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The Corticotropin-Releasing Factor$_1$ Receptor Antagonist R121919 Attenuates the Behavioral and Endocrine Responses to Stress

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ABSTRACT

Corticotropin-releasing factor (CRF) is the major physiological regulator of the hypothalamic-pituitary-adrenal (HPA) axis and serves to coordinate the mammalian endocrine, autonomic, and behavioral responses to stress. Considerable literature from clinical and preclinical data suggests that hypersecretion of hypothalamic and/or extrahypothalamic CRF systems is a major factor in the pathogenesis of affective and anxiety disorders. Based on this premise, a CRF_1 receptor antagonist has been hypothesized to possess anxiolytic and/or antidepressant properties. In this study, an acute dose of the lipophilic CRF_1 receptor antagonist 3-[6-(dimethylamino)-4-methyl-pyrid-3-yl]-2,5-dimethyl-*N*,*N*-dipropyl-pyrazolo[2,3-*a*]pyrimidin-7-amine (R121919), administered i.v. to rats with surgically implanted jugular cannula 60 min before a 5-min restraint stress, dose dependently attenuated peak plasma adrenocorticopin hormone (ACTH) and corticosterone concentrations by 91 and 75%, respectively. In a second study, acute administration of R121919 reduced measures of anxiety in a rodent defensive withdrawal paradigm. R121919 dose dependently decreased latency to exit the tube, and total time spent in the tube 60 min after a single subcutaneous administration. In addition, the ACTH and corticosterone response to novelty was decreased by 82 and 97%, respectively, at the 10-mg/kg dose of R121919. In another study, this dose was associated with approximately an 85% occupancy of the CRF_1 receptor in the cortex measured 75-min postsubcutaneous injection. These data confirm that R121919 acts as a CRF_1 receptor antagonist in vivo, attenuates HPA axis responsivity, and possesses anxiolytic properties.

Since its discovery and characterization by Vale et al. (1981), a burgeoning database has accrued concerning the neural and endocrine roles of corticotropin-releasing factor (CRF). A preeminent role of CRF as the key regulator of the hypothalamo-pituitary-adrenal (HPA) axis, as well as the behavioral, immune, and autonomic aspects of the stress response has been clearly established (for review, see Owens and Nemeroff, 1991).

Two CRF receptor subtypes, CRF_1 and CRF_2, with distinct anatomical localization and receptor pharmacology have been identified (Chang et al., 1993; Chen et al., 1993; Lovenberg et al., 1995; Chalmers et al., 1996; Grigoriadis et al., 1996). The CRF_1 receptor is the predominant receptor in the pituitary, cerebellum, and neocortex in the rat (Primus et al., 1997), whereas CRF_2 receptors are more prevalent in subcortical regions, such as the ventromedial hypothalamus, lateral septum, and dorsal raphe nucleus. A growing body of evidence has shown that CRF_1 receptors may specifically mediate some of the anxiogenic-like behaviors observed after administration of CRF (Heinrichs et al., 1997). Several additional members of the CRF peptide family, including urocortin, urocortin II, and urocortin III have also recently been identified (Lewis et al., 2001).

Numerous studies have attempted to isolate the role of individual CRF receptor subtypes in mediating distinct behaviors. Mice with targeted knockouts of the CRF_1 receptor demonstrate an impaired stress response (Timpl et al., 1998). In contrast to the CRF_1 receptor knockouts, mice lacking CRF_2 receptors demonstrate increased anxiety-like behaviors and hypersensitivity to stress (Bale et al., 2000; Kishimoto et al., 2000). These results lead to the intriguing possibility that there are two separate CRF systems as suggested by their distinct anatomical distribution and pharmacology, and furthermore that activation of CRF_1-type receptors may be anxiogenic, whereas CRF_2 receptor activation may modu-

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ABBREVIATIONS: CRF, corticotropin-releasing factor; HPA, hypothalamic-pituitary-adrenal; ACTH, adrenocorticopin hormone; CORT, corticosterone; ANOVA, analysis of variance; SNK, Student-Newman-Keuls.

late stress-coping mechanisms. Clinical data also support dysregulation of the CRF system in the pathogenesis of affective and anxiety disorders (Arborelius et al., 1999).

The synthesis of the first small-molecule CRF antagonist, buytyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl]-ethylamine (CP-154,526), was reported by Chen et al. (1996). The compound demonstrated activity in blocking CRF-mediated ACTH responses (Schulz et al., 1996), in learned helplessness models (Mansbach et al., 1997), in the defensive withdrawal paradigm (Arborelius et al., 2000), and in attenuating stress-induced relapse to drug seeking in cocaine- and heroin-trained rats (Shaham et al., 1998). Antalarmin, a methyl analog of CP-154,526, attenuated the behavioral, neuroendocrine, and autonomic responses to stress in adult nonhuman primates (Habib et al., 2000). CRA1000 and CRA1001, two other small-molecule CRF antagonists, were able to reverse the swim stress-induced reduction of the time spent in the light area in the light/dark exploration, although they were ineffective in non-stressed animals in the same paradigm (Okuyama et al., 1999). DMP695, another small-molecule CRF antagonist produced by DuPont, has also been demonstrated to show anxiolytic-like activity in animal models (Millan et al., 2001). The development of lipophilic small-molecule CRF_1 receptor antagonists has been recently reviewed (McCarthy et al., 1999; Owens and Nemeroff, 1999).

R121919 is a potent small-molecule CRF_1 receptor antagonist with high affinity for the CRF_1 receptor (K_i = 2–5 nM) and over 1000-fold weaker activity at the CRF_2 receptor, CRF-binding protein, or 70 other receptor types (Grigoriadis et al., 2000; Keck et al., 2001). Recently, the effectiveness of R121919 in major depression was demonstrated in a small open-label clinical study (Zobel et al., 2000). R121919 reduced measures of both anxiety and depression in the depressed patients. In addition, two recent studies have demonstrated anxiolytic-like effects of R121919 in rodents (Keck et al., 2001; Heinrichs et al., 2002).

In the present set of experiments, we examined the ability of R121919 to block restraint-stress induced activation of the HPA axis, and whether it possesses activity in the defensive withdrawal test. Finally, receptor autoradiography was used to examine the blood-brain barrier penetrability and occupancy of central CRF receptors by R121919 after peripheral administration and the interaction between receptor occupancy and behavioral parameters was evaluated.

Materials and Methods

Animals. Male Sprague-Dawley rats (225–250 g on arrival; Harlan, Prattville, AL) were housed two per cage unless otherwise noted, with food and water available ad libitum, in an environmentally controlled animal facility with a 12-h light/dark cycle (lights on at 7:30 AM). Animals were allowed at least 7 days to acclimate to the animal facility before initiation of experimentation. The Emory University Institutional Animal Care and Use Committee approved of all the animal protocols utilized in these studies.

Drug Preparation. For the restraint stress, R121919 was dissolved in an aqueous 70% (v/v) polyethylene glycol 400 solution, and serially diluted in this vehicle to the appropriate concentrations. The drug was injected i.v. in a volume of 1 ml/kg. For the defensive withdrawal experiments, R121919 solutions were made fresh the night before each experiment. R121919 was dissolved in a vehicle consisting of 5% (v/v) polyethoxylated castor oil (Alkamuls EL-620; Aventis, Strasbourg, France) in 0.3% tartaric acid (Sigma-Aldrich, St. Louis, MO). The solution was sonicated for at least an hour to allow the drug to dissolve. Small amounts of glacial acetic acid (approximately 15–20 μl in a volume or 4–5 ml of solution) were added to increase solubility, until the powder was completely in solution. Both the vehicle and all concentrations of the antagonists used in the studies were adjusted to a final pH of ~4.5. R121919 was a gift of Janssen Pharmaceuticals (Beerse, Belgium).

Restraint Stress. For the restraint stress study, the rats were cannulated 5 days before the experiment. Rats were implanted with chronic jugular venous cannulae under aseptic conditions, as described in detail previously (Thrivikraman et al., 2000). Briefly, animals were anesthetized with a mixture of acepromazine (1.5 mg/kg s.c.; Tech America, Fermenta Animal Health Co., Kansas City, MO), ketamine (37 mg/kg s.c.;Vetalar, Aveco Co. Inc., Fort Dodge, IA), and xylazine (7.4 mg/kg s.c.; Rompum, Miles Laboratories Inc., Shawnee, KS), and the jugular vein was exposed by blunt dissection and a small incision made using iridectomy scissors. The cannula, consisting of a piece of polyethylene 50 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 subcutaneously to emerge from the neck of the animal. The wounds were closed with metal clips and 200 to 250 μl of 120 μg/ml gentamicin was added to the cannula (Schein Pharmacy, Port Washington, NY) and flushed every 3rd day. The rats were then housed singly for 2 days to recover from the surgery.

Three days after surgery, the animals were moved from their cage into a polyethylene bucket (28 cm in diameter and 37 cm in height) with food and water available ad libitum. The animals were weighed and then allowed to acclimate to the bucket for 2 days before testing. The bucket was used to allow convenient access to the jugular cannula for blood sampling. On testing day, a blood sample (300 μl) was obtained 60 min before restraint stress to determine basal ACTH and corticosterone (CORT) values, immediately before administering either vehicle (70% polyethylene glycol), or varying concentrations of R121919 (0.33, 1.0, 3.3, or 10 mg/kg; n = 8–10/group) via the i.v. cannula. An additional blood sample was then collected before placing the rodent in a plastic restraint cone for 5 min. A third blood sample was collected immediately after restraint, before the animal was removed from the restraint cone and returned to its bucket. Additional samples were collected at 10, 15, 30, 60, and 90 min after beginning the restraint. The blood volume removed during sample collection was replaced with an equal volume of isotonic saline. Animals were decapitated at 3 or 8 h after initiation of the restraint stress, and the brains were immediately removed and frozen on dry ice, and then kept frozen at −80°C until slicing for autoradiography. One animal treated with 10 mg/kg R121919 was removed from analysis because ACTH and CORT values were greater than 2 standard deviations from the mean at all time points measured. This animal had ACTH and CORT values greater than 200 pg/ml and 200 ng/ml, respectively, at all time points measured, including the point immediately before drug administration, indicating that the elevated ACTH and CORT in this animal were not a direct effect of R121919 administration.

Receptor Autoradiography. Using the atlas by Paxinos and Watson (1986), brains from the restraint stress and defensive withdrawal studies were sectioned at the level of the prefrontal cortex and lateral septum to determine CRF_1 and CRF_2 receptor binding, respectively. The prefrontal cortex has been shown via in situ hybridization to express predominantly CRF_1 receptors, whereas the lateral septum only expresses CRF_2 receptors in rodents (Van Pett et al., 2000). For both experiments, 15-μm rat brain sections containing the prefrontal cortex and the lateral septum were sectioned at approximately −20°C and mounted on Superfrost Plus Slides (Fisher Scientific, Pittsburgh, PA) and stored at −80°C until the assay.

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. Sections were removed from the −80°C freezer and allowed to warm to room temperature in a desiccator. Brain sections were fixed for 2 min in 0.1% paraformaldehyde, pH 7.5, followed by a 15-min incubation in assay buffer (50 mM Tris, 10 mM $MgCl_2$, 2 mM EGTA, 0.1% bovine serum albumin, 0.1 mM bacitracin, and 0.1% aprotinin, pH 7.5). Next, triplicate slides containing adjacent brain sections were incubated in one of three conditions: 1) 0.1 nM radiolabeled ^{125}I-sauvagine (PerkinElmer Life Sciences, Boston, MA) to determine total binding at both the CRF_1 and CRF_2 receptor subtypes; 2) 0.1 nM radiolabeled ^{125}I-sauvagine + 1 μM CP-154,526 (Pfizer, Groton, CT), a CRF_1-receptor specific antagonist, to determine CRF_2 receptor-specific binding; or 3) 0.1 nM radiolabeled ^{125}I-sauvagine + 1 μM unlabeled sauvagine (American Peptide Co., Inc., Sunnyvale CA) to determine nonspecific binding. After a 2-h incubation, unbound radioligand was removed by two 5-min rinses in ice-cold phosphate-buffered saline + 1% bovine serum albumin on a rotating platform at 60 rpm, followed by two brief dips in ice-cold distilled, deionized H_2O. Slides were then rapidly dried using a cold air blow dryer and apposed to Kodak Biomax MR film (Eastman Kodak, Rochester, NY) with ^{125}I-microscale standards (Amersham Biosciences, Inc., Piscataway, NJ) for 80 to 90 h.

Image Analysis. Images from the receptor autoradiography films were digitized with a CCD-72 (Dage-MTI, Michigan City, IN) image analysis system equipped with a Nikon camera. Semiquantitative analysis was performed using AIS software (version 4.0; Imaging Research, St. Catherines, ON, Canada). Optical densities were calibrated against coexposed ^{125}I-microscale standards (Amersham Biosciences, Inc.) and expressed in terms of nanocuries per gram of tissue equivalent. CRF_1 receptor-specific binding was calculated as total binding − CRF_2 receptor binding, and CRF_2 receptor-specific binding was calculated as CRF_2 receptor binding − nonspecific binding. Receptor binding data are expressed as a percentage of the control values. The percentage of binding was subtracted from 100 to estimate percentage of receptor occupancy by R121919 relative to vehicle-treated rats. In all cases, three 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.

Statistics. Significant differences were evaluated by one- or two-tailed t tests or one-way ANOVA followed by Student-Newman-Keuls (SNK) post hoc analysis as appropriate. ACTH and CORT values were log transformed before statistical analysis. Details of data analysis are provided in the corresponding figure legends. All data are expressed as the mean ± S.E.M. All statistics were performed using SigmaStat (version 2.03; SPSS Science, Chicago, IL).

Defensive Withdrawal. The rats were handled daily before the experiments starting 7 days before behavioral testing. On testing day, the rats were transported from the animal facility to the testing area in a covered cart before their normal light cycle began (between 6:30 and 7:00 AM). Sixty minutes before testing, rats received subcutaneous injections of either vehicle or R121919 (0.33, 1.0, 3.33, or 10 mg/kg; n = 7–8/group). The animals were then immediately returned to their cage and left undisturbed until testing. An additional group of rats that had been housed and handled as described above but not tested in defensive withdrawal were killed concurrently with the tested rats to obtain basal ACTH and CORT values. All experiments were conducted between 8:00 AM and 12:00 PM, which allowed ACTH and CORT to be obtained at a similar time of day as in restraint stress experiments.

For the defensive withdrawal experiments, a 100 × 100-cm white Plexiglass arena with 50-cm-high walls was used. The bottom of the arena was painted a flat gray and grid lines were drawn at 20-cm intervals to facilitate scoring. On testing day, the light level was adjusted to 600 to 750 lux across the entire arena using a light meter (VWR Scientific, Atlanta, GA). To begin the trial, the rat was placed in front of a black polyvinyl chloride tube (10 cm in diameter × 21 cm in length, closed at one end) and allowed to walk in unassisted. The tube was then placed into the field at a distance of 20 cm from a corner, with the open end of the tube facing the corner. Each trial lasted 10 min and was videotaped. After the conclusion of the trial, the animal was returned to its home cage. Five minutes later, the animal was transported to an adjacent room and immediately decapitated with a guillotine. Whole blood was collected in 1.5-ml microcentrifuge tubes and glass Vacutainer tubes containing EDTA (BD Biosciences, Franklin Lakes, NJ) on ice for ACTH and CORT measurements, respectively. Blood samples were centrifuged (3100g CORT/1900g ACTH, 10 min, 4°C), and the plasma and serum were collected and stored at −80°C until ACTH and CORT determinations were performed (Thrivikraman and Plotsky, 1993). Brains were quickly removed after decapitation and placed on dry ice cortex side up, with dry ice powder layered on top to preserve cortical morphology, and then kept frozen at −80°C until slicing. ACTH was measured in duplicate samples of rat plasma by a two-site immunoradiometric assay (Nichols Diagnostics, San Juan Capistrano, CA) with coefficient of variation of 5% and sensitivity of 1 pg/ml. Corticosterone was assayed in duplicate samples of rat serum by a double antibody radioimmunoassay (ICN Pharmaceuticals, Costa Mesa, CA) with a coefficient of variation of 6% and a sensitivity of 1.2 ng/ml.

Behavioral Analysis. Latency to exit the tube was determined by recording the time of onset of the first four-paw transition from inside the tube into the arena. The rat was considered to have returned to the tube when it had completely returned to the tube interior. The time of each excursion was then summed and subtracted from the number of seconds of the experiment (600) to determine the total time spent inside the tube. The sessions were videotaped, and then analyzed separately by two experienced raters who were blind to treatment. The inter-rater reliability was greater than 0.9.

Results

Restraint Stress: Endocrine Measures. ACTH and CORT responses to R121919 and to restraint stress are presented in Fig. 1, A and B. Pretreatment baseline values of ACTH and CORT were not different between groups, and the mean averages were 0.96 ± 0.34 pg/ml and 18.22 ± 6.9 ng/ml, respectively. R121919 had no effect on baseline ACTH or CORT levels measured 1 h after the single injection. Ten minutes after a 5-min restraint stress both ACTH and CORT were significantly increased in vehicle-treated animals, with nearly a 40- and 11-fold increase, respectively, relative to prerestraint concentrations. The maximum or peak responses in ACTH and CORT in vehicle animals were at 10 and 15 min, respectively. R121919 dose dependently decreased ACTH and CORT responses to restraint stress. Peak plasma ACTH and corticosterone concentrations at a dose of 10 mg/kg R121919 were 9 and 25%, respectively, of vehicle controls.

Restraint Stress: Ex Vivo Binding. Calculated inhibition of binding ex vivo, which was used as a surrogate for receptor occupancy, at 3 and 8 h after i.v. administration of R121919 are displayed in Fig. 1C. At the highest dose tested, 10 mg/kg, R121919 occupied approximately 75% of CRF_1 receptors in the cortex at 3 h, and 45% at 8 h. At both time points an increase in CRF_1 ex vivo binding occurred with increasing dosage. In contrast, CRF_2 ex vivo binding by R121919 in the lateral septum remained under 20% and was not statistically different from controls (at any dose), and did not exhibit dose dependence.

Defensive Withdrawal: Behavior. One-hour pretreatment with a single subcutaneous injection of R121919 produced a dose-dependent decrease in the latency to the first

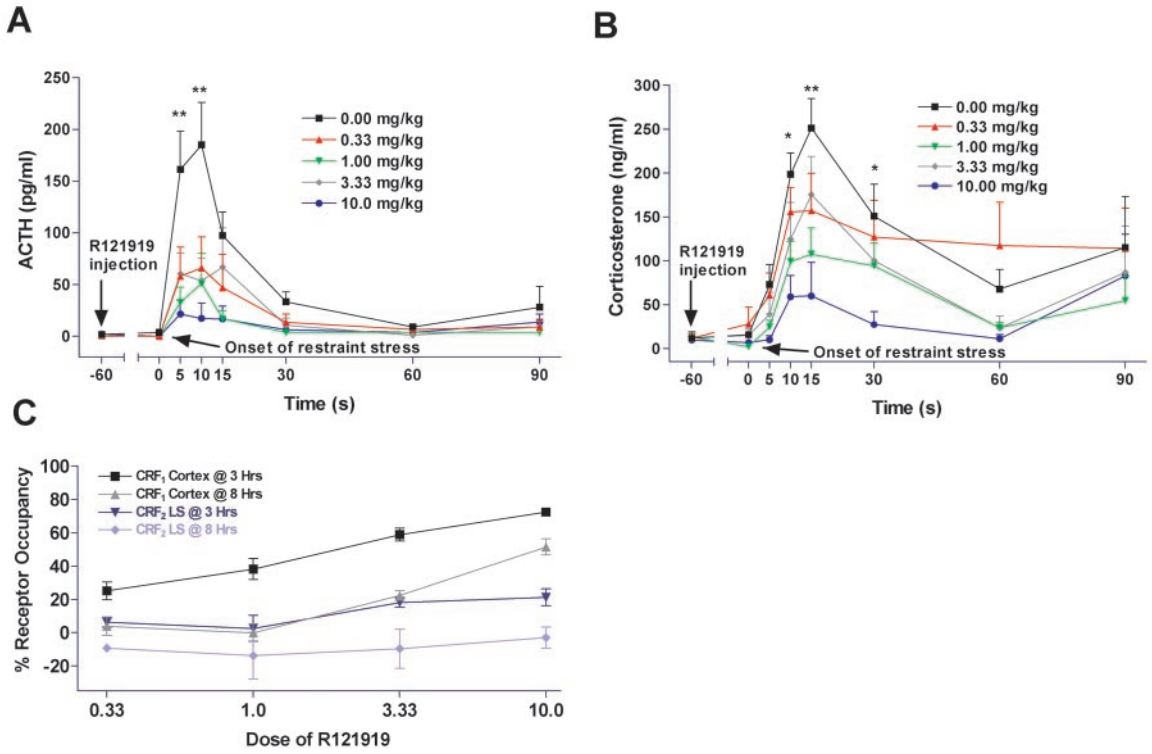

Fig. 1. A, ACTH response to 5 min of restraint stress 60 min after i.v. injection of R121919. **, $p < 0.01$ compared with all other doses at that time point via two-way ANOVA with repeated measures on time followed by SNK post hoc analysis. B, CORT response to 5 min of restraint stress 60 min after i.v. injection of R121919. *, $p < 0.05$ versus 10.0-mg/kg dose at that time point; **, $p < 0.01$ versus 1.0 and 10.0-mg/kg doses; $p < 0.05$ versus 0.33-mg/kg dose at that time point. Comparisons made by two-way ANOVA with repeated measures on time followed by SNK post hoc analysis. C, calculated receptor occupancy 3 and 8 h after a single i.v. administration of R121919. CRF_1 receptor occupancy was estimated in the cortex, and CRF_2 receptor occupancy was estimated in the lateral septum (LS). See text for specific details of calculation.

four-paw transition in defensive withdrawal testing (Fig. 2A). For the highest dosage tested (10 mg/kg), the latency to the first transition decreased by over 55% compared with vehicle-treated rats. Moreover, 10.0 mg/kg R121919 produced a 5-fold decrease in the total amount of time spent in the tube compared with control (Fig. 2B). Thirty-seven percent of the vehicle-treated animals failed to make a single transition during the 600-s trial, although 100% of the animals receiving 10 mg/kg R121919 made at least one transition. Total time spent in the tube was also significantly decreased after treatment with R121919 (Fig. 2B). The average length per transition was also calculated, and showed a nonsignificant trend toward increased trip duration with increasing dosage (data not shown).

Defensive Withdrawal: Endocrine. R121919 dose dependently attenuated ACTH and CORT concentrations obtained 5 min after the termination of the 10-min defensive withdrawal test (Fig. 2D). ACTH and CORT concentrations in the 10-mg/kg treatment group were 16 and 2%, respectively, of the control group values. This effect was statistically significant ($p < 0.01$) at both the 3.3- and 10.0-mg/kg groups versus control for CORT, and at the 10.0-mg/kg dosage for ACTH measurements. Basal concentrations of ACTH and CORT taken from animals raised under the same conditions as the test groups were 37.3 ± 12.3 pg/ml and 2.6 ± 2.4 ng/ml, respectively.

Defensive Withdrawal: Ex Vivo Binding. R121919 (3.33 and 10 mg/kg) occupied nearly 80 to 85% of cortical CRF_1 receptors 75 min after a single s.c. injection (Fig. 2C). In contrast, CRF_2 ex vivo occupancy in the lateral septum ranged from 5 to 20% at the 0.33, 1.0, and 3.33 mg/kg R121919. CRF_2 ex vivo occupancy increased to approximately 24% at the 10.0-mg/kg dose, but this was not statistically different from any other treatment group. Representative autoradiographs are shown in Fig. 3.

Discussion

Although several small-molecule CRF_1 receptor antagonists have been developed over the past 5 years, much of the pioneering preclinical work in the field used peptide-based antagonists that needed to be directly infused into target brain regions. α-Helical CRF_{9-41} was one of the first compounds used as a CRF receptor antagonist and demonstrated activity in several animal models of anxiety. These and many other studies clearly demonstrated that CRF_1 receptor antagonists exhibit anxiolytic activity in a variety of animal models (Arborelius et al., 1999; Holsboer, 1999).

Exploratory behavior, as measured by the time an animal spends investigating objects in a novel environment, is often used as a measure of innate fearfulness or anxiety in rodents. Prior exposure to stressors such as restraint or shock reduces the amount of exploratory behavior. Direct i.c.v. injection of CRF similarly reduces exploratory behavior in this paradigm. This effect can be reversed by pretreatment with α-helical-CRF_{9-41}. Furthermore, α-helical-CRF_{9-41} also attenuates the response to laboratory stressors, e.g., restraint stress and defensive withdrawal, in the absence of exogenous CRF, presumably by blocking the effects of endogenously released CRF (Berridge and Dunn, 1987; Berridge and Dunn,

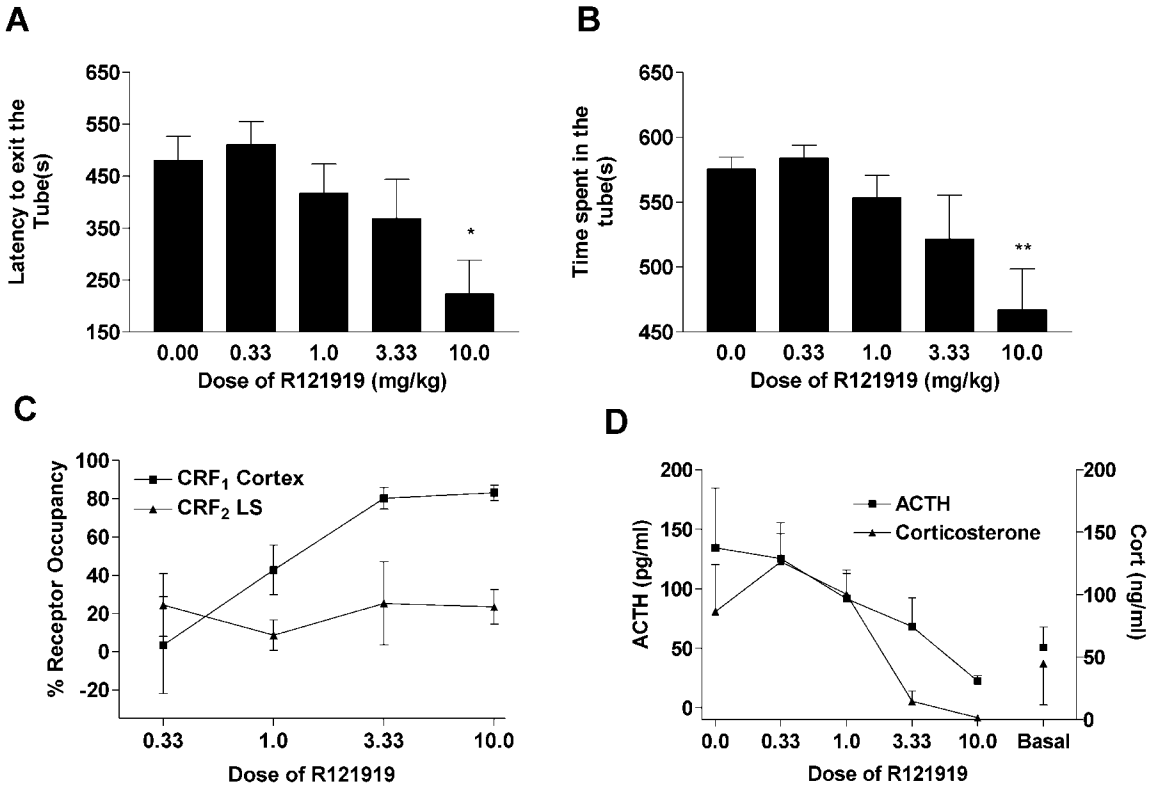

Fig. 2. A, latency to exit the tube in the defensive withdrawal paradigm was significantly reduced by treatment with 10.0 mg/kg R121919 60 min before the onset of defensive withdrawal. *, $p < 0.05$ versus 0.00 (vehicle) and 0.33-mg/kg doses; one-way ANOVA, followed by SNK post hoc analysis. B, total time spent in the tube was significantly decreased after treatment with 10.0 mg/kg R121919 60 min before the onset of defensive withdrawal. **, $p < 0.01$ versus 0.00 (vehicle), 0.33 mg/kg, $p < 0.01$ versus 1.00-mg/kg doses; one-way ANOVA, followed by SNK post hoc analysis. C, estimated receptor occupancy as determined by autoradiography 75 min after defensive withdrawal. D, ACTH and CORT responses 15 min after the onset of 10 min of defensive withdrawal. An additional group of rats (basal) that did not undergo defensive withdrawal is included for comparison. Both ACTH and CORT were significantly decreased relative to vehicle-treated animals that underwent defensive withdrawal. *, $p < 0.05$.

1989; Takahashi et al., 1989). These data clearly support a preeminent role for CRF in modulating the behavioral response to stress and provided one of the major rationales for the development of small-molecule CRF antagonists with enhanced bioavailability.

Small-molecule lipophilic CRF antagonists exhibit important pharmacological differences compared with peptide antagonists due to the widespread distribution of the former class of compounds both peripherally and throughout the central nervous system. Small-molecule antagonists may exert both central and peripheral effects on CRF systems. Peptides, because of their polar nature and relative inability to permeate the blood-brain barrier, need to be infused directly, either i.c.v. or into specific brain nuclei, which limit the neuroanatomically accessible targets.

As noted above, the results from transgenic studies with knockout mice have indicated that CRF_1 receptors seem to specifically mediate the anxiogenic affects of CRF. CRF_1 receptor knockout mice exhibit a reduced stress response, whereas CRF_2 receptor knockout mice seemed to be more anxious than controls (Timpl et al., 1998; Bale et al., 2000; Kishimoto et al., 2000), although this view is by no means universal. There is a general consensus that CRF_1 receptor antagonists may represent a new class of psychotherapeutic agents for the treatment of mood and anxiety disorders (Holsboer, 1999).

Ideally, any clinically useful CRF_1 receptor antagonist should demonstrate efficacy in attenuating anxiety and/or depressive-like symptomatology, possess a favorable pharmacokinetic and bioavailability profile, and exhibit a low incidence of side effects. R121919 is a small-molecule CRF_1 receptor-selective antagonist that shows good solubility and readily crosses the blood-brain barrier (Keck et al., 2001). Concurrent with our own research, the first open-label study with R121919 in human patients with major depressive disorder has recently been completed (Zobel et al., 2000). This study showed an overall improvement in anxiety and depression measures after 30 days of treatment with R121919, and a worsening of affective symptomatology after drug discontinuation. Although this was a small open-label study, taken together with the present findings in rodent models, it supports CRF antagonists as novel treatments for both depression and anxiety disorders.

Because little data are currently in the public domain regarding the pharmacology of R121919, our first goal in this experiment was to characterize the ability of R121919 to block restraint stress-induced HPA axis activation. Restraint stress is a potent activator of the HPA axis that has been blocked via CRF antagonists in past studies (Krahn et al., 1986; Berridge and Dunn, 1987, 1989; Cole et al., 1990; Smagin et al., 1996). R121919 dose dependently attenuated restraint-induced HPA axis activation (Fig. 1, A and B). Both basal levels before infusion of drug, and 1 h after administration were nearly identical between the treatment groups, indicating R121919 did not affect ACTH and CORT levels under basal, nonstressed conditions. Significant effects of

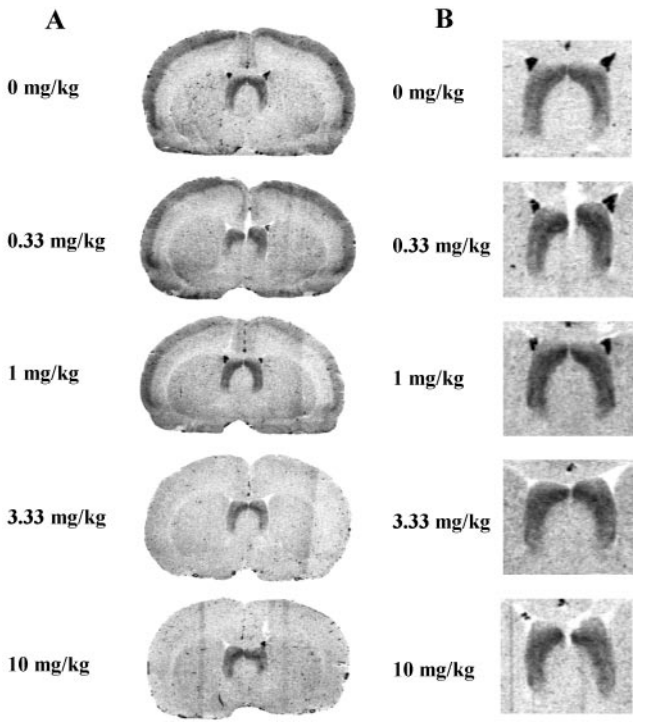

Fig. 3. Dose-dependent occupancy of CRF_1 receptors without significant occupancy of CRF_2 receptors 75 min after the subcutaneous administration of the CRF_1 antagonist R121919. A, representative images of CRF_1 receptor binding in the frontal/parietal cortex after autoradiography with 0.1 nM ^{125}I-sauvagine. B, representative images of CRF_2 receptor binding in the lateral septum (LS) after autoradiography with 0.1 nM ^{125}I-sauvagine + 1 μM CP-154,526.

R121919 on HPA axis activity were seen only after restraint stress. At all doses tested, the animals mounted a response to the restraint stress. Although the amplitude of the response was significantly attenuated, the time course was similar in all conditions. This provides evidence that R121919 attenuated the HPA axis response, but did not lead to adrenal insufficiency or completely block the stress response, at least after acute administration.

From a physiological and clinical standpoint, a complete absence of adrenocortical hormones under basal conditions or in response to stress is an undesirable outcome. Glucocorticoids are necessary to maintain normal body homeostasis and energy utilization. In both clinical (Heim et al., 2000) and preclinical (Ladd et al., 2000) models of HPA axis hyperactivity, nonstressed ACTH and cortisol (human)/CORT (rat) concentrations are not significantly different between control and experimental groups. The differences manifest after exposure to acute stressors. Thus, the ability of R121919 to attenuate HPA axis activity during stressful conditions, but not basal conditions is advantageous.

After demonstrating R121919 was efficacious in blocking restraint-induced HPA axis activation, we sought to determine its efficacy in an animal model of anxiety. Defensive withdrawal has been previously used to explore the connection between CRF systems and anxiety (Takahashi et al., 1989; Smagin et al., 1996). Our results are consistent with the activity of i.c.v. α-helical CRF_{9-41} and CP-154,526 in the defensive withdrawal paradigm (Takahashi et al., 1989; Arborelius et al., 2000); R121919 significantly increased the amount of time rats spent exploring the novel environment upon initial testing (Fig. 2, A and B).

In our experiments, R121919 clearly attenuated the scored measures of anxiety in the novel environment. Although the effect was only statistically significant compared with vehicle control at the highest dose tested (10.0 mg/kg), both total time spent in the tube and latency to exit showed clear dose-dependent effects (Fig. 2, A and B). Regression analysis yielded an R^2 value of approximately 0.93 between dosage and both latency and time spent in the tube, further validating the dose dependence of this observation. It seemed that optimal behavioral affects occurred at a receptor occupancy at CRF_1 receptors between 60 and 80%. Furthermore, other studies have shown that R121919 does not seem to have sedating effects in the doses used, so these effects do not seem to be related to changes in spontaneous locomotor activity (Keck et al., 2001). Preliminary studies in our laboratory have also not shown gross changes in spontaneous locomotor activity after acute administration of R121919.

After successfully demonstrating that R121919 possesses anxiolytic activity in the defensive withdrawal paradigm and the ability to block restraint stress-induced HPA axis activation, we also examined the HPA axis response to defensive withdrawal. A separate set of rats was handled and maintained in the animal facility under conditions identical to the experimental group, but did not undergo defensive withdrawal. Figure 2D clearly demonstrates that the novel defensive withdrawal arena acted as a fairly potent stressor that activated the HPA axis under our testing conditions; furthermore, R121919 dose dependently attenuated the HPA response to defensive withdrawal (Fig. 2D).

Because the cannula in the restraint stress experiments allowed venous access without having to disturb the animal, we used polyethylene glycol as our initial vehicle. For the defensive withdrawal experiments, the tartaric vehicle proved more amenable to subcutaneous injection for the concentrations we used. In spite of the differences in route of administration and differences in the vehicles used between the two experiments, these data are revealing on the pharmacokinetics of R121919.

First, R121919 seems to fairly rapidly cross the blood-brain barrier. Second, even 8 h after a single administration there was still a marked blockade of CRF_1 receptors in the cortex (Figs. 1C, 2C, and 3). Thus, R121919 and/or an active metabolite retain its ability to block central CRF_1 receptors for a significant amount of time. In contrast, CRF_2 receptors were not significantly occupied in the dose range tested, indicating that the activity of R121919 in modulating the stress response is mediated primarily through blockade of the CRF_1 receptor subtype.

Our findings with R121919 further support the hypothesis that peripherally administered small-molecule CRF_1 receptor antagonists act as anxiolytics in animals. Currently, we are exploring the effects of long-term administration of R121919 on behavioral and endocrine measures, as well as the concomitant changes in CRF receptor function.

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