Effects of Corticotropin-Releasing Factor 1 Receptor Antagonism on the Hypothalamic-Pituitary-Adrenal Axis of Rodents

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ABSTRACT
Corticotropin-releasing factor (CRF) is the major hypothalamic neuropeptide responsible for stimulation of the hypothalamic-pituitary-adrenal axis (HPAA), resulting in the synthesis and release of glucocorticoids from the adrenal cortex. In a recent study, we reported the discovery of the CRF1 receptor antagonist, 3-(4-chloro-2-morpholin-4-yl-thiazol-5-yl)-8-(1-ethylpropyl)-2,6-dimethyl-imidazo[1,2-b]pyridazine (MTIP), which has efficacy in preclinical models of stress-induced alcohol consumption. Because CRF1 is important in HPAA activation, we evaluated the effects of MTIP administration on rodent HPAA function. Initial studies established the MTIP doses required for brain and pituitary CRF1 occupancy and those associated with the inhibition of intracerebroventricular CRF on the HPAA in mice. Then, rat basal plasma corticosterone (CORT) concentrations were measured hourly by radioimmunoassay for 24 h after three daily doses of MTIP or vehicle. In these studies, the early phase of the nocturnal CORT surge was reduced; however, the area under the CORT curve was identical for the 24-h period. In subsequent studies, increases in plasma CORT due to direct pharmacological manipulation of the HPAA axis or by stressors were evaluated after MTIP treatment in mice. MTIP attenuated CORT responses generated by immediate bolus administration of insulin or ethanol; however, MTIP did not affect activation of the HPAA by other stressors and pharmacological agents. Therefore, MTIP can modulate basal HPAA activity during the CORT surge and reduced activation after a select number of stressors but does not produce a lasting suppression of basal CORT. The ability of MTIP to modulate plasma CORT after hyperinsulinemia may provide a surrogate strategy for a target occupancy biomarker.

Introduction
Corticotropin-releasing factor (CRF) is an important regulator of the endocrine, immune, behavioral, and autonomic responses to stress (Vale et al., 1981). This peptide produces its biological effects by binding to two pharmacologically distinct G protein-coupled receptors, CRF1 and CRF2 (Bale and Vale, 2004). CRF exhibits high affinity for CRF1 but substantially lower affinity for CRF2. Although CRF1 is abundant in the pituitary, CRF2 predominates in tissues such as the heart, skeletal muscle, and gastrointestinal tract. In the brain, CRF1 is found in the cerebral cortex, amygdala, cerebellum, and brainstem, whereas CRF2 is only abundant in the lateral septum and hypothalamus. Hypothalamically derived CRF is a key regulator of the hypothalamo-pituitary-adrenal axis (HPAA) through stimulation of pituitary release of adrenocorticotropin stimulating the release of corticosterone (CORT) by the rat adrenal (Vale et al., 1981). Whereas the role of CRF at the level of the pituitary is well established (Timpl et al., 1998; Preil et al., 2001), CRF also has distinct actions via the central nervous system (Müller et al., 2003). Central administration of CRF produces activation of the HPAA, and little is known about the ability of CRF1 antagonists to modulate this response (Song et al., 1995). Disturbances in the central CRF system have been proposed to play an important role in the etiology of major depression (Holsboer, 2000) and alcoholism (Sommer et al., 2008). In addition, there is a correlation between remission of depressive symptoms and a normalization of HPAA function (Holsboer, 2000). The discovery of the related urocortin family of peptides in the lateral septum and hypothalamus.

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ABBREVIATIONS: CRF, corticotropin releasing factor; CRF1, corticotropin-releasing factor receptor type 1; CRF2, corticotropin-releasing factor receptor type 2; CORT, corticosterone; HPAA, hypothalamic-pituitary-adrenal axis; MTIP, 3-(4-chloro-2-morpholin-4-yl-thiazol-5-yl)-8-(1-ethylpropyl)-2,6-dimethyl-imidazo[1,2-b]pyridazine; R121919, 2,5-dimethyl-3-(6-dimethylamino-4-methylpyridin-3-yl)-7-dipropylaminopyrazolo[1,5-a]pyrimidine; SSR125543, 4-(2-chloro-4-methoxy-5-methylphenyl)-N-[(1S)-2-cyclopropyl-1-(3-fluoro-4-methylyphenyl)ethyl]-5-methyl-N-prop-2-ynyl-1,3-thiazol-2-amine; RIA, radioimmunoassay; ANOVA, analysis of variance; AST, astressin; NBI-34041, 2-(2,4-dichlorophenyl)-4-methyl-6-(1-propylbutyl)-7,8-dihydro-6H-1,3,6,8a-tetraaza-acenaphthylene.
revealed additional peptides that interact with CRF receptors (Bale and Vale, 2004). Urocortin I has high affinity for both CRF1 and CRF2, whereas urocortin II and urocortin III exhibit high affinity and selectivity for CRF2. CRF2-selective peptides suppress feeding while having a more modulatory effect on stress-like responses (Hashimoto et al., 2004; Jamieson et al., 2006).

Additional insight into the roles of CRF receptors in the neuroendocrine response can be found in observations using CRF1- and CRF2-deficient mice. CRF1-deficient mice have decreased adrenocorticotropin and CORT responses after restraint stress compared with wild-type controls (Smith et al., 1998; Timpl et al., 1998); however, they exhibit very little difference in basal plasma CORT concentrations. It should be noted that the CRF1-KO exhibited adrenal agenesis attributed to insufficient adrenocorticotropin during development (Smith et al., 1998), and, therefore, it is not surprising that these animals cannot mount an appropriate endocrine stress response. CRF2-deficient mice (Bale et al., 2000; Coste et al., 2000) exhibit normal basal plasma adrenocorticotropin and CORT and a normal circadian hormone rhythm. In contrast to the CRF1-deficient mice, CRF2-deficient mice exhibit an increased endocrine responsiveness to restraint stress and, in some studies, have exhibited a prolongation of the CORT response to stress (Coste et al., 2000). Finally, CRF1- and CRF2-deficient mice (Preil et al., 2001; Bale et al., 2002) exhibit minor changes in plasma adrenocorticotropin and CORT in response to restraint stress. Whereas there was no difference in basal CORT, these animals exhibited a reduction in morning CORT. Based on these results, it is thought that both CRF1 and CRF2 participate in the endocrine stress response although CRF1 has the major stimulatory role subsequent to physiological stressors.

The behavioral effects of centrally administered CRF along with the behavioral phenotype of the deficient mice have made CRF1 an attractive target for drug discovery and development (Nielsen, 2006). Preclinical antagonist studies indicate the potential for antidepressant- and anxiolytic-like activities (Kehne and De Lombaert, 2002) as well as the potential for the treatment of alcoholism (Gehlert et al., 2007) and substance abuse (Koob and Kreek, 2007). An early indication that both CRF1 and CRF2 participate in the endocrine stress response although CRF1 has the major stimulatory role subsequent to physiological stressors.

Effect of CRF1 Antagonists on Plasma CORT after Intracerebroventricular Administration of CRF

Male C57BL6 mice (Harlan, Indianapolis, IN) were administered vehicle (2 ml/kg, 3% dimethyl sulfoxide and 20% Emulphor in water) or MTP, 2,5-dimethyl-3-(6-dimethylamino-4-methylpyridin-3-yl)-7-dipropylaminopyrazolo[1,5-a]pyrimidine (R121919), or 3, 4-(2-chloro-4-methoxy-5-methylphenyl)-N-((1S)-2-cyclopropyl-1-(3-fluoro-4-methylphenyl)ethyl)-5-methyl-N-prop-2-ynyl-1,3-thiazo-l-2-amine (SSR125543) (30 mg/kg p.o. in vehicle) for 3 days (all three compounds were synthesized at Eli Lilly and Company (Gehlert et al., 2007)). The in vitro potency, in vivo potency, and single-dose pharmacokinetics of all these compounds have been reported previously (Gehlert et al., 2007) (Supplemental Table 1). The 30 mg/kg dose was selected because this was the maximal dose that provided a suspension suitable for a single bolus oral dose with all three compounds, and all three compounds had similar in vitro affinity for the receptor. One hour after the final dose, animals were administered 3 μg of CRF via direct, vertical, free-hand intracerebroventricular injection to a depth of 3.5 mm below bregma with a cuffed 27-gauge needle attached to a 25-μl Hamilton syringe. Mice were sacrificed 15 min later, and trunk blood was collected. In some experiments, randomly assigned mice were gently restrained by hand and injected with artificial cerebrospinal fluid or the CRF1/2 antagonist astressin (various doses in a total volume of 3 μl). After sacrifice, trunk blood was collected, and the plasma was separated by centrifugation (5 min at 7000 rpm in an Eppendorf tabletop centrifuge). Plasma was frozen at −80°C until assayed for CORT by radioimmunoassay (RIA) (MP Biomedicals, Salon, OH). In some experiments, plasma adrenocorticotropin was also assessed using RIA (MP Biomedicals). Data were analyzed and plotted using Microsoft Excel (Microsoft, Redmond, WA) and GraphPad Prism (GraphPad Software Inc., San Diego, CA).

Measurement of Plasma CORT and Adrenocorticotropin by RIA

Plasma corticosterone and adrenocorticotropin were measured with the corresponding ImmunoChem Double Antibody 125I radioimmunoassay kit from MP Biomedicals. In all experiments in which plasma CORT or adrenocorticotropin was measured, blood was collected in EDTA-containing tubes and kept on ice until centrifuged for plasma collection. The plasma was stored at −80°C until assayed following the standard protocol provided with the kit.

The range of detection of the corticosterone kit is approximately 0.7 to 1000 ng/ml with inter- and intra-assay coefficients of variation of less than 5% in our laboratory. The reported specificity of the assay by the manufacturer indicates less than 0.1% cross-reactivity with any other steroids commonly found in plasma.

For the adrenocorticotropin kit, the range of detection is approximately 3.7 to 1000 pg/ml with inter- and intra-assay coefficients of variation of less than 8%. The manufacturer reports that the assay is specific for adrenocorticotropin1, 39 as well as adrenocorticotropin1, 24.
Ex Vivo Binding of $^{125}$I-Tyr$^3$-Sauvagine

The method for CRF-1 receptor binding ex vivo using rat cerebellum has been described previously (Gehlert et al., 2005). Rat cerebellum was selected because it had been previously shown to contain predominantly CRF-1 receptors at a density suitable to perform binding assays. In addition, this tissue is within the blood-brain barrier and allowed us to estimate central occupancy by the compound. Rats were gavaged with vehicle (2 ml/kg, 3% dimethyl sulfoxide and 20% Emulphor in water) or compound in vehicle for 3 consecutive days. One hour after the last dose, rats were decapitated and pituitary glands and cerebella were removed, frozen on dry ice, and stored at −70°C. On the day of the assay, tissue was thawed, homogenized in buffer (50 mM Tris-HCl, 2 mM EGTA, and 10 mM MgCl$_2$) and incubated at 37°C for 1 h. For the binding assay, approximately 200 µg of prepared homogenate was combined with a final concentration of 0.175 nM $^{125}$I-Tyr$^3$-Sauvagine (PerkinElmer Life and Analytical Sciences, Waltham, MA) in assay buffer with 0.1% bovine serum albumin, 0.1% bacitracin, and 100 kU/ml aprotinin. Nonspecific binding was determined by addition of 1 µM ovine CRF (American Peptide Company, Sunnyvale, CA). After incubation at room temperature for 120 min, the assay was terminated by centrifugation, and binding was assessed using a gamma counter. ED$_{50}$ values were derived from specific binding calculated in GraphPad Prism using the four-parameter sigmoidal dose-response model.

Effect of MTIP on Basal CORT in the Rat

To determine this, we used the BASi Culex API system that allows for administration of the compound via gastric cannulae and blood sampling via implanted jugular cannulae. This method eliminates the need to restrain the animal to accomplish these tasks and the resulting perturbations in stress hormones. For the following studies, all animals were surgically modified and dosed at BASi facilities (West Lafayette, IN) using the Culex API system as described previously (Bohs et al., 2000; Peters et al., 2000).

**BASI Study LLY-4767.** Twenty-two male Sprague-Dawley rats (300–350 g; Harlan) were used. All rats were surgically implanted with gastric catheters, allowed 7 days of recovery, and then implanted with femoral vein catheters. The rats were divided into two groups of 11 for the experiment and placed in the Culex system at BASi. Group 1 received bolus infusions of vehicle (0.5% Tween 80 in 0.1 N HCl, pH 4.0) via the intragastric catheter, and group 2 received MTIP at 30 mg/kg in a volume of 7.5 ml/kg. All animals were dosed daily at noon for 3 days. After the last dose of compound, 75-µl blood samples were taken hourly for 24 h via the Culex system and stored at 4°C until the end of the study. The blood samples were centrifuged and plasma was collected and stored at −80°C until assayed for CORT. Because of technical issues with the blood collection, 7 of the original 22 animals did not complete the study, and data points from those animals were excluded from the study. Fifteen animals completed the study (six vehicle-treated and nine MTIP-treated). Plasma CORT was measured using RIA. All samples were run in duplicate, and the assay was conducted according to kit instructions. The resulting CORT levels were averaged by group (mean ± S.E.M.) for each time point and statistically evaluated by a repeated-measures, two-way analysis of variance (ANOVA) using Lilly internal Discovery Statistics software.

**BASI Study LLY-4783.** Thirty-six male Sprague-Dawley rats (350–375 g; Harlan) were used for the study. All animals had intragastric and femoral artery catheters implanted before the initiation of the study. Arterial catheters were used for blood collection in this experiment because BASi historically has had fewer catheter problems with arterial than with venous lines, particularly in longer duration studies. The rats were divided into two groups of 18 and placed in the Culex chambers for the duration of the study. In an initial study, one group of rats was administered three daily doses of vehicle, and a second group of rats were given three daily doses of MTIP. Blood samples were taken hourly thereafter for 24 h. In a second study, the dose-response relationship was explored. For this experiment, the study design consisted of three consecutive cycles of dosing for 3 days followed by blood sample collection at eight time points during the following 24 h. For each cycle, group 1 received vehicle (0.5% Tween 80 in 0.1 N HCl, pH 4.0) via the intragastric catheter, and group 2 received MTIP in an ascending dose per cycle of 0.1, 1.0, or 10 mg/kg in a volume of 7.5 ml/kg. At the end of each blood collection, the samples were centrifuged, and plasma was collected and stored at −80°C until the end of the study.

Measurement of Plasma MTIP Concentrations

The plasma concentrations of MTIP were determined by positive TurbolonSpray liquid chromatography-mass spectrometry using a Sciex 4000 system (AB Sciex, Framingham, MA). Samples were prepared by methanol precipitation and centrifuged to remove particulate matter. An aliquot of the supernatant was transferred and diluted with 80% water-20% methanol. The lower limit of quantitation was 1 ng/ml. Pharmacokinetic parameters were calculated by noncompartmental analysis, using the trapezoidal rule for area under the curve calculation using a validated pharmacokinetic calculation program (WinPTK; Eli Lilly and Co., Indianapolis, IN). To determine the plasma concentration of MTIP associated with the maximal concentration ($C_{\text{max}}$), a single oral 10 mg/kg dose was given to rats, and the $C_{\text{max}}$ was determined to be 2400 ng/ml. Dose linearity was assumed, and the plasma concentration associated with 1.5 mg/kg (the single-dose ED$_{50}$) was determined to be 360 ng/ml.

Effect of MTIP on Plasma CORT after Predator Odor or Treatment with Pharmacological Agents

**Hyperinsulinemia.** C57BL/6 mice (male, 22–26 g; Harlan) were handled for 3 days before the experiment. The mice were dosed orally with vehicle (1% carboxymethyl cellulose) or MTIP (3, 10, or 30 mg/kg, 10 ml/kg) for 3 days ($n = 8/group$. On the 3rd day, insulin (Humalin R, 1 U/kg, 10 ml/kg; i.p.; Eli Lilly and Company) or vehicle (saline intraperitoneally) was injected 1 h after the compound dose. Trunk blood was collected 1 h after insulin administration and kept on ice until the plasma was separated by centrifugation (10 min at 8000 rpm in an Eppendorf tabletop centrifuge). Plasma was frozen at −80°C until assayed for CORT. Blood glucose was measured at time of sacrifice with an AccuCheck Advantage monitor and Comfort Curve strips (Roche, Indianapolis, IN).

**Predator Odor Stress.** Animals were handled for 3 days before the experiment day ($n = 10/group$). The mice were dosed intracerebroventricularly 45 min before predator odor stress. Mice were exposed to predator odor (soiled rat bedding) for 10 min and then were returned to their home cage for 20 min. The home cage control group was briefly picked up and then returned to their home cage for 30 min. Trunk blood was collected in EDTA tubes at 30 min after oral exposure and kept on ice until centrifuged to collect plasma (10 min at 8000 rpm in an Eppendorf table top centrifuge) Plasma was frozen at −80°C until assayed for CORT.

**Metyrapone.** Animals were handled for 3 days before the experiment day and were dosed orally with 30 mg/kg MTIP ($n = 8/group$). On the experiment day animals were administered metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone, 75 mg/kg i.p.; Sigma-Aldrich, St. Louis, MO) 1 h after MTIP (orally) and 60 min before sacrifice. A control group was dosed intracerebroventricularly with astressin (1 µg/3 µl) 45 min before metyrapone. Trunk blood was collected and kept on ice until the plasma was separated by centrifugation (10 min at 8000 rpm in an Eppendorf tabletop centrifuge). Plasma was collected and frozen at −80°C until assayed for CORT and adrenocorticotropic.

**Caffeine.** Animals were handled for 3 days before the experiment. The mice were administered MTIP (30 mg/kg p.o.) or vehicle for 3 days. On the final experiment day, MTIP or vehicle were dosed 2 h before caffeine. Astressin was administered immediately (1 µg/3 µl i.c.v.) 30 min before caffeine. Caffeine was injected (30 mg/kg, i.p.) 30
min before sacrifice and blood collection. Plasma was collected and frozen at −80°C until assayed for CORT.

**Yohimbine.** Mice were dosed for 3 days with vehicle (1% carboxymethyl cellulose) or MTIP (30 mg/kg p.o.). Two hours after the last MTIP dose, the mice were administered yohimbine (2.0 mg/kg i.p.; Sigma-Aldrich) and sacrificed 30 min later. Two additional groups were administered astressin (1 μg/kg i.c.v.) 30 min before intraperitoneal vehicle or yohimbine (n = 6–9/group). Trunk blood was collected in tubes containing EDTA and kept on ice until the plasma was separated by centrifugation (5 min at 7000 rpm in an Eppendorf tabletop centrifuge). Plasma was frozen at −80°C until assayed for CORT.

**Ethanol.** Animals were dosed for 3 days with MTIP (30 mg/kg p.o.) On the 3rd day, the animals were dosed with MTIP 1 h before ethanol (Decon Laboratories, King of Prussia, PA; orally, 32% in H₂O) or vehicle (H₂O) (n = 8/group). The animals were administered ethanol or water 30 min before sacrifice. Trunk blood was collected and kept on ice until the plasma was separated by centrifugation (10 min at 8000 rpm in an Eppendorf tabletop centrifuge). Plasma was frozen at −80°C until assayed for CORT. Blood alcohol content was measured with an ethanol L3K assay kit (Diagnostic Chemicals, Ltd., Charlottetown, PE, Canada).

## Results

The pharmacological and pharmacokinetic properties of the CRF1 antagonists used in the present study were published previously (Gehlert et al., 2007) and are summarized in Supplemental Table 1. We first examined the effects of R121919, SSR125543, and MTIP on plasma CORT concentrations after central CRF administration. Because these compounds have similar in vitro and in vivo potencies (Supplemental Table 1), we used a maximal dose of 30 mg/kg for both mouse and rat studies. We were limited to this as a maximal dose because higher concentrations in this vehicle were too viscous to deliver through a syringe and needle. In addition, this dose provided near maximal receptor occupancy in the ex vivo binding assays (Supplemental Table 1). In these studies, the nonpeptide CRF1 antagonists were administered orally for 3 days and then CRF was administered into the lateral cerebral ventricle using a freehand technique. Thirty minutes later, the animals were sacrificed, and plasma CORT was measured by RIA. The peptide CRF1/CRF2 antagonist, astressin was administered intracerebroventricularly before CRF in some animals to determine the relative contributions of CRF1 and CRF2 receptor stimulation. The results from these experiments are summarized in Fig. 1. At a dose of 30 mg/kg, MTIP was the only CRF1 antagonist that could produce a statistically significant reduction in plasma CORT. Central administration of 3 μg of astressin produced complete inhibition of the CRF-induced increase in CORT. It was interesting to note that the CRF1 antagonists appeared to produce a reduction in basal concentrations of CORT, but these were not statistically significant in this study. Because MTIP produced the greatest reduction in CRF-stimulated CORT, subsequent studies were conducted using MTIP. First, we evaluated the ability of MTIP to occupy the pituitary CRF1 receptor. Using this method, we found that three days of orally administered MTIP inhibited 125I-sauvagine binding to rat pituitary glands ex vivo with an ED₅₀ of 7.5 mg/kg compared with 7.8 mg/kg observed when using the cerebella from the same animals (Fig. 2). These values were somewhat greater than that seen in single-dose studies (Gehlert et al., 2007). Therefore, a dose of 30 mg/kg

![Fig. 1. Effect of CRF1 antagonists (30 mg/kg p.o., 3-day dosing) on intracerebroventricular CRF-induced plasma CORT concentrations. In these studies, mice were treated with maximal doses of three nonpeptide CRF1 receptor antagonists for 3 days. One hour after the final dose, animals were administered 3 μg of CRF intracerebroventricularrly, and blood was collected 15 min later. Plasma CORT concentrations were measured by radioimmunoassay. Astressin was administered in the opposite ventricle 30 min before intracerebroventricular CRF. In these studies, Astressin completely antagonized the increase in plasma concentrations of CORT produced by intracerebroventricular CRF. MTIP was the only nonpeptide antagonist that produced a statistically significant reduction in CORT, and this antagonist was selected for further study. In addition to the reduction in CRF-stimulated CORT, there was also a reduction in basal CORT noted that was studied in the rat (Fig. 3). *p < 0.05 versus vehicle (veh)/CRF group, ANOVA with Bonferroni post hoc testing (n = 8).](image)

![Fig. 2. Inhibition of 125I-Tyr³-sauvagine binding to rat pituitary ex vivo. Rats were administered various doses of MTIP by gavage, and the specific binding of 125I-Tyr³-sauvagine was assessed as described under Materials and Methods. In these studies, MTIP produced a dose-dependent decrease in the binding of 125I-Tyr³-sauvagine to the pituitary (A) ex vivo with an estimated ED₅₀ of 7.5 mg/kg compared with an ED₅₀ of 7.8 mg/kg for the cerebellum (B) (n = 5).](image)
MTIP produced a substantial inhibition of the ex vivo binding to the brain and pituitary CRF1 receptors. Next, we evaluated the effects of MTIP on the basal plasma levels of CORT. Using the Culex system, we administered 30 mg/kg MTIP once daily for 3 days to Sprague-Dawley rats and began blood sampling 1 h after the third dose. The resulting plasma samples were assayed for CORT and MTIP concentrations. These results are summarized in Figs. 3 and 4. MTIP produced a marked suppression in plasma CORT for 3 h after administration compared with that for the vehicle control. When assessed 8 and 9 h after administration, a significant increase in the plasma levels of CORT was noted. Overall, the total amount of CORT (area under the curve) secreted over 24 h was unchanged (Supplemental Fig. 3). Because there was a significant suppression of CORT at the early time points, we focused on 1 h after administration for the additional studies. Using the plasma samples from these animals, we also assessed the concentrations of MTIP (Fig. 4), and these were maintained at a relatively high level throughout the 9-h period during which changes in CORT were observed. The plasma levels were greater than that associated with the ED50 concentrations determined in a single-dose study as denoted by the dashed line. Pharmacokinetic parameters determined from this study are found in Supplemental Table 2. Compared with the t1/2 for the single-dose pharmacokinetics (Supplemental data Table 1), the t1/2 for MTIP was similar to that observed with a single dose, whereas the Tmax was later (4.11 h versus 2.2 h). A dose-response study was then conducted using a cumulative dosing paradigm for the 0.1, 1, and 10 mg/kg doses administered over 3 days. The results from the 30 mg/kg dose were incorporated from the original study. In this study, significant reductions in basal CORT were observed at 10 and 30 mg/kg 1 h after the third dose (Fig. 5). The reduction seen in CORT was similar between the 10 and 30 mg/kg doses, indicating that this was probably the maximal effect.

Subsequent studies were conducted to establish the effects of MTIP on the stress-induced increases in CORT using a number of different provocations. These studies were conducted in mice to allow for freehand intracerebroventricular injections in some experiments. In the first of these experiments, we evaluated the effects of an injection of insulin on plasma CORT 1 h after injection. A dose-response study was conducted to optimize the dose of insulin and a 1 U/kg dose was found to produce a robust decrease in plasma glucose and robust increase in plasma CORT (Supplemental Fig. 2). The baseline blood concentrations of glucose were consistent with published values for C57BL/6 mice (Goren et al., 2004). MTIP was evaluated by dosing 1 h before the insulin injection. These data are summarized in Fig. 6. MTIP produced a significant decrease in plasma CORT at 30 mg/kg, whereas lower doses did not produce a significant effect. In the next set of experiments, we evaluated the effect of metyrapone on plasma adrenocorticotropin. In mice, metyrapone inhibits the 11β-hydroxylase of desoxycorticosterone to CORT, resulting in decreased plasma and brain concentrations of CORT. The loss of feedback inhibition on CRF synthesis...
increases hypothalamic CRF, resulting in increased plasma adrenocorticotropin in a physiological attempt to increase plasma CORT. The dose of metyrapone required to suppress CORT was optimized at 75 mg/kg i.p. (Supplemental Fig. 1). As shown in Fig. 7B, metyrapone produced a pronounced decrease in plasma CORT, and this was unaffected by pre-treatment with MTIP. As seen in the rat studies, MTIP produced a reduction in the basal concentrations of plasma CORT. When the effects of adrenocorticotropin were evaluated, metyrapone produced an approximate doubling of plasma adrenocorticotropin that was unaffected by 30 mg/kg p.o. of MTIP (Fig. 7A). To understand the potential contribution of the CRF2 receptor to this increase in adrenocorticotropin, we administered the peptidic CRF1/CRF2 antagonist astressin at a dose of 1 μg i.c.v. and observed a partial reduction in the plasma adrenocorticotropin concentrations (Fig. 7A) suggesting that a combination of central CRF1 and CRF2 receptors may be necessary to see reversal of this effect. The dose of 1 μg was found to completely suppress the activation of the HPAA produced by 3 μg of CRF (Fig. 1).

To evaluate the effects of MTIP on a putative psychological stressor, we exposed mice to predator odor (rat urine). Exposure to predator odor produced a robust increase in the plasma CORT that was reduced by prior intracerebroventricular administration of astressin (Fig. 8A). On the other hand, pretreatment with 30 mg/kg MTIP did not affect the increase in plasma CORT (Fig. 8B). In the subsequent experiments, the effects of MTIP on caffeine-induced increases in plasma CORT were assessed. MTIP did not affect the increase when given 2 h before caffeine (Fig. 9). In this study, the benzodiazepine anxiolytic alprazolam was also assessed and did not affect the increases in CORT produced by caffeine. We also evaluated the effects of CRF antagonists on the increases in plasma CORT produced by the α-2 antagonist yohimbine (Fig. 10). In these studies, intracerebroventricular pretreatment with astressin produced a significant reduction in yohimbine-stimulated CORT whereas MTIP had no significant effect. Finally, MTIP was found to reduce the increase in plasma CORT after the immediate administration of ethanol (Fig. 11).

Discussion

These experiments were conducted to understand the effects of CRF1 antagonism on basal and stress-induced CORT secretion in vivo. CRF is the major hypothalamic secretagogue that regulates the secretion of adrenocorticotropin and consequently, the secretion of CORT in the adrenal gland. CRF1 has the highest affinity for CRF and is abundant in the brain and pituitary gland (Bale and Vale, 2004), and CRF1-deficient mice exhibit a pronounced basal corticosterone deficiency, a loss in the diurnal CORT rhythm, and a blunted endocrine response to stressors (Smith et al., 1998; Timpl et al., 1998). Although a few studies have examined the effect of short-term CRF1 antagonist administration on the HPAA, the impact of longer term administration of these compounds on the HPAA or the effects of central blockade are not well understood. In initial experiments, we evaluated the ability of several CRF1 receptor antagonists to block the increase in plasma corticosterone produced by central injection of CRF. The purpose of these experiments was to determine which CRF1 antagonist would have the most profound effect on the centrally administered CRF-induced CORT secretion. The peptide CRF1/2 antagonist, astressin, produced a complete inhibition when administered intracerebroven-
particularly before the CRF infusion. Compared with R121919 and SSR125543, MTIP produced the most pronounced, although incomplete, antagonism and was used in all subsequent studies. Using ex vivo binding, a 30 mg/kg dose of MTIP was found to produce a substantial occupancy of both cerebellar (within the blood-brain barrier) and pituitary (outside the blood-brain barrier) CRF1 receptors, and this dose was used in subsequent experiments. Three days of MTIP treatment was found to produce a pronounced reduction in the initial evening (rats) CORT surge compared with that in vehicle-treated controls. There appeared to be compensation for this effect with a prolongation of the corticosterone elevation seen at the 8:00 PM and 9:00 PM sampling times. These findings would be consistent with a rebound activation of the HPAA, presumably at the level of the hypothalamus via a CRF mechanism that comes into play when the effective concentrations of MTIP are reduced. Total 24-h CORT secretion was not statistically different between the MTIP- and vehicle-treated animals, suggesting that there was no apparent lasting HPAA suppression. To assess the plasma concentrations of MTIP in this study, we evaluated the plasma levels of the parent compound using liquid chromatography-mass spectrometry. After three daily gastric infusions, plasma MTIP concentrations were maintained above that required for greater than 50% occupancy for 22 of the first 24 h. In a subsequent dose-response study, doses of 10 and 30 mg/kg appeared to produce maximal suppression of basal CORT the 1st h after administration. These doses

Fig. 8. Effect of astressin (A) (*, p < 0.001 compared with vehhc group; #, p < 0.05 compared with veh/POS group, ANOVA, Tukey post hoc test) or MTIP (B) (*, p < 0.05 compared with vehhc group, ANOVA, Tukey post hoc test) on predator odor stress (POS) induced CORT in male C57BL/6 mice (n = 10). veh, vehicle; hc, home cage control group.

Fig. 9. Effect of MTIP [30 mg/kg p.o., 2 h before caffeine (caff)] or alprazolam (alpra, 1 μg/μl i.c.v., 30 min before caffeine) on caffeine-induced CORT in male C57BL/6 mice. Blood was collected 30 min after caffeine. n = 7 to 8; *, p < 0.05 compared with the respective vehicle (v) group; ANOVA, Tukey post hoc test.

Fig. 10. Effect of MTIP [30 mg/kg p.o., 2 h before yohimbine (yeh)] or astressin (ast; 1 μg/μl i.c.v.) on yohimbine-induced CORT in male C57BL/6 mice. Blood was collected 30 min after yohimbine. n = 6 to 9; *, p < 0.01 compared with all vehicle groups; #, p < 0.05 compared with all yohimbine-treated groups; ANOVA, Tukey post hoc test.

Fig. 11. Effect of MTIP on ethanol-induced increases in plasma CORT. A, 2 g/kg ethanol (e) produces a significant increase in plasma CORT 30 min after administration in vehicle-pretreated animals (v) compared with vehicle (V)-treated animals. n = 8; *, p < 0.05; ANOVA, Tukey post hoc. B, pretreatment of mice with 30 mg/kg MTIP had no significant effect on blood alcohol concentrations (BAC) 30 min after oral gavage of 2 g/kg ethanol (n = 8).
correlated with the occupancy measures and the ability of MTIP to antagonize the elevation of plasma CORT after intracerebroventricular CRF. The compensation for the suppression of CORT in the later dark hours may be the result of lower levels of receptor occupancy or compensation by other pathways, such as vasopressin (Pinnock and Herbert, 2001), that can influence the secretion of CORT.

Having performed initial studies to understand the effects of CRF1 antagonism on basal CORT secretion, we conducted studies in various animal models of stress. We used a variety of types of pharmacological stressors to fully evaluate the potential of this compound class to modulate the HPAA. The most robust of the MTIP responses was suppression of the increase in CORT seen after an immediate injection of insulin. At a dose of 30 mg/kg, MTIP produced a significant reduction in the increase in plasma CORT while not affecting the reduction in blood glucose produced by insulin. Lower doses of MTIP did not produce an effect that reached significance. MTIP also produced a significant reduction in the plasma CORT concentrations produced by a bolus infusion of ethanol. Of interest, in a previous study, we demonstrated that MTIP also suppressed the behavioral consequences of ethanol withdrawal (Gehlert et al., 2007). MTIP did not produce a discernable effect on plasma CORT in response to several other stressors including caffeine and predator odor. We also tested the effects of metyrapone, an inhibitor of steroid 11β-hydroxylase that reduces the circulating concentrations of CORT (Carballeira et al., 1976). In vivo, this compound produces a loss of feedback inhibition on hypothalamic CRF synthesis and release, resulting in increased adrenocorticotropin release from the pituitary. Antagonism of brain and pituitary CRF receptors would theoretically reduce the increase in adrenocorticotropin produced by metyrapone. Central administration of AST resulted in a partial reversal of the adrenocorticotropin response, whereas MTIP had no measurable effect. Therefore, antagonism of both CRF1 and CRF2 receptors may be required to affect this response. Alternatively, a very high percentage of brain CRF1 receptors may need to be antagonized to reduce this response, and this can only be achieved by direct central administration. Unfortunately, the limited aqueous solubility of the currently available CRF1 antagonists precluded testing this hypothesis by intracerebroventricular administration. A similar phenomenon was observed in the evaluation of yohimbine activation of the HPAA, for which centrally administered AST produced a substantial reduction in plasma CORT whereas MTIP did not. Also interesting was the observation that AST could not produce complete reversal of the CORT response in the models evaluated, even though the intracerebroventricular dose of AST was capable of completely reversing the endocrine effects of intracerebroventricular CRF. Therefore, it is likely that there are other pathways, such as vasopressin-activated, that contribute to the CORT response and are not antagonized by the compounds used in the present study.

The present study is the first comprehensive preclinical evaluation of the effects of a centrally active CRF1 antagonist on the HPAA. On the basis of ex vivo binding studies, a relatively high degree of receptor occupancy was associated with the doses required to prevent the activation of the HPAA by centrally administered CRF. Previous preclinical studies have evaluated the HPAA effects of the CRF1 antagonist, SR125543A. This compound produced an inhibition of adrenocorticotropin secretion after intravenous administration of CRF for up to 6 h after administration of a dose of 30 mg/kg to rats (Gully et al., 2002). In the present study, we were not able to observe antagonism of the CORT response to centrally administered CRF in mice, suggesting that the primary effect of SR125543A is probably pituitary CRF1 antagonism and not via antagonism of the central effects of CRF. The CRF1 antagonist, R121919, has been shown to reduce adrenocorticotropin and CORT after a peripherally administered CRF challenge both preclinically and clinically (Ising et al., 2007). It has also been shown to reduce endocrine activation after the defensive withdrawal procedure in rats (Gutman et al., 2003). In this study, a dose-dependent reduction in both adrenocorticotropin and CORT was observed that correlated with the occupancy and behavioral measures. Although we did not conduct defensive withdrawal procedures in the present study, we did evaluate the effects of MTIP on predator odor activation of the HPAA and did not see a statistically significant effect. Intracerebroventricular administration of astressin was able to prevent the increase in CORT, and this would be consistent with the requirement of both CRF1 and CRF2 antagonism to prevent this response. Further study with a broader spectrum of behavioral stressors will be required to understand the potential subtleties of CRF1 receptor blockade by various antagonists in these paradigms.

The secondary goal of the present study was to identify a pharmacological agent that could activate the HPAA and be antagonized by a centrally active CRF1 antagonist. If such a paradigm were identified, it may be a useful biomarker to evaluate central CRF1 receptor occupancy in clinical studies. We detected a suppression of the CORT response to an immediate bolus of insulin as a promising method to pursue in clinical studies evaluating CRF1 receptor antagonists. Further studies will need to be conducted to determine how well this method could translate from mouse to human. The endocrine effects of the CRF1 antagonist 2-(2,4-dichlorophenyl)-4-methyl-6-(1-propylbutyl)-7,8-dihydro-6H-tetraaza-acenaphthylene (NBI-34041) have been explored preclinically and clinically. In rats, there was a dose-dependent reduction in plasma adrenocorticotropin subsequent to intravenous CRF or a 10-min intermittent footshock. In the clinic, NBI-34041 reduced the adrenocorticotropin but not the cortisol response to intravenous CRF. It was reported to produce a statistically significant diminution of the increase in plasma adrenocorticotropin and cortisol subsequent to the Trier Social Stress Test after 9 days of treatment (Ising et al., 2007). There was no significant effect on basal cortisol observed in this study. Based on these results, modulation of the HPAA may present a translatable biomarker to estimate brain CRF1 receptor occupancy although additional work is needed to optimize the methodology and paradigm to make this a robust measure.

In summary, we have found that CRF1 antagonism produces a reduction in the first 3 h of the CORT surge and reduces the CORT increase produced by ethanol and insulin. The compound had little effect on the endocrine response to predator odor or other pharmacological stressors. Therefore, CRF1 antagonism in rodents produces subtle alterations in HPAA function that may be related to high levels of receptor occupancy. In addition, the ability of MTIP to antagonize the
CORT response to insulin may provide a biomarker strategy to assess CRF1 target engagement in future human studies.

Authorship Contributions

Participated in research design: Gehlert, Cramer, and Morin.

Conducted experiments: Cramer and Morin.

Performed data analysis: Gehlert, Cramer, and Morin.

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