Chronic Administration of the Selective Corticotropin-Releasing Factor 1 Receptor Antagonist CP-154,526: Behavioral, Endocrine and Neurochemical Effects in the Rat

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ABSTRACT
Corticotropin-releasing factor 1 (CRF1) receptor antagonists may represent a novel group of drugs for the pharmacotherapy of depression and/or anxiety disorders. We have investigated the behavioral, endocrine, and neurochemical effects of chronic administration of a selective CRF1 receptor antagonist, CP-154,526. After 9 to 10 days of treatment with CP-154,526 (3.2 mg/kg/day), defensive withdrawal behavior was significantly decreased suggesting anxiolytic activity. In animals treated for 14 days with the low dose of CP-154,526, serum corticosterone concentrations returned to baseline levels faster after application of an airpuff startle. Using in situ hybridization, no changes in CRF1 receptor mRNA expression were detected in parietal cortex, basolateral amygdala, or cerebellum after chronic treatment with CP-154,526. A dose-dependent decrease in CRF mRNA expression was observed in the hypothalamic paraventricular nucleus (PVN) and the Barrington’s nucleus, an effect that was significant at the high but not the low dose of CP-154,526. CP-154,526 did not alter central CRF2A receptor binding or mRNA expression, or urocortin mRNA expression. The present findings suggest that chronic administration of CP-154,526 produces anxiolytic-like effects but no evidence of adrenal insufficiency. Previous postmortem studies revealed increased CRF peptide and mRNA levels in the PVN of depressed patients, which may mediate the hyperactivity of the hypothalamic-pituitary-adrenal axis observed in such patients. In view of a possible use for CRF1 receptor antagonists in the treatment of depression, the present finding that CP-154,526 decreases CRF synthesis in the PVN is of considerable interest.

The neuropeptide, corticotropin-releasing factor (CRF) coordinates the endocrine response to stress through its action as the major physiological regulator of the hypothalamic-pituitary-adrenal (HPA) axis [see Owens and Nemeroff (1991) for review]. In addition to the hypothalamic paraventricular nucleus (PVN), CRF-containing neurons are also localized to extrahypothalamic limbic structures and brainstem nuclei that subserve behavioral and autonomic regulatory functions. There is overwhelming evidence 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 (Owens and Nemeroff, 1991).

Recently, the existence of two distinct CRF receptor subtypes with contrasting neuroanatomical distributions has been demonstrated: CRF1 and CRF2 [see Chalmers et al. (1996) for review]. In rats, CRF1 is the predominant receptor within the pituitary, cerebellum, and neocortex. The CRF2 receptor consists of at least two major splice variants, CRF2A and CRF2B. The former is more prevalent in subcortical regions, particularly the lateral septum (LS), ventromedial hypothalamus (VMH), and dorsal raphe nucleus (DRN), 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., 1996a,b). Urocortin binds with equal affinity to both CRF receptor subtypes, but demonstrates approximately 10-fold higher affinity for CRF2 receptors than does CRF itself [see Chalmers et al. (1996) for review]. In the rat, urocortin-containing perikarya and urocortin mRNA expression are...
most prominent in the Edinger-Westphal nucleus 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 DRN (Vaughan et al., 1995; Wong et al., 1996). It is of interest to note that these two regions nearly exclusively express CRF3 mRNAs. 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.

There now exists a large body of evidence supporting the hypothesis that CRF is hypersecreted from hypothalamic as well as extrahypothalamic neurons in some patients with affective disorders (for reviews, see Arborelius et al. (1999) and Owens et al. (1999)). After antidepressant treatment, measures of hyperactivity HPA axis and CRF function appear to normalize suggesting that hyperactivity of CRF neurons is a state marker for depression (Plotsky et al., 1998; Holsober, 1999).

The hypothesis that CRF may also play a role in the pathophysiology of anxiety disorders derives mainly from preclinical findings (for review, see Arborelius et al. (1999)). It is well known that central administration of CRF increases anxiety-like behaviors in rodents and transgenic mice that overexpress CRF exhibit anxiogenic behavior. Conversely, CRF receptor antagonists or CRF antisense oligonucleotides produce anxiolytic-like effects in the rat. Studies using CRF1 receptor knockout mice and CRF1 and CRF2 receptor antisense oligonucleotides have revealed that the anxiogenic effect of CRF appears to be mediated by the CRF1 receptor subtype (Heinrichs et al., 1997; Smith et al., 1998; Timpl et al., 1998). In humans, elevated concentrations of cerebrospinal fluid CRF have been reported in patients with anxiety disorders, including post-traumatic stress disorder, obsessive-compulsive disorder, and Tourette's syndrome (see Plotsky et al., 1998; Holsober, 1999).

Based on findings such as those described above, it has been hypothesized that CRF1 receptor antagonists may represent a novel class of drugs for treatment of depression and/or anxiety disorders (Owens and Nemeroff, 1999). Recently, a selective, nonpeptide CRF1 receptor antagonist, CP-154,526, was synthesized and characterized (Chen et al., 1997). CP-154,526 displays high affinity to CRF1 receptors ($K_i < 10 \text{ nM}$) and blocks CRF-stimulated adenylate cyclase activity in rodent pituitary and cortical membranes (Lundkvist et al., 1996; Schulz et al., 1996). As expected, acute administration of CP-154,526 or its analog antalarmin blocks CRF- and stress-induced elevations in plasma adenocorticotropin (ACTH) and shows anxiolytic activity (Lundkvist et al., 1996; Schulz et al., 1996; Webster et al., 1996; Chen et al., 1997; Deak et al., 1999). Moreover, CP-154,526 exhibits antidepressant-like activity in the learned helplessness paradigm (Mansbach et al., 1997). Interestingly, but in contrast to standard antidepressant drugs that require repeated administration before clinical effects are observed, CP-154,526 showed antidepressant-like activity after a single dose. However, in the treatment of human depression and anxiety disorders drugs are usually administered during a prolonged period of time. Thus, the purpose of the present study was to investigate whether the anxiolytic effect and the blockade of the endocrine stress response observed after acute administration of CP-154,526 persists during chronic treatment. Moreover, does chronic blockade of CRF1 receptors lead to adaptive alterations in central CRFergic function as assessed by measures of CRF and urocortin mRNA expression and CRF receptor mRNA expression and receptor binding?

### Materials and Methods

**Animals.** Male Sprague-Dawley rats (200–250 g body weight at the beginning of the study; Charles River laboratories, Raleigh, NC) were housed two to three per cage under controlled laboratory conditions (12:12 h light:dark cycle, lights on at 7:00 AM) with water and food available ad libitum. The behavioral experiments and the stress tests were performed between 8:00 AM and noon.

**Drug Treatment.** CP-154,526 (butyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-ethylamine) was provided by Pfizer Inc. (Groton, CT).

In the acute endocrine experiments CP-154,526 was dissolved in a vehicle consisting of saline:ethoxylated castor oil (emulphor 620): dimethyl sulfoxide in the ratio 90:5:5. In the chronic experiments, rats were treated s.c. with either 80% polyethylene glycol 400 (vehicle), 3.2 mg/kg/day, or 32 mg/kg/day CP-154,526 (in vehicle) delivered via Alzet osmotic minipumps (model 2 ML2; Alza Corp., Palo Alto, CA) for 14 days. The rats were handled daily during the course of the treatment. There were no significant differences in body weight between the treatment groups on day 14 (data not shown). The defensive withdrawal test was performed on days 9 or 10, and the airpuff startle tests were performed in the same rats on days 14 or 15. For the neurochemical experiments another set of rats were chronically treated with CP-154,526 as described above and sacrificed on day 14 (data not shown). The defensive withdrawal test was performed on days 9 or 10, and the airpuff startle tests were performed in the same rats on days 14 or 15. For the neurochemical experiments another set of rats were chronically treated with CP-154,526 as described above and sacrificed on day 14 by decapitation. Their brains were quickly dissected, frozen on dry ice, and stored at −80°C until cryostat sectioning. From these animals the adrenal gland was dissected, frozen, and stored at −80°C until weighing.

**Defensive Withdrawal Paradigm.** The apparatus consisted of an open field (75 x 75 cm) with 50-cm high side walls made of white plastic. Each rat was placed in a black polyvinyl chloride tube (10 cm in diameter x 21 cm) that was closed at one end and placed at a distance of 20 cm from a corner of the open field with the open end of the tube facing the corner. The behavior of the rat was recorded by videotape for 10 min. The following parameters were monitored: 1) latency until the animal exited into the open field, 2) total time the animal stayed in the tube, and 3) number of entries into the open field. Entries were defined as all four paws in the open field (Takahashi et al., 1989). The light intensity in the open field was approximately 600 lux during the experiment. Because of the great inter-individual variability in this model, we used a 95% confidence interval.

**Airpuff Startle.** Rats were implanted with chronic jugular vein cannulas 2 days before the stressor under aseptic conditions as previously described (Thrivikraman and Plotsky, 1993). Briefly, animals were anesthetized with an i.p. injection of a mixture of acepromazine (1.5 mg/kg; Tech America, Fermenta Animal Health Co., KS City, MO), ketamine (37 mg/kg; Vetalar, Aveco Co. Inc., Fort Dodge, IA), and xylazine (7.4 mg/kg; Rompun, Miles Laboratory Inc., Shawnee, KS). The jugular vein was exposed by blunt dissection, and a small incision was made using iridectomy scissors. The cannula, consisting of a piece of PE50 tubing (Clay Adams, Sparks, MD) with a tip of silicone tubing (T5715-3; Baxter, McGaw Park, IL), was inserted into the vein approximately 3 cm in the caudal direction, ligated to the vessel, and tunneled s.c. to emerge from the neck of the animal. The wounds were closed with metal clips, and the cannula was filled with sterile saline containing gentamicin (2.5 μg/100 g of body weight; Schein Pharmacy, Port Washington, NY). Thereafter, animals were individually housed in polyethylene buckets (28 cm in diameter and 37 cm high) containing regular wood chip bedding, under controlled laboratory conditions (12:12 h light:dark cycle, lights on at 7:00 AM) with free access to food and tap water.
Blood samples (0.3 ml) were taken from the unrestrained rat under resting conditions, i.e., immediately before application of the stressor, time point 0, and at 5, 10, 15, 30, and 60 min after the stressor and collected in EDTA-containing tubes. Air puff started as presented an episode of three blocks of airpuffs directed toward the side of the head. Each block consisted of three 5-s blasts from pressurized cans (Dust-off; Fisher Scientific, Atlanta, GA) separated by a 10-s interval. A 1-min interval separated each block of airpuffs. Application of the airpuff to the rats induced a typical startle response.

Individual tissue samples, dissected as described for the CRF RIA and the supernatant was collected and stored at −20°C until determination of ACTH and corticosterone levels. Plasma ACTH and corticosterone levels were determined using commercially available radioimmunoassay (RIA) kits (ACTH; Nichols Institute Diagnostics, San Juan Capistrano, CA; corticosterone: ICN Biomedicals, Costa Mesa, CA) with a detection limit of 5 pg/ml for ACTH and 5 ng/ml for corticosterone.

**CRF RIA.** Regional brain samples for CRF RIA were stored at −80°C until assay. The CRF concentrations were determined by RIA as previously described in detail (Ladd et al., 1996). Briefly, duplicate aliquots from each sample were extracted in 1 M HCl, lyophilized, and then reconstituted in 200 μl of SPEAB buffer (100 mM NaCl, 50 mM Na₂HPO₄, 25 mM EDTA, 0.1% sodium azide, 0.1% BSA, and 0.1% Triton X-100, pH 7.3) and incubated at 4°C for 18 h with 100 μl of rabbit anti-CRF (Peninsula, Belmont, CA) at a final dilution 1:70,000 in SPEAB buffer containing 1.0% normal rabbit serum. Next, 50 μl (17,000–20,000 cpm) of radiolabeled 125I-Tyr⁻⁻⁻rat/human CRF (New England Nuclear, Boston, MA) were added to each tube. After incubation with radiolabeled CRF for 24 h at 4°C, 10 μl of goat anti-rabbit serum (Amersham, Arlington Heights, IL) was added to each tube. Application of the airpuff to the rats induced a typical startle response.

**CRF Receptor Binding.** Single point CRF receptor binding assays were performed as previously described (Ladd et al., 1996) on individual tissue samples, dissected as described for the CRF RIA (vide supra) at a near-saturating concentration of 125I-labeled ovine CRF (1 nM final concentration; 0.1 nM 125I-labeled ovine CRF plus 0.9 nM ovine CRF). Specific CRF receptor binding was calculated by subtracting the mean counts per minute in triplicate pellets incubated with 125I-labeled ovine CRF in the presence of 1 μM unlabeled rat CRF. The Kᵦᵦᵦᵦ of 125I-labeled ovine CRF binding in our laboratory ranges from 0.25 to 0.6 nM. Tissue samples were homogenized in 4 ml of buffer (50 mM Tris-HCl, 10 mM MgCl₂, and 2 mM EDTA, pH 7.2, at 22°C) containing the peptidase inhibitors aprotinin (0.1%) and bacitracin (0.1%) and 0.1% BSA using a Polytron homogenizer (model 3100; Brinkmann Instruments, Westbury, NY) at 20,000 rpm for 10 s, followed by centrifugation at 32,000g for 10 min at 4°C. This procedure was repeated either two or four times, and the sample pellets were washed, capped, and frozen at −70°C. On the day of the assay, samples were resuspended and homogenized in buffer to yield a final concentration of approximately 150 μg of protein/100 μl.

Aliquots (100 μl) of membrane homogenate were incubated for either 90 min or 3 h with 100 μl of 125I-labeled ovine CRF (0.1 nM final concentration; New England Nuclear), 50 μl ovine CRF (0.9 nM final concentration), and 50 μl of either rat CRF (1 μM final concentration) to define nonspecific binding or incubation buffer (total binding). Specific binding as a percentage of total binding varies slightly among brain regions and represents 60% to 70% of total binding. Aliquots of the tissue homogenate were used to determine total protein content using BSA as the standard. After incubation, samples were microcentrifuged for 3 min at 12,000g; aspirated, then washed in ice-cold PBS (pH 7.4) containing 0.01% Triton X-100. The supernatant was removed, and the pellet was counted in an gamma counter (LKB, Rockville, MD) at 86% efficiency.

**CRF Receptor Autoradiography.** In vitro CRF receptor autoradiography as previously described (Skelton et al., 2000) was performed on 15-μm rat brain sections mounted on SuperFrost Plus slides (Fisher Scientific). Brain sections were fixed for 2 min in 0.1% paraformaldehyde followed by a 15-min incubation in assay buffer (50 mM Tris, 10 mM MgCl₂, 2 mM EGTA, 0.1% BSA, 0.1 mM bacitracin, and 0.1% aprotinin; pH 7.5) to remove endogenous CRF. Next, triplicate slides containing adjacent brain sections were incubated for 2 h at room temperature in one of three conditions: 1) 0.1 nM radiolabeled 125I-sauvagine (DuPont-NEN) to determine total binding at both the CRF₁ and CRF₂ receptor subtypes, 2) 0.1 nM radiolabeled 125I-sauvagine + 1 μM CP-154,526 to determine CRF₂ receptor-specific binding, or 3) 0.1 nM radiolabeled 125I-sauvagine in 1 μM unlabeled sauvagine (American Peptide Company Inc., 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 ddH₂O. Slides were then rapidly dried with a blow dryer on the coldest setting and apposed to Kodak (Rochester, NY) Biomax MR film with 125I microscale standards (Amersham Pharmacia Biotech, Piscataway, NJ) for 90 to 160 h.

**In Situ Hybridization.** Serial coronal brain sections (15 μm) were prepared on a cryostat at −18°C, thaw-mounted onto SuperFrost Plus slides under RNase-free conditions, and stored with Hemi-Cap desiccant capsules (Life Technologies, Grand Island, NY) 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 step-wise manner to room temperature, postfixed in 4% paraformaldehyde (pH 7.4) for 20 min, and rinsed twice in 10 mM PBS (pH 7.4) for 2 min. Next, the slides were treated for 15 min with proteinase K (Promega Life Science, Madison, WI; 10 μg/ml in 0.1 M Tris with 50 mM EDTA) at room temperature, followed by a quick rinse in deionized water, 2.5 min in 0.1 M triethanolamine (pH 8.0), 10 min of acetylation (0.5% acetic anhydride in 0.1 M triethanolamine, pH 8.0), two rinses in 2× SSC, and dehydration through a graded ethanol series. The sections were then air-dried for at least 1 h 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 pGEM4 plasmid. The urocortin riboprobe was constructed from an approximately 0.6-kb EcoRI fragment ligated into a pBluescript II-SK⁺ plasmid (W. Vale, Salk Institute for Biological Studies, La Jolla, CA). The insert includes ~70 bp of 5′-untranslated cDNA, 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 urocortin receptor, followed by ~130 bp of 3′-untranslated cDNA. The CRF₁ riboprobe was constructed from a 275-bp fragment, encoding the N-terminal region of the CRF₁ receptor, ligated into a pBluescript II-SK⁺ plasmid (T. Lovenberg, Neurocrine Biosciences Inc., San Diego, CA). Radiolabeled antisense cRNAs were synthesized by incorporating α-35S-UTP (DuPont NEN) into the CRF and urocortin probes, α-35S-CTP plus α-35S-UTP into the CRF₁ receptor probe, and [α-32P]CTP into the CRF₂ receptor probe. The transcription reactions were performed utilizing the Ambion MAXiscript kit (Austin, TX) with SP6 (CRF), T7 (urocortin and CRF₁), or T3 (CRF₂) 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 through ethanol precipitation and/or gel filtration using a G-50 Sephadex Quick Spin column (Roche Molecular Biochemicals).

The brain sections were hybridized overnight at 58–60°C with 1 × 10⁶ cpm of α-32P- or α-35S-labeled cRNA probe diluted into hybridization buffer [50% formamide, 10% dextran sulfate, 0.3 M NaCl, 1× Denhardt’s solution, 10 mM Tris, 1 mM EDTA, 2 mg/ml yeast tRNA, 10 mM dithiothreitol (DTT)] in humidified Nunc trays (Nalge Nunc Inc., Naperville, IL). The next day, slides were allowed to cool to room temperature before being washed four times in 4× standard saline
citrate (SSC) for 5 min on a rotating platform at 60 rpm. The sections were then treated with 250 μg/ml RNase A (Promega) for 30 min at 37°C. Subsequently, the slides underwent a series of SSC washes (supplemented with 1 mM DTT) with salt concentrations decreasing from 2× to 0.5×, followed by a 60-min high stringency wash with 0.1× SSC + 1 mM DTT at 60°C, then dehydration through a graded ethanol series. The slides were air-dried for at least 1 h and then apposed to Kodak Biomax MR film for 12 h to 21 days (depending on the probe and brain region examined) with 14C standards (Amer-sham Pharmacia Biotech). Controls have previously been performed in the laboratory to establish the specificity of the signal included hybridization with sense strand probes as well as predigestion with RNase A.

**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; Frederick, MD) or Analytical Imaging Station software (Imaging Research, Inc., St. Catherines, Ontario, Canada). Optical densities were calibrated against 14C standards (in situ hybridization films) or 125I microscale standards (receptor autoradiography films) and expressed in terms of nanocuries per gram 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: CRF1 receptor-specific binding was calculated by subtracting CRF2 receptor binding from total binding, and CRF2 receptor specific binding was calculated by subtracting nonspecific binding from CRF2 receptor binding. In all cases, two to six 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.

**Statistical Analysis.** Adrenal weight, basal levels of ACTH and corticosterone, and the neurochemical data were analyzed by ANOVA. The effects on ACTH and corticosterone levels before and after airpuff startle were analyzed by two-way repeated measures ANOVA followed by Tukey's post hoc test. The different parameters of the defensive withdrawal test were analyzed using Kruskal-Wallis one-way ANOVA on ranks or the Mann-Whitney rank sum test. A P value of <.05 was considered significant. All values are presented as mean ± S.E.

**Results**

**Effects of CP-154,526 on Defensive Withdrawal.** Acute treatment with CP-154,526 did not affect the latency to leave the tube, total time spent in tube, or number of entries into open field in the defensive withdrawal test (Table 1). Animals treated chronically with the low dose of CP-154,526 (3.2 mg/kg/day) showed a decreased latency to initially leave the tube compared with vehicle-treated rats, although this effect did not reach statistical significance (Fig. 1A). However, the total time spent withdrawn in the tube was significantly decreased in CP-154,526-treated animals compared with vehicle-treated rats (Fig. 1A). There was no difference in the number of entries into the open field between animals treated with CP-154,526 and vehicle (Fig. 1B), suggesting that locomotor activity was not affected by the drug treatment. The animals receiving chronic treatment with the high dose of CP-154,526 (32 mg/kg/day) revealed signs of sickness behavior and stayed withdrawn in the tube most of the time. These rats were not included in the analyses.

**Effects of CP-154,526 on Endocrine Responses to Airpuff Startle.** As previously shown (Engelmann et al., 1996), airpuff startle induced a time-dependent increase in both plasma ACTH and corticosterone concentrations in vehicle-treated animals (Fig. 2, A and B; Fig. 3, A and B). An acute dose of 32, but not 3.2 mg/kg of CP154,526, completely

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**TABLE 1**

Behavioral effects in the defensive withdrawal test following acute treatment with 3.2 mg/kg CP-154,526 (CP 3.2; n = 6), 32 mg/kg CP-154,526 (CP 32; n = 5), or vehicle (n = 4).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Latency (s)</th>
<th>Duration (s)</th>
<th>No. of Entries</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>173.5 ± 45.0</td>
<td>414.8 ± 54.2</td>
<td>8.0 ± 0.7</td>
</tr>
<tr>
<td>CP 3.2</td>
<td>102.7 ± 26.7</td>
<td>343.7 ± 65.5</td>
<td>7.7 ± 1.0</td>
</tr>
<tr>
<td>CP 32</td>
<td>64.0 ± 27.7</td>
<td>438.7 ± 47.6</td>
<td>9.0 ± 1.2</td>
</tr>
</tbody>
</table>

The latency of the animals to initially leave the tube (latency), the total time the animals stayed in the tube (duration), and the number of entries into the open field indicates the locomotor activity of the animals. Data are expressed as means ± S.E.
blocked airpuff-induced increases of plasma ACTH and corticosterone levels (Fig. 2, A and B).

Basal concentrations of plasma ACTH or corticosterone were not altered by chronic treatment with CP-154,526. In animals treated chronically with CP-154,526 (3.2 mg/kg/day), airpuff startle produced a time-dependent increase in plasma ACTH, which was not different from that in vehicle-treated animals (Fig. 3A). However, in CP-154,526-treated rats, the plasma corticosterone returned to baseline levels at 60 min after the airpuff, whereas in vehicle-treated animals corticosterone levels were still significantly elevated at this time point (Fig. 3B). In rats receiving the high dose of CP-154,526 (32 mg/kg/day) the veins were noted to be very fragile, and at the time of the experiment blood samples could only be collected from two of the eight rats. In these two rats the airpuff startle still produced a marked, time-dependent increase in plasma ACTH and corticosterone that was indistinguishable from that seen in rats receiving the low dose of CP-154,526 (data not shown).

Effects of CP-154,526 on Adrenal Weight. There was no significant difference in adrenal weight between animals treated chronically with vehicle (41.6 ± 1.2 mg, n = 8), 3.2 mg/kg/day CP-154,526 (43.3 ± 1.8 mg, n = 9), or 32 mg/kg/day CP-154,526 (46.4 ± 2.0 mg, n = 10).

Effects of CP-154,526 on CRF$_1$ Receptor Binding and mRNA Expression. Chronic administration of CP-154,526 produced a significant and dose-dependent decrease in CRF$_1$...
receptor binding in the parietal cortex as demonstrated by ex vivo autoradiography (PC; Fig. 4, A and B). A significant decrease in CRF₁ receptor binding was also observed in cerebellum homogenates; however, this effect was abolished after more washes and a longer incubation time (Fig. 5). These changes almost certainly represent residual drug bound to the receptors.

As shown in Table 2, CRF₁ receptor mRNA expression in parietal cortex, basolateral amygdala, or cerebellum (see Fig. 6) was not affected by chronic administration of CP-154,526.

**Effects of CP-154,526 on CRF Content and mRNA Expression.** Acute administration of CP-154,526 (3.2 or 32 mg/kg) did not affect CRF peptide content in the prefrontal cortex, frontal/parietal cortex, hippocampus, amygdala, ME, DRN, locus ceruleus (LC), and parabrachial nucleus. In addition, CRF gene expression in the PVN and the central nucleus of amygdala (CeA) did not change after a single dose of the drug (data not shown).

Chronic treatment with CP-154,526 dose dependently decreased CRF mRNA expression in the PVN and Barrington’s nucleus, an effect that reached significance at the high dose (Fig. 7, A–C). CRF mRNA expression was not changed in the PC, CeA, or the bed nucleus of stria terminalis (BNST).

**Effects of CP-154,526 on CRF₂ Receptor Binding and mRNA Expression and Urocortin mRNA Expression.** CRF₂ receptor binding in LS, VMH, and DRN was not altered

**TABLE 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PC (n)</th>
<th>BLA (n)</th>
<th>Cerebellum (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>130.5 ± 18.3</td>
<td>33.4 ± 5.4</td>
<td>579.6 ± 58.7</td>
</tr>
<tr>
<td>CP 3.2</td>
<td>127.6 ± 12.8</td>
<td>35.3 ± 8.7</td>
<td>459.7 ± 43.5</td>
</tr>
<tr>
<td>CP 32</td>
<td>130.5 ± 15.9</td>
<td>28.6 ± 6.7</td>
<td>478.4 ± 30.8</td>
</tr>
</tbody>
</table>

Data are expressed as means ± S.E.
Discussion

Previous studies investigating possible anxiolytic activity of a single dose of CP-154,526 or antalarmin have sometimes provided inconsistent results. For instance, CP-154,526 attenuates fear-potentiated startle (Schulz et al., 1996; Chen et al., 1997) and shows anxiolytic-like activity in the elevated plus-maze (Lundkvist et al., 1996), however, in one study CP-154,526 was without effect in this model (Griebel et al., 1998). Moreover, in the light/dark test and the mouse defensive test battery, CP-154,526 has been shown to have anxiolytic-like activity but not in conflict tests, i.e., punished lever pressing and punished drinking tests (Griebel et al., 1998). In the present study we used the defensive withdrawal test, because central CRFergic systems appear to be involved in the mediation of the behaviors in this model (Takahashi et al., 1989; Smagin et al., 1996). We observed that acute administration of either dose of CP-154,526 did not significantly affect defensive withdrawal behaviors. The lack of anxiolytic-like behaviors of CP-154,526 in contrast to previous findings where central administration of the CRF receptor antagonist a-helical CRF 8–41 produced clear anxiolytic-like behaviors in this model (Takahashi et al., 1989). This may be related to the different route of administration of the CRF receptor antagonists used in the two studies, i.e., s.c. versus i.c.v. However, in the present study we observed a tendency for a decrease in latency to initially leave the tube after acute treatment with both doses of CP-154,526 (Table 1). The lack of statistical significance of this effect may be related to the low number of animals in the control group. In contrast, rats treated chronically with the low dose of CP-154,526 spent significantly less time withdrawn in the tube than did control animals, suggesting anxiolytic activity of the compound during chronic treatment (Fig. 1A). As assessed using ex vivo autoradiography, chronic treatment with the low dose of CP-154,526 produced about a 25% blockade of CRF1 receptors in the parietal cortex (Fig. 4B). Thus, it is reasonable to believe that approximately the same degree of CRF1 receptor blockade occurs in other brain regions, i.e., the amygdala which appears to mediate the anxiolytic effects of CRF1 receptor antagonists (Swiergiel et al., 1993; Liebsch et al., 1995, 1999). Acute administration of CP-154,526, at least after the high dose, would also produce a substantial blockade of central CRF1 receptors for a period of time; however, no effect in the defensive withdrawal model was observed. This suggests that other mechanisms in addition to CRF1 receptor blockade may be involved in the anxiolytic-like effect observed during chronic treatment with the low dose of CP-154,526. One possible mechanism could be through a decrease in CRF synthesis (mRNA expression) as observed in Barrington’s nucleus and the PVN of animals treated chronically with CP-154,526 (Fig. 7C). Neurons in Barrington’s nucleus have been demonstrated to send projections to the LC, the origin of the main noradrenergic projections to the forebrain, some of which contain CRF (Valentino et al., 1996). Because local injection of CRF into the LC increases defensive withdrawal behavior (Butler et al., 1990) and a peptide antagonist attenuates it (Smagin et al., 1996), a decrease in CRFergic input to the LC from the Barrington nucleus may contribute to the anxiolytic-like effect observed in rats treated chronically with CP-154,526.

As previously reported, airpuff startle induced a robust and time-dependent increase in plasma ACTH and corticosterone (Engelmann et al., 1996). This effect was markedly attenuated by acute administration of the high, but not the low dose of CP-154,526. Previous studies have shown that a single dose of CP-154,526 or antalarmin attenuates CRF-induced elevations of plasma ACTH concentrations (Schulz et al., 1996; Webster et al., 1996) and foot shock-induced increase in plasma ACTH but not that of corticosterone (Deak et al., 1999; Owens and Nemeroff, 1999). In contrast to a previous study (Bornstein et al., 1998), no change in basal concentrations of ACTH or corticosterone was observed after chronic administration of CP-154,526 in the present study. The main difference between the two studies is the different route of administration used, i.e., two daily s.c. injections in the study by Bornstein et al. (1998) and osmotic minipumps in the present study.

During chronic treatment with the low dose of CP-154,526, application of an airpuff startle induced a similar time-dependent rise in plasma ACTH as seen in vehicle-treated animals. However, the corticosterone response returned to baseline levels faster than in control animals. Because this effect was not observed after a single dose of CP-154,526, it is probably not primarily mediated through CRF1 receptor blockade but through some other mechanism developed during the chronic treatment. For example, corticosterone syn-

Fig. 6. Representative autoradiographs showing CRF mRNA expression in coronal sections of rat brain in the parietal cortex (PC) and the basolateral amygada (BLA; upper panel), and in the cerebellum (Cer; lower panel) observed with in situ hybridization.
thesis and release is subjected to a negative feedback at several different levels.

CRF₁ receptor binding densities in parietal cortex and cerebellum were dose dependently decreased by chronic CP-154,526 treatment. This effect could be washed off from cerebellar homogenates, suggesting that this effect is almost certainly due to residual drug bound to the receptors. In the present study, whether the decrease in CRF₁ receptor binding observed in the parietal cortex could be washed off was not studied. However, it is most likely that this effect in the parietal cortex is also a result of residual drug bound to CRF₁ receptors. Moreover, no change in gene expression of the CRF₁ receptor in the parietal cortex, basolateral amygdala, or the cerebellum suggest that the synthesis of extrahypothalamic CRF₁ receptors are not subjected to a negative feedback mechanism by CRF. This lack of a compensatory up-regulation of CRF receptors after chronic blockade may contribute to the lack of tolerance to the anxiolytic effects demonstrated by CP-154,526. However, it should be noted that down-regulation of cell surface receptors by drug administration can occur in the absence of changes in mRNA expression (e.g., receptor internalization).

CRF mRNA expression decreased in the PVN and Barrington's nucleus after chronic administration of CP-154,526. CRF synthesis in the PVN appears to be subjected to a positive feedback mechanism through CRF₁ receptors (Imaki et al., 1996). Thus, it is possible that the observed decrease in CRF mRNA expression in the PVN in animals treated chronically with CP-154,526 may be due to a chronic blockade of this feedback mechanism. Whether a similar positive feedback mechanism through CRF₁ receptors also exists for CRF synthesis in the Barrington's nucleus is not known. Post-mortem studies have revealed increased CRF peptide and mRNA levels in the PVN in depressed patients (Raadsheer et al., 1994, 1995) and have been postulated to play a role in the hyperactivity of the HPA axis observed in many depressed patients. In view of the possible use of CRF₁ receptor antagonists in the treatment of depression, the present finding that a
14-day administration with CP-154,526 decreases CRF mRNA expression in the PVN is of considerable interest.

CRF<sub>2</sub> receptor binding or mRNA expression was not changed by chronic administration of CP-154,526 suggesting that chronic treatment with a selective CRF<sub>1</sub> receptor antagonist does not modify CRF<sub>2</sub> receptor functioning (Owens and Nemeroff, 1999; Smart et al., 1999). Similarly, urocortin mRNA expression in the Edinger-Westphal nucleus was not altered by CP-154,526 administration. Although CP-154,526 lacks appreciable affinity for the CRF<sub>2</sub> receptor, until recently it has been suggested that the urocortin-CRF<sub>2</sub> receptor system might represent a parallel stress-regulating system similar to the CRF-CRF<sub>1</sub> receptor system and that there might be considerable cross talk between them. The lack of chronic CP-154,526 administration to alter this system suggests that the above hypothesis may not be true. Indeed, we have recently observed evidence that these two systems might represent “antiparallel” systems (Skelton et al., 2000).

### Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LS (nCi/g)</th>
<th>VMH (nCi/g)</th>
<th>DRN (nCi/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>439.5 ± 22.1</td>
<td>344.1 ± 10.9</td>
<td>620.5 ± 31.7</td>
</tr>
<tr>
<td>CP 3.2</td>
<td>444.4 ± 30.3</td>
<td>333.0 ± 26.5</td>
<td>664.6 ± 51.4</td>
</tr>
<tr>
<td>CP 32</td>
<td>473.4 ± 29.8</td>
<td>294.7 ± 22.2</td>
<td>584.2 ± 25.5</td>
</tr>
</tbody>
</table>

Data are expressed as means ± S.E.

### Table 4

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LS (nCi/g)</th>
<th>DRN (nCi/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>123.8 ± 7.9</td>
<td>116.1 ± 6.0</td>
</tr>
<tr>
<td>CP 3.2</td>
<td>123.2 ± 11.1</td>
<td>122.3 ± 9.5</td>
</tr>
<tr>
<td>CP 32</td>
<td>124.6 ± 6.3</td>
<td>121.8 ± 9.1</td>
</tr>
</tbody>
</table>

Data are expressed as means ± S.E.
TABLE 5  
Urocortin mRNA expression (nCi/g) in the Edinger-Westphal nucleus during chronic administration of 3.2 mg/kg/day CP-154,526 (CP 3.2: n = 10), 32 mg/kg/day CP-154,526 (CP 32: n = 11), or vehicle (n = 10) Data are expressed as means ± S.E.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Edinger-Westphal nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>1928.2 ± 104.9</td>
</tr>
<tr>
<td>CP 3.2</td>
<td>1800.8 ± 111.7</td>
</tr>
<tr>
<td>CP 32</td>
<td>1745.3 ± 84.7</td>
</tr>
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</table>

Although CP-154,526 is a potent CRF1 antagonist, it is of interest to note that, in this study and others, plasma ACTH and corticosterone concentrations can still increase in response to stress and that basal concentrations are not greatly affected. Therefore, any concerns regarding potential serious adverse effects such as adrenal insufficiency, which might hinder clinical development, appear to be minimal at present.

References


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