense-strand probes, as well as predigestion with RNase A.

CRF receptor autoradiography

After a modification of the techniques of De Souza et al. (1985) and Primus et al. (1997), *ex vivo* CRF receptor autoradiography was performed on 15 μm rat brain sections mounted on SuperFrost Plus slides (Fisher) and stored as described above until assay. Brain sections were fixed for 2 min in 0.1% paraformaldehyde followed by a 15 min incubation in assay buffer (50 mmol/l Tris, 10 mmol/l MgCl_2 , 2 mmol/l EGTA, 0.1% BSA, 0.1 mmol/l bacitracin, and 0.1% aprotinin, pH 7.5) to remove endogenous CRF. Next, triplicate slides containing adjacent brain sections were incubated for 2 hr at room temperature in one of three conditions: (1) 0.1 nmol/l radiolabeled ^{125}I -sauvagine (DuPont NEN) to determine total binding at both the CRF_1 and CRF_2 receptor subtypes, (2) 0.1 nmol/l radiolabeled ^{125}I -sauvagine + 1 $\mu\text{mol/l}$ CP-154,526 (CRF_1 -specific antagonist) to determine CRF_2 receptor-specific binding, or (3) 0.1 nmol/l radiolabeled ^{125}I -sauvagine + 1 $\mu\text{mol/l}$ unlabeled sauvagine (American Peptide Company, Sunnyvale, CA) to determine nonspecific binding. After the incubation, unbound radioligand was removed by two 5 min rinses in ice-cold (4°C) PBS + 1% BSA on a rotating platform at 60 rpm, followed by two brief dips in ice-cold distilled, deionized H_2O . Slides were then rapidly dried with a blow dryer on the coldest setting and apposed to Kodak Biomax MR film (Eastman Kodak) with ^{125}I -microscale standards (Amersham) for 80–90 hr.

Image analysis

Images from the *in situ* hybridization and receptor autoradiography films were digitized with a Dage-MTI CCD-72 (Michigan City, IN) image analysis system equipped with a Nikon camera. Semiquantitative analysis was performed using Scion Image (version 3.0b) software. Optical densities were calibrated against ^{14}C -standards (*in situ* hybridization films) or ^{125}I -microscale standards (receptor autoradiography films) and expressed in terms of nCi/gm of tissue equivalent. For the purpose of quantifying mRNA levels, specific signal density was determined relative to neutral background density present in the same brain section. For the purpose of quantifying CRF receptor levels, CRF_1 receptor-specific binding was calculated as “total binding – CRF_{2A} receptor binding,” and CRF_{2A} receptor-specific binding was calculated as “ CRF_{2A} receptor binding – nonspecific binding.” In all cases, two to four sections per region were matched for rostrocaudal level according to the atlas of Paxinos and Watson (1986) and used to produce a single value for each animal.

Drugs

Alprazolam was a gift from Upjohn (Kalamazoo, MI). Flumazenil was a gift from Hoffmann-LaRoche (Basel, Switzerland). FG7142 was purchased from Research Biochemicals. CP-154,526 was synthesized and provided by Dr. Mark M. Goodman (Emory University, Atlanta, GA).

Statistics

Significant differences were evaluated by one- or two-tailed *t* tests or one-way ANOVA as appropriate. Exact details of data analysis are provided in the corresponding figure legends. All data are expressed as the mean $\pm$ SEM.

Figure 1. CRF concentrations in the LC after acute administration of compounds active at the benzodiazepine receptor. All drugs were administered subcutaneously, 90 min before death. CRF concentrations were determined via radioimmunoassay in LC homogenates. Each vertical bar represents the mean $\pm$ SEM ($n = 12$ rats per group). * $p < 0.05$ after one-tailed statistical analysis of the ANOVA, based on the presumption of an alprazolam-induced decrease in LC CRF.

RESULTS

CRF concentrations after acute benzodiazepine administration

We have observed previously that acute administration of the benzodiazepine full agonist alprazolam (1 mg/kg) decreases CRF concentrations in the LC. To characterize this action further, dose–response curves after acute administration were generated for three drugs spanning the spectrum of intrinsic efficacy for the benzodiazepine receptor (agonist, antagonist, and partial inverse agonist). The brains of male Sprague Dawley rats were dissected into 14 regions after acute administration of drugs active at the benzodiazepine receptor: median eminence, prefrontal cortex, frontal/parietal cortex, cingulate cortex, BNST, septum, amygdala, piriform cortex, hypothalamus, hippocampus, entorhinal cortex, dorsal raphe, cerebellum, and LC. Only in the LC were CRF concentrations altered in a manner consistent with the pharmacological action of the drug at the benzodiazepine receptor. Alprazolam, a full agonist, dose-dependently decreased CRF concentrations in the LC by up to 32% [$p < 0.05$ via one-way ANOVA with one-tailed analysis based on the results of our previous data (Owens et al., 1989, 1991) yielding the hypothesis that CRF concentrations would decrease in the LC after alprazolam treatment; Fig. 1]. Conversely, FG7142, a partial inverse agonist, caused a trend to dose-dependently increase CRF concentrations in this region by up to 37%. The benzodiazepine antagonist flumazenil did not acutely alter CRF concentrations in the LC at any dose tested.

CRF concentrations and pituitary–adrenal axis activity after chronic benzodiazepine administration

Figure 2 illustrates the diminished basal activity of the HPA axis after 2 weeks of treatment with alprazolam. Plasma ACTH concentrations were decreased by 51% (8.6 ± 1.7 vs 17.7 ± 5.1 pg/ml), and serum corticosterone concentrations were decreased by 77% (2.5 ± 0.4 vs 10.7 ± 3.8 ng/ml; $p < 0.05$) in the alprazolam-treated rats compared with the control rats. It is also

Figure 2. Chronic alprazolam decreases serum corticosterone, suggesting decreased basal activity of the HPA axis. Plasma ACTH and serum corticosterone were measured via radioimmunoassay after 14 d of incorporation of alprazolam into the diet of male Sprague Dawley rats at a concentration of 90 mg·kg⁻¹·d⁻¹. Each vertical bar represents the mean ± SEM (*n* = 16 per group). **p* < 0.05 versus controls after analysis by a two-tailed *t* test.

Figure 3. Chronic alprazolam decreases CRF concentrations in the locus coeruleus. After 14 d of incorporation of alprazolam into the diet of male Sprague Dawley rats at a concentration of 90 mg·kg⁻¹·d⁻¹, CRF concentrations were measured via radioimmunoassay in locus coeruleus micropunch samples (*n* = 4 per group) and amygdala homogenates (*n* = 8 per group). Each vertical bar represents the mean ± SEM; circles represent individual values of CRF concentration in the locus coeruleus. **p* < 0.05 versus controls after analysis by a one-tailed *t* test, based on the presumption of an alprazolam-induced decrease in LC CRF.

important to note that the low ACTH and corticosterone levels in the controls demonstrate that these rats were nonstressed at the time of death. Therefore, the changes in CRF neuronal activity reported in the alprazolam-treated rats are thought to represent alterations in baseline activity rather than an attenuation of stress-induced responses.

Similar to the findings in the acute studies, CRF concentrations were decreased in the LC by ~25% after chronic alprazolam treatment (Fig. 3, 132.5 ± 11.6 vs 171.6 ± 12.9 pg of CRF/mg of protein; *p* < 0.05 after analysis by a one-tailed *t* test based on the hypothesis described above). No changes in CRF concentrations were demonstrated in the amygdala.

CRF mRNA expression

CRF mRNA expression was examined in the BNST, PVN, CeA, and Barrington's nucleus. These regions have been implicated in CRF-induced anxiogenic behavior (Krahn et al., 1988; Heinrichs et al., 1992; Lee and Davis, 1997) and/or exhibit altered CRF

Figure 4. Chronic alprazolam decreases CRF mRNA expression in the CeA. After 14 d of incorporation of alprazolam into the diet of male Sprague Dawley rats at a dose of 90 mg·kg⁻¹·d⁻¹, CRF mRNA expression was measured via densitometric analysis subsequent to *in situ* hybridization performed in the BNST, PVN, CeA, and Barrington's nucleus (Bar Nu). Each vertical bar represents the mean ± SEM (*n* = 8 per group). Two to four slices were quantified per region per rat. ***p* < 0.01 versus controls after analysis by a two-tailed *t* test.

Figure 5. Chronic alprazolam increases urocortin mRNA expression in the Edinger-Westphal nucleus. After 14 d of incorporation of alprazolam into the diet of male Sprague Dawley rats at a dose of 90 mg·kg⁻¹·d⁻¹, urocortin mRNA expression was measured via densitometric analysis subsequent to *in situ* hybridization performed in the Edinger-Westphal nucleus. Each vertical bar represents the mean ± SEM (*n* = 8 per group). Three slices were quantified per rat. ****p* < 0.001 versus controls after analysis by a two-tailed *t* test.

mRNA expression after stress (Chappell et al., 1986; Imaki et al., 1991; Kalin et al., 1994; Hatalski et al., 1998; Hsu et al., 1998). Within the CeA, CRF mRNA expression was decreased by 30% in the alprazolam-treated rats compared with the controls (Fig. 4, 83.4 ± 8.0 vs 118.8 ± 4.7 nCi/g; *p* < 0.01). No alterations in CRF mRNA expression were demonstrated in the other three regions examined.

Urocortin mRNA expression

Urocortin mRNA expression was examined in the EWN, the region of highest expression of this peptide within the brain, and was found to be increased by 107.3% in the rats that received chronic alprazolam treatment compared with the controls (Fig. 5, 1246.5 ± 92.0 vs 601.3 ± 26.7 nCi/g; *p* < 0.001).

CRF receptor binding and mRNA expression

CRF₁ receptor mRNA expression was found to be slightly (12.5%) but significantly decreased in the frontal/parietal cere-

Figure 6. Alterations in CRF₁ receptor function after chronic alprazolam treatment. *a*, Chronic alprazolam decreases CRF₁ receptor mRNA in the basolateral amygdala (BLA) and frontal/parietal cerebral cortex (F/P CTX). After 14 d of incorporation of alprazolam into the diet of male Sprague Dawley rats at a dose of 90 mg·kg⁻¹·d⁻¹, CRF₁ receptor mRNA expression was measured via densitometric analysis subsequent to *in situ* hybridization in the BLA, cerebellum (CB), and F/P CTX. Each vertical bar represents the mean ± SEM (*n* = 8 per group). Three to four slices were quantified per region per rat. **p* < 0.05 and ***p* < 0.01 versus controls after analysis by a two-tailed *t* test. *b*, Chronic alprazolam decreases CRF₁ receptor-binding density in the BLA and CB. After 14 d of incorporation of alprazolam into the diet of male Sprague Dawley rats at a dose of 90 mg·kg⁻¹·d⁻¹, CRF₁ receptor binding was measured by densitometric analysis subsequent to receptor autoradiography performed in the CB, BLA, and F/P CTX. Total binding was determined using 0.1 nmol/l [¹²⁵I]-sauvagine. CRF_{2A} binding was determined in the presence of 1 μmol/l CP-154,526 (CRF₁-selective antagonist). Nonspecific binding was determined in the presence of 1 μmol/l sauvagine. CRF₁ receptor-specific binding was calculated as total binding – CRF_{2A} binding. Each vertical bar represents the mean ± SEM (*n* = 8 per group). Three to four slices were quantified per region per rat. **p* < 0.05 and ****p* < 0.001 versus controls after analysis by a two-tailed *t* test.

bral cortex of the alprazolam-treated rats compared with controls (Fig. 6*a*, 38.3 ± 1.8 vs 43.8 ± 1.6 nCi/g; *p* < 0.05). The most robust decrease in CRF₁ mRNA was observed in the basolateral amygdala, in which the expression of this CRF receptor subtype was found to be decreased 32.3% by chronic alprazolam treatment (17.2 ± 1.2 vs 25.3 ± 1.8 nCi/g; *p* < 0.01). No significant changes in CRF₁ expression were found in the cerebellum, although the CRF₁ mRNA signal was decreased by ~10% in this region.

In contrast to the decreases in expression of the CRF₁ receptor, chronic alprazolam treatment led to an increase of 41.6% in the expression of CRF_{2A} mRNA within the LS (Fig. 7*a*, 17.0 ± 0.9 vs 12.0 ± 1.5 nCi/g; *p* < 0.05). There was also a 34.8% increase in

Figure 7. Alterations in CRF_{2A} receptor function after chronic alprazolam treatment. *a*, Chronic alprazolam increases CRF_{2A} receptor mRNA in the lateral septum. After 14 d of incorporation of alprazolam into the diet of male Sprague Dawley rats at a dose of 90 mg·kg⁻¹·d⁻¹, CRF_{2A} receptor mRNA expression was measured via densitometric analysis subsequent to *in situ* hybridization in the lateral septum and VMH. Each vertical bar represents the mean ± SEM (*n* = 8 per group). Two to three slices were quantified per region per rat. **p* < 0.05 versus controls after analysis by a two-tailed *t* test. *b*, Chronic alprazolam increases CRF_{2A} receptor-binding density in the lateral septum and VMH. After 14 d of incorporation of alprazolam into the diet of male Sprague Dawley rats at a dose of 90 mg·kg⁻¹·d⁻¹, CRF_{2A} receptor binding was measured by densitometric analysis subsequent to receptor autoradiography performed in the lateral septum and VMH. Total binding was determined using 0.1 nmol/l [¹²⁵I]-sauvagine. CRF_{2A} binding was determined in the presence of 1 μmol/l CP-154,526 (CRF₁-selective antagonist). Nonspecific binding was determined in the presence of 1 μmol/l sauvagine. CRF_{2A} receptor-specific binding was calculated as CRF_{2A} binding – nonspecific binding. Each vertical bar represents the mean ± SEM (*n* = 8 per group). Three slices were quantified per region per rat. **p* < 0.05 and ***p* < 0.01 versus controls after analysis by a two-tailed *t* test.

the expression of CRF_{2A} mRNA within the VMH (21.6 ± 2.0 vs 16.0 ± 2.2 nCi/g), which did not reach statistical significance.

Alterations in CRF receptor binding demonstrated via autoradiography paralleled the changes in CRF receptor mRNA expression reported above. Chronic alprazolam treatment produced decreases in CRF₁ receptor binding, while causing concomitant increases in the density of CRF_{2A} receptor binding (Figs. 6*b*, 7*b*). This effect on CRF₁ receptors was most prominent in the BLA. Within this region, CRF₁ receptor binding was decreased 45.0% from that detected in control rats (Fig. 6*b*, 1266.2 ± 116.0 vs 2301.1 ± 178.2 nCi/g; *p* < 0.001). As with the mRNA expression, smaller decreases in CRF₁ receptor binding were demon-

strated in the frontal/parietal cortex and cerebellum (16.0 and 30.3%, respectively).

CRF_{2A} receptor-binding density was markedly increased in both the LS and the VMH (Fig. 7b). Comparable with the increase in its mRNA expression, CRF_{2A} receptor binding was increased by 39.0% in the LS (826.6 ± 44.9 vs 594.5 ± 74.4 nCi/gm; $p < 0.05$). An even greater enhancement of CRF_{2A} receptor binding was observed in the VMH. Within this region, binding to CRF_{2A} receptors was increased by 66.0% compared with binding in control rats (571.1 ± 41.2 vs 344.04 ± 49.3 nCi/gm; $p < 0.01$).

DISCUSSION

Pharmacological agents with a high affinity for the benzodiazepine receptor can produce a spectrum of activity from profound sedation to anxiolysis, to anxiogenesis, and even to proconvulsant actions (Owens et al., 1997). These studies confirm and extend our previous reports (Owens et al., 1989, 1991) that acute administration of the benzodiazepine full agonist alprazolam decreases CRF concentrations within the LC and that a partial inverse agonist at the benzodiazepine receptor may cause a reciprocal increase in CRF concentrations within this region. Although the magnitude of increase is not as great, the effect of the partial inverse agonist FG7142 is similar to that observed after acute or chronic stress (Chappell et al., 1986).

In the areas studied, the chronic administration of alprazolam resulted in a generalized decrease in CRF production, release, and functions mediated via the CRF₁ receptor. Specifically, there were reductions in CRF mRNA expression within the CeA, CRF concentrations within the LC, CRF₁ receptor mRNA expression and binding within the BLA, and basal HPA axis activity. In contrast and unexpectedly, our measures of function in the “second” CRF-like system revealed that urocortin mRNA expression within the EWN and CRF_{2A} receptor function in the LS and VMH were both increased. This intriguing finding suggests that rather than urocortin and CRF_{2A} receptors representing a second parallel CRF-like system, perhaps they represent “antiparallel” stress systems. Indeed, opposite effects of CRF₁ and CRF_{2A} receptor systems have been demonstrated recently with respect to learning and memory consolidation in fear conditioning (Radulovic et al., 1999). Until further information is obtained regarding the neurobiology of urocortin and CRF_{2A} receptors, it is difficult to interpret the findings any further.

One of the primary brain regions where this extensive benzodiazepine-induced modulation of CRF neuronal function occurs is the amygdala, which appears to be an important site for the anxiogenic effects of CRF. A high density of CRF-expressing neurons that communicate widely throughout the brain, particularly to brainstem autonomic nuclei and other limbic structures, is contained within the CeA, and a rich population of CRF receptors composed predominantly of the CRF₁ subtype is found within the BLA (Gray and Bingaman, 1996). Studies have demonstrated that stress upregulates the production of CRF mRNA within the CeA (Kalin et al., 1994; Albeck et al., 1997; Hatałski et al., 1998; Hsu et al., 1998) and, furthermore, that basal levels of CRF mRNA expression in rats are increased in both a genetic model of anxiety, as well as in the maternal separation anxiety model based on adverse early-life experience (Altemus et al., 1994–1995; Ladd et al., 2000). Within the amygdala, stress and anxiety are associated with increases in the production and release of CRF, a neuropeptide with demonstrated anxiogenicity in this region, particularly via actions at the CRF₁ receptor subtype

(Rassnick et al., 1993; Swiergiel et al., 1993; Liebsch et al., 1995; Pich et al., 1995; Richter and Weiss, 1999). We have found that chronic administration of alprazolam reduces both the expression of CRF mRNA within the CeA, the major output nucleus of the amygdala, and the expression and binding to the CRF₁ receptor within the BLA, an amygdaloid nucleus that receives input from widespread regions of the brain. This decrease in the responsiveness of the amygdala to CRF, as well as in the subsequent output of CRF from this important center of fear and anxiety, could be an important/relevant aspect of the mechanism of anxiolytic efficacy of benzodiazepines.

Because the decrease in CRF mRNA within the CeA that we observed was not accompanied by a decrease in local CRF concentrations, it appears likely that these affected CRF-expressing neurons project to targets outside the amygdala. One probable target is the LC. The amygdala is a major source of CRF input to the LC (Koegler-Muly et al., 1993), and recent studies have provided clear evidence that CRF-immunoreactive neurons with cell bodies located in the CeA project to and form synapses on tyrosine hydroxylase-positive LC dendrites in the rostralateral peri-LC area (Van Bockstaele et al., 1996, 1998). Substantial evidence has accumulated indicating that CRF serves as an excitatory neurotransmitter in the LC (Valentino et al., 1993; Conti and Foote, 1996; Smagin et al., 1997). As in the amygdala, the LC has long been implicated as an essential component of the neural substrates that underlie fear and anxiety (Redmond, 1987; Charney et al., 1995).

Both acute and chronic stress have been shown to produce a twofold increase in LC CRF concentrations (Chappell et al., 1986). Additionally, both CRF concentrations and CRF receptor binding are elevated in two rodent models of adverse early-life experience, which produce persistent increases in anxiety (Caldji et al., 1998; Ladd et al., 2000). As with the amygdala, CRF has been demonstrated to produce behavioral evidence of anxiety when directly injected in the LC (Butler et al., 1990; Weiss et al., 1994). Conversely, stress-induced anxiety and the accompanying norepinephrine release in LC terminal fields are reduced by low doses of CRF antagonists administered directly within the LC (Swiergiel et al., 1992; Smagin et al., 1996, 1997). Taken together, the evidence supports the hypothesis that CRF is endogenously released into the LC in response to certain stressors, perhaps from terminals originating within the CeA, and that it can act within this region as a mediator of anxiety and fear responses. Our results indicate that both acute and chronic treatment with alprazolam decreases CRF concentrations within the LC by ~30% (Owens et al., 1991). This decrease in CRF stimulatory input to the LC noradrenergic system, plausibly secondary to decreased CRF mRNA expression in neurons of the CeA–LC pathway, may contribute to the anxiolytic effects of benzodiazepines. In view of this, it is particularly interesting to note that both acute and chronic stress produces a similar magnitude of increase in LC CRF content. Likewise, both acute and chronic administration of alprazolam produces similar decreases in LC CRF concentrations (Chappell et al., 1986). Thus, tolerance does not seem to develop to the effects of stress or alprazolam on LC CRF concentrations. If a reduction in LC CRF concentrations is relevant to the anxiolytic action of benzodiazepines, this may be a reason why, unlike other benzodiazepine effects, such as sedation and muscle relaxation, tolerance does not develop to the anxiolytic effects of benzodiazepines.

Like the Caldji et al. (1998) study cited above, which reported parallel increases in CRF concentrations and receptor density in

the LC, our current results demonstrate that CRF concentrations/mRNA expression and CRF₁ receptor binding/mRNA expression are concurrently decreased by chronic alprazolam administration. Similarly, urocortin mRNA expression and CRF_{2A} receptor binding/mRNA expression are coordinately upregulated. This runs contrary to the expected inverse relationship between CRF and CRF receptors that is evident within the HPA axis, where CRF receptors in the anterior pituitary are downregulated by CRF or stress (Owens et al., 1991). However, this is not necessarily the case with the extrahypothalamic CRF neuronal systems that are believed to mediate the behavioral and autonomic responses to stress. In separate studies, stress has been shown to upregulate both CRF mRNA expression and/or concentrations and CRF receptor mRNA expression and/or binding in the PVN (Imaki et al., 1996; Kiss et al., 1996), CeA (Fuchs and Flugge, 1995; Pich et al., 1995; Albeck et al., 1997), and LC (Chappell et al., 1986; Caldjji et al., 1998). Additionally, CRF, itself, has been shown to upregulate directly the expression of mRNA for its CRF₁ receptor subtype (Imaki et al., 1996; Mansi et al., 1996). These parallel changes in ligand availability and receptor density suggest a possibility for positive feedback regulation to increase net neurotransmission through these CRF neuronal circuits. This is teleologically appropriate for a system that evolved to react swiftly and effectively to coordinate an animal's behavioral and autonomic responses to stressors.

One can postulate that after the stressor is no longer present, other mechanisms, such as GABAergic inhibition, may act on the CRF neuronal systems to reset them to baseline activity. We have provided evidence here that potentiating the activity of GABAergic inhibitory neurotransmission with alprazolam causes a coordinate decrease in CRF mRNA expression and CRF₁ receptor binding/expression within the amygdala, as well as perhaps in cerebral cortex and cerebellum. The resultant decrease in CRF activity mediated via the CRF₁ receptor would be expected to result in diminished expression of anxiety and, therefore, may be a significant mechanism by which benzodiazepines mediate their anxiolytic effects. Most evidence yielded from CRF₁ receptor knock-out mice, as well as the administration of CRF₁-selective antagonists and antisense, indicates that the CRF₁ receptor is the predominant subtype involved in mediating the anxiogenic effects of CRF (Liebsch et al., 1995, 1999; Lundkvist et al., 1996; Heinrichs et al., 1997b; Griebel et al., 1998; Timpl et al., 1998).

Although the effects of a parallel downregulation of CRF and its CRF₁ receptor are relatively straightforward to deduce, the effects of the parallel upregulation of CRF_{2A} receptor binding and mRNA expression for urocortin, the putative endogenous CRF_{2A} receptor ligand, have less clear neurobiological consequences. Because of the relatively recent discovery of urocortin and the CRF_{2A} receptor, as well as the lack of selective CRF_{2A} antagonists, it has been difficult to determine the physiological or behavioral functions mediated or modulated via the CRF_{2A} receptor. Available data suggest a role of urocortin and the CRF_{2A} receptor in the central regulation of appetite, food intake, and water balance (Spina et al., 1996; Hara et al., 1997a,b; Martinez et al., 1998; Smagin et al., 1998). However, our current results are not readily attributable to alterations in osmoregulation or nutrient balance.

Some studies have also demonstrated a role for CRF_{2A} receptors outside of the regulation of food and water balance. Most relevant to our current results is a study in which CRF_{2A} receptor mRNA expression was found to be decreased in the VMH by >60% after maternal deprivation of rats pups, a manipulation

that produces behavioral evidence of heightened anxiety in adulthood. Within the VMH, this decrease in CRF_{2A} receptor mRNA was returned to control levels, not by selective restoration of food intake, but rather by selective restoration of surrogate grooming and handling (Eghbal-Ahmadi et al., 1997, 1999). The handling of rat pups is a manipulation that has been demonstrated previously to produce less anxious rats in adulthood (Levine, 1957; Denenberg, 1964; Francis et al., 1996). Another recent study reported that although the subchronic infusion of CRF₂ receptor antisense into the lateral ventricle of male rats did not produce an anxiolytic effect as measured in the standard elevated-plus-maze paradigm, it did increase immobility in a forced-swim test. The authors believe that this result demonstrates that CRF₂ receptors may selectively influence coping behaviors in response to stress (Liebsch et al., 1999). Thus, although the CRF–CRF₁ receptor system appears to mediate the expression of anxiety as measured in the classic behavioral paradigms, the urocortin–CRF₂ receptor system may play a role in stress-coping behaviors. Therefore, our evidence that alprazolam upregulates binding and expression of the CRF_{2A} receptor, as well as its putative ligand urocortin, could indicate that an aspect of the efficacy of benzodiazepines in treating anxiety disorders is their ability to increase activity of a neurobiological system involved in coping effectively in stressful or anxiety-provoking situations.

If, as the literature suggests, CRF is involved in the pathogenesis of anxiety disorders, then drugs that effectively treat anxiety may alter the components of CRF neuronal systems that underlie the symptomatology of these disorders. Our results support this view. Here we report that the anxiolytic drug alprazolam altered CRF concentrations and mRNA expression, as well as CRF₁ and CRF_{2A} receptor mRNA expression in a manner precisely opposite to lifelong changes observed in rat models of anxiety, based either on genetics (fawn-hooded rat) or on early adverse life experience, whether externally induced by the experimenter (maternal separation) or occurring naturally (low licking–grooming and arched-back nursing). Finally, our results suggest the intriguing possibility that CRF neuronal systems may be composed of two separate, but interrelated, subdivisions (CRF–CRF₁ and urocortin–CRF₂) that can be coordinately and inversely regulated by stress, anxiety models, or anxiolytic drugs.

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