JPET Fast Forward. Published on March 8, 2012 as DOI: 10.1124/jpet.111.189753
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JPET #189753

The Effects of Subchronic CRF1 Receptor Antagonism on the Hypothalamic-Pituitary-Adrenal Axis of Rodents

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Running Title: CRF-1 Receptor Antagonism and the HPAA

Abbreviations: CRF, Corticotropin releasing factor; CRF1, Corticotropin releasing factor receptor type 1; CRF2, Corticotropin releasing factor receptor type 2; 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-methylphenyl)ethyl]-5-methyl-N-prop-2-ynyl-1,3-thiazol-2-amine, CORT, corticosterone; HPAA, hypothalamic-pituitary-adrenal axis; CRF1-KO, mice deficient in Corticotropin releasing factor receptor type 1; CRF2-KO, mice deficient in Corticotropin releasing factor receptor type 2; CRF1-KO, mice deficient in both Corticotropin releasing factor receptor type 1 and type 2; ACTH, adrenocorticotropic hormone; RIA, radioimmunoassay; DMSO, dimethylsulfoxide

Number of pages: 39

Number of tables: 0

Number of figures: 11

Number of references: 38

Words in Abstract: 248

Words in Introduction: 673

Words in Discussion: 1499

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Abstract

Corticotropin-releasing factor (CRF) is the major hypothalamic neuropeptide responsible for the stimulation of hypothalamic-pituitary-adrenal axis (HPAA) resulting in the synthesis and release of glucocorticoids from the adrenal cortex. Recently, 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) that has efficacy in preclinical models of stress-induced alcohol consumption. Since CRF1 is important in HPAA activation, we evaluated the effects of subchronic MTIP administration on rodent HPAA function. Initial studies established MTIP doses required for brain and pituitary CRF1 occupancy and associated with the inhibition of intracerebroventricular CRF on the HPAA in mice. Subsequently, rat basal plasma corticosterone (CORT) concentrations were measured hourly by radioimmunoassay for 24 hours following 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 hour 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 acute 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 following hyperinsulinemia may provide a surrogate strategy for a target occupancy biomarker.

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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. While 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, while 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 adrenocortotropin hormone (ACTH) stimulating the release of corticosterone (CORT) by the rat adrenal (Vale et al., 1981). While 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 (Muller 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 revealed additional peptides that interact with CRF receptors (Bale and Vale, 2004).

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Urocortin I has high affinity for both CRF1 and CRF2 while 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 (CRF1-KO) have decreased ACTH and CORT responses after restraint stress compared to 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 ACTH during development (Smith et al., 1998) and, therefore, it is not surprising that these animals cannot mount an appropriate endocrine stress response. CRF2-KO (Bale et al., 2000; Coste et al., 2000) exhibit normal basal plasma ACTH and CORT and a normal circadian hormone rhythm. In contrast to the CRF1-KO, CRF2-KO 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, CRF1/2-KO (Preil et al., 2001; Bale et al., 2002) exhibit minor changes in plasma ACTH and CORT in response to restraint stress. While 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 though CRF1 has the major stimulatory role subsequent to physiological stressors.

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The behavioral effects of centrally administered CRF along with the behavioral phenotype of the deficient mice have made the 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 open-label clinical study indicated the potential for antidepressant activity (Zobel et al., 2000) and improved sleep (Held et al., 2004), however, recent clinical studies did not observe anxiolytic (Coric et al., 2010) or antidepressant (Binneman et al., 2008) activities. Presently, there is no clinical occupancy biomarker to establish central CRF1 receptor occupancy in the human studies and it is unclear whether there was adequate brain or pituitary CRF1 occupancy in these clinical trials. Recently, we disclosed a novel CRF1 antagonist 3-(4-Chloro-2-morpholin-4-yl-thiazol-5-yl)-8-(1-ethylpropyl)-2,6-dimethyl-imidazo[1,2-b]pyridazine (MTIP) that reduced ethanol consumption in preclinical models (Gehlert et al., 2007). Brain receptor occupancy was estimated using ex vivo autoradiography and would not be suitable for clinical studies (Heinrichs et al., 2002; Gehlert et al., 2007). In the present study, we first established that MTIP could reduce the activation of the HPAA following central administration of CRF. Subsequently, we evaluated the effects of MTIP on basal and stress-induced CORT secretion to better understand the influence of brain and pituitary CRF1 occupancy on measures of HPAA functionality. To activate the HPAA, we used several strategies that were shown to increase plasma CORT by central and/or peripheral mechanisms including caffeine (Spindel et al., 1983),

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yohimbine (Marinelli et al., 2007), ethanol (Zgombick and Erwin, 1988), predator odor stress (Anisman et al., 2001) and insulin (Muret et al., 1992).

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Methods

All studies adhered to the NIH Guide for the Care and Use of Laboratory Animals and were performed with approval from the Lilly Animal Care and Use Committee.

Effect of CRF1 antagonists on plasma CORT after icv administration of CRF

Male, C57BL6 mice (Harlan, Indianapolis, IN) were administered vehicle (2 ml/kg, 3% dimethylsulfoxide (DMSO), 20% Emulphor in water) or MTIP, R121919 or SSR125543 (30 mg/kg, p.o. in vehicle) for three 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 previously been reported (Gehlert et al., 2007) (Supplementary Table 1). The 30 mg/kg dose was selected since 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 following the final dose, animals were administered 3 ug of CRF via direct, vertical, free-hand i.c.v. 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 minutes later and trunk blood collected. In some experiments, randomly assigned mice were gently restrained by hand and injected with aCSF or the CRF1/2 antagonist Astressin (various doses in a total volume of 3 µl). After sacrifice, trunk blood was collected by the plasma was separated by centrifugation (5 min at 7000 rpm in an Eppendorf table top centrifuge). Plasma was frozen at -80^{o}C until assayed for CORT by radioimmunoassay (RIA) (MP Biomedicals Salon, OH). In some experiments, plasma

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ACTH was also assessed using RIA (MP Biomedicals Salon, OH). Data were analyzed and plotted using Microsoft Excel and Graphpad Prism.

Measurement of plasma CORT and ACTH by RIA

Plasma corticosterone or ACTH were measured with the corresponding ImmunoChem Double Antibody ^{125}I radioimmunoassay kit from MP Biomedicals (Salon, OH). In all experiments where plasma CORT or ACTH were measured, blood was collected in EDTA containing tubes and kept on ice until centrifuged for plasma collection. The plasma was stored at -80^{o}C until assayed following the standard protocol provided with the kit.

The range of detection of the corticosterone kit is approximately 7.7 -1000 ng/ml with inter- and intra- assay coeffiecent of variation (cv) 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 ACTH kit the range of detection is approximately 5.7-1000 pg/mL with an inter- and intra assay cv of less than 8%.The manufacturer reports the assay is specific for ACTH $^{1-39}$ as well as ACTH$^{1-24}$.

Ex vivo binding of ^{125}I-Tyr^{0}-Sauvagine

The method for CRF-R1 binding *ex vivo* has been previously described using rat cerebellum (Gehlert et al., 2005). Rat cerebellum was selected because it had been previously shown to contain predominantly CRF-R1 receptors at a density suitable to perform binding assays. In addition, this tissue is within the blood brain barrier and

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allowed us to estimate central occupancy by the compound. Rats were gavaged with vehicle (2 ml/kg, 3% dimethylsulfoxide (DMSO), 20% Emulphor in water) or compound in vehicle for three consecutive days. One hr after the last dose, rats were decapitated and pituitaries and cerebella removed, frozen on dry ice and stored at –70ºC. On the day of the assay, tissue was thawed, homogenized in buffer (50 mM Tris HCl, 2 mM EGTA, 10 mM $MgCl_2$) and incubated at 37^{o}C for 1 hr. For the binding assay, approximately 200 ug of prepared homogenate was combined with a final concentration of 0.175 nM ^{125}I-Tyr^{0}-Sauvagine (Perkin Elmer, Boston, MA) in assay buffer with 0.1% bovine serum albumin, 0.1% bacitracin and 100 kU/ml aprotinin. Non-specific 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 assessed using a gamma counter. ED_{50} values were derived from specific binding calculated in Graph Pad Prism using the 4 parameter sigmoidal dose response model.

Effect of MTIP on basal CORT in the rat.

To accomplish 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 in West Lafayette, Indiana using the Culex APIsystem as previously described (Bohs et al., 2000; Peters et al., 2000).

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BASi Study LLY-4767

Twenty two male Sprague Dawley rats (Harlan, Indianapolis, IN, 300-350 g) 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 2 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 while Group 2 received MTIP at 30 mg/kg in a volume of 7.5ml/kg. All animals were dosed daily at 12:00 hours for 3 days. Following the last dose of compound, 75 uL blood samples were taken hourly for 24 hours via the Culex system and stored at 4°C until the end of the study. The blood samples were centrifuged, plasma collected and stored at -80°C until assayed for CORT. Due to 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 (6 vehicle treated and 9 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 + SEM) for each time point and statistically evaluated using a repeated measures, 2-way analysis of variance (ANOVA) using Lilly internal Discovery Statistics software.

BASi Study LLY-4783

Thirty-six male Sprague Dawley rats (Harlan, 350-375 g) were used for the study. All animals had intragastric and femoral artery catheters implanted prior to the initiation of the study. Arterial catheters were used for blood collection in this experiment as BASi

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historically has fewer catheter problems with arterial versus 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 while a second group of rats were given three daily doses of MTIP. Blood samples were taken hourly thereafter for 24 hours. In a second study, the dose-response relationship was explored. For this experiment, the study design consisted of three consecutive cycles of dosing for three days followed by blood sample collection at eight time points during the following 24 hours. For each cycle, Group 1 received vehicle (0.5% Tween-80 in 0.1 N HCl, pH 4.0) via the intragastric catheter while 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, plasma collected and stored at -80^{o}C until the end of the study.

Measurement of plasma MTIP concentrations

The plasma concentrations of MTIP were determined by positive turbo ionspray LC/MS/MS using a Sciex 4000. 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 non-compartmental analysis, using the trapezoidal rule for AUC calculation using a validated pharmacokinetic calculation program (WinPTK, Eli Lilly and Co., Indianapolis, IN). To determine the plasma concentration of MTIP associated with the Cmax, a single oral 10 mg/kg dose was given

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to rats and the Cmax was determined to be 2400ng/ml. Dose linearity was assumed and the plasma concentration associated with a 1.5 mg/kg (the single dose ED_{50}) was determined to be 360 ng/ml.

Effect of MTIP on plasma CORT following predator odor or treatment with pharmacological agents

Hyperinsulinemia

C57Bl/6 mice (male, 22-26g, Harlan, Indianapolis, IN) were handled for 3 days prior to experiment. The mice were dosed po with veh (1% CMC) or MTIP (3, 10 or 30 mg/kg, 10 ml/kg) for three days(n=8 per group). On the third day, insulin (Humalin R, Eli Lilly and Company, 1 U/kg, 10 ml/kg, ip) or Veh (saline, ip) was injected 1 hour following compound dose. Trunk blood was collected one hour after insulin administration and kept on ice until the plasma was separated by centrifugation (10 min, 8000 rpm Eppendorf table top centrifuge). Plasma was frozen at -80oC until assayed for CORT.

Blood glucose was measured at time of sacrifice with AccuCheck Advantage monitor and Comfort Curve strips (Roche, Indianapolis, IN).

Predator Odor Stress

Animals were handled for 3 days prior to experiment day. N=10 per group. The mice were dosed icv 45 min prior to predator odor stress. Mice were exposed to predator odor (soiled rat bedding) for 10 min then returned to their home cage for 20 min. The home cage control group was briefly picked up then returned to their home cage for 30 min. Trunk blood was collected in EDTA tubes at 30 min post po exposure and kept on

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ice until centrifuged to collect plasma. (10 min, 8000 rpm Eppendorf table top centrifuge) Plasma was frozen at -80^{o}C until assayed for CORT.

Metyrapone

Animals were handled for 3 days prior to experiment day and dosed po with 30 mg/kg MTIP, n=8 per group. On experiment day animals were administered metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone, Sigma-Aldrich, St. Louis MO, ip, 75 mg/kg) one hour after MTIP (po) and 60 min prior to sacrifice. A control group was dosed icv with astressin (1ug/3uL) 45 min prior to metyrapone. Trunk blood was collected and kept on ice until the plasma was separated by centrifugation (10 min, 8000 rpm Eppendorf table top centrifuge). Plasma was collected and frozen at -80^{o}C until assayed for CORT and ACTH.

Caffeine

Animals were handled for 3 days prior to experiment. The mice were administered MTIP (po, 30 mg/kg) or vehicle for 3 days. On the final experiment day, MTIP or vehicle were dosed 2 hours prior to caffeine. Astressin was administered acutely (icv, 1 ug/3 ul) 30 min prior to caffeine. Caffeine was injected (30 mg/kg, i.p.) 30 min prior to sacrifice and blood collection. Plasma was collected and frozen at -80^{o}C until assayed for CORT.

Yohimbine

Mice were dosed for 3 days with vehicle (1% CMC) or MTIP (30 mg/kg, po). Two hours following the last MTIP dose the mice were administered vehicle (saline) or yohimbine (Sigma, St. Louis,MO, 2.0 mg/kg, ip) and sacrificed 30 min later. Two additional groups were administered astressin (1ug/3uL, icv) 30 min prior to ip vehicle or yohimbine. N=6-

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9 per 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 table top centrifuge). Plasma was frozen at -80^{o}C until assayed for CORT.

Ethanol

Animals were dosed for 3 days with MTIP (po, 30 mg/kg) On the third day the animals were dosed with MTIP one hour prior to ethanol (po, Dacon, 32% in H_2O) or vehicle (H_2O). N=8 per group. The animals were administered ethanol or water 30 minutes prior to sacrifice. Trunk blood was collected and kept on ice until the plasma was separated by centrifugation (10 min at 8000 rpm in an Eppendorf table top centrifuge). Plasma was frozen at -80oC until assayed for CORT. Blood alcohol content measured with Ethanol L3K assay kit (Diagnostic Chemicals, Ltd, Charlottetown, PE,Canada)

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Results

The pharmacological and pharmacokinetic properties of the CRF1 antagonists used in the present study have been previously published (Gehlert et al., 2007) and are summarized in the supplementary data (Supplementary Table 1). We first examined the effects of R121919, SSR125543 and MTIP on plasma CORT concentrations after central CRF administration. Since these compounds have similar in vitro and in vivo potencies (Supplementary 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 since 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 (Supplementary Table 1). In these studies, the nonpeptide CRF1 antagonists were administered orally for three 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 measured by RIA. The peptide CRF1/CRF2 antagonist, Astressin was administered i.c.v. prior to CRF in some animals to determine the relative contributions of CRF1 and CRF2 receptor stimulation. The results from these experiments are summarized in Figure 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 ug Astressin produced a 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. Since MTIP produced the greatest reduction in CRF-stimulated CORT, subsequent studies were conducted using MTIP. First, we

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evaluated the ability of MTIP to occupy the pituitary CRF1 receptor. Using this method, we found that three days of orally administered MTIP inhibited ^{125}I-sauvagine binding to rat pituitary ex vivo with an ED_{50} of 7.5 mg/kg compared to 7.8 mg/kg observed when using the cerebella from the same animals (Figure 2). These values were somewhat greater than that seen in single dose studies (Gehlert et al., 2007). Therefore, a dose of 30 mg/kg 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 three days to Sprague-Dawley rats and began blood sampling one hour after the third dose. The resulting plasma samples were assayed for CORT and MTIP concentrations. These results are summarized in Figures 3 and 4. MTIP produced a marked suppression in plasma CORT for three hours after administration when compared to vehicle control. When assessed 8 and 9 hours after administration, a significant increase in the plasma levels of CORT was noted. Overall, the total amount of CORT (AUC) secreted over 24 hours was unchanged (Supplemental Figure 3). Since there was a significant suppression of CORT at the early time points, we focused on one hour after administration for the additional studies. Using the plasma samples from these animals, we also assessed the concentrations of MTIP (Figure 4) and these were maintained at a relatively high level throughout the nine hour period where changes in CORT were observed. The plasma levels were greater than that associated with the ED_{50} concentrations determined in a single dose study as denoted by the dashed line. Pharmacokinetic parameters determined from this study are found in Supplementary Table 2. Compared to the single dose pharmacokinetics

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(Supplementary data Table 1), the $t_{1/2}$ for MTIP was similar to that observed with a single dose while the Tmax was later (4.11 hours versus 2.2 hours). Subsequently, a dose-response study was conducted using a cumulative dosing paradigm for the 0.1, 1 and 10 mg/kg doses administered over three 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 mg/kg and 30 mg/kg one hour following the third dose (Figure 5). The reduction seen in CORT was similar between the 10 and 30 mg/kg doses indicating this was likely 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 i.c.v. injections in some experiments. In the first of these experiments, we evaluated the effects of an acute injection of insulin on plasma CORT one hour after injection. A dose-response study was conducted to optimize the dose of insulin and a 1U/kg dose was found to produce a robust decrease in plasma glucose and robust increase in plasma CORT (Supplementary Figure 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 hour prior to the insulin injection. These data are summarized in Figure 6. MTIP produced a significant decrease in the plasma CORT at 30 mg/kg while lower doses did not produce a significant effect. In the next set of experiments, we evaluated the effect of metyrapone on plasma ACTH. In mice, metyrapone inhibits the 11-beta hydroxylation of desoxycorticosterone to CORT resulting in decreased plasma and brain concentrations

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or CORT. The loss of feedback inhibition on CRF synthesis increases hypothalamic CRF resulting in an increased plasma ACTH in a physiological attempt to increase plasma CORT. The dose of metyrapone required to suppress CORT was optimized at 75 mg/kg, i.p. (Supplementary Figure 1). As shown in Figure 7B, metyrapone produced a pronounced decrease in plasma CORT and this was unaffected by pretreatment with MTIP. As seen in the rat studies, MTIP produced a reduction in the basal concentrations of plasma CORT. When evaluating the effects of ACTH, metyrapone produced an approximate doubling of plasma ACTH that was unaffected by 30 mg/kg, p.o. of MTIP (Figure 7A). To understand the potential contribution of the CRF2 receptor to this increase in ACTH, we administered the peptidic CRF1/CRF2 antagonist Astressin at a dose of 1 ug i.c.v. and observed a partial reduction in the plasma ACTH concentrations (Figure 7A) suggesting that a combination of central CRF1 and CRF2 receptors may be necessary to see reversal of this effect. The dose of 1 ug was found to completely suppress the activation of the HPAA produced by 3 ug CRF (Figure 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 icv administration of astressin (Figure 8A). On the other hand, pretreatment with 30 mg/kg MTIP did not affect the increase in plasma CORT (Figure 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 two hours prior to caffeine (Figure 9). In this study, the

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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 in the increases in plasma CORT produced by the alpha-2 antagonist yohimbine (Figure 10). In these studies, icv pretreatment with astressin produced a significant reduction in yohimbine-stimulated CORT while MTIP had no significant effect. Finally, MTIP was found to reduce the increase in plasma CORT following the acute administration of ethanol (Figure 11).

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Discussion

These experiments were conducted to understand the effects of subchronic CRF1 antagonism on basal and stress-induced CORT secretion in vivo. CRF is the major hypothalamic secretogogue that regulates the secretion of ACTH and consequently, the secretion of CORT in the adrenal. CRF1 has the highest affinity for CRF and is abundant in the brain and pituitary (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). While a few studies have examined the effect of acute CRF1 antagonist administration on the HPAA, it is not well understood what the impact of longer term administration of these compounds on the HPAA or the effects of central blockade. 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 i.c.v. prior to the CRF infusion. When compared to R121919 and SSR125543, MTIP produced the most pronounced, though 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 three hours of the evening (rats) CORT surge when compared to

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vehicle treated controls. There appeared to be compensation for this effect with a prolongation of the corticosterone elevation seen at 8 pm and 9 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 hour CORT secretion was not statistically different between the MTIP and vehicle treated animals suggesting 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 LC-MS. After three daily gastric infusions, plasma MTIP concentrations were maintained above that required for greater than 50% occupancy for 22 of the first 24 hours. In a subsequent dose-response study, doses of 10 mg/kg and 30 mg/kg appeared to produce a maximal suppression of basal CORT the first hour after administration. These doses correlated with the occupancy measures and the ability of MTIP to antagonize the elevation of plasma CORT after icv 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 utilized 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 a suppression of the increase in CORT seen following an acute injection of insulin. At a

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dose of 30 mg/kg, MTIP produced a significant reduction in 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. Interestingly, 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 11-beta-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 ACTH release from the pituitary. Antagonism of brain and pituitary CRF receptors would theoretically reduce the increase in ACTH produced by metyrapone. Central administration of AST resulted in a partial reversal of the ACTH response while 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 i.c.v. administration. A similar phenomenon was observed when evaluating yohmibine activation of the HPAA where centrally administered AST produced a substantial reduction in plasma CORT while MTIP did not. Also interesting was that AST could not produce a complete reversal of the CORT response in the

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models evaluated, even though the i.c.v. dose of AST was capable of completely reversing the endocrine effects of i.c.v. CRF. Therefore it is likely that there are other pathways such as vasopressin activated that contribute to the CORT response that 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. Based on 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 ACTH secretion following intravenous administration of CRF for up to 6 hours 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 likely pituitary CRF1 antagonism and not via antagonism of the central effects of CRF. The CRF1 antagonist, R121919, has been shown to reduce ACTH and CORT following a peripherally administered CRF challenge both preclinically and clinically (Ising et al., 2007). It has also been shown to reduce endocrine activation following the defensive withdrawal procedure in rats (Gutman et al., 2003). In this study, a dose-dependent reduction in both ACTH and CORT were observed that correlated with the occupancy and behavioral measures. While 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

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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 will be required in a broader spectrum of behavioral stressors 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 useful biomarker to evaluate central CRF1 receptor occupancy in clinical studies. We detected a suppression of the CORT response to an acute bolus of insulin as a promising method to pursue in clinical studies when 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 NBI34041 has been explored preclinically and clinically. In rats, there was a dose-dependent reduction in plasma ACTH subsequent to intravenous CRF or a 10 minute intermittent footshock. In the clinic, NBI34041 reduced the ACTH but not the cortisol response to intravenous CRF. It was reported to produce a statistically significant diminution of the increase in plasma ACTH 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

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CRF1 receptor occupancy though additional work will need 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 three hours 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.

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Authorship Contributions

Participated in research design: Gehlert, Cramer, Morin
Conducted experiments: Cramer, Morin
Contributed new reagents or analytic tools:
Performed data analysis: Gehlert, Cramer, Morin
Wrote or contributed to the writing of the manuscript: Gehlert, Cramer, Morin

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Figure Legends

Figure 1. Effect of CRF1 antagonists (30 mg/kg, p.o., three day dosing) on icv CRF induced plasma CORT concentrations. In these studies, mice were treated with maximal doses of three non-peptide CRF1 receptor antagonists for three days. One hour following the final dose, animals were administered 3 ug CRF, icv and blood collected 15 minutes later. Plasma CORT concentrations were measured by radioimmunoassay. Astressin was administered in the opposite ventricle 30 minutes prior to icv CRF. In these studies, Astressin completely antagonized the increase in plasma concentrations of CORT produced by i.c.v. 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 (see Figure 3). *$p<0.05$ versus veh/CRF group, ANOVA with Bonferroni post hoc testing. n=8

Figure 2. Inhibition of ^{125}I-Tyr^{0}-Sauvagine binding to rat pituitary ex vivo. Rats were administered various doses of MTIP by gavage and the specific binding of ^{125}I-Tyr^{0}-Sauvagine assessed as described in the Methods section. In these studies, MTIP produced a dose dependent decrease in the binding of ^{125}I-Tyr^{0}-Sauvagine to the pituitary ex vivo with an estimated ED_{50} of 7.5 mg/kg compared to an ED_{50} of 7.8 mg/kg for the cerebellum (n=5).

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Figure 3. The effect of 30 mg/kg MTIP by bolus administration through a gastric fistula for three days on the plasma concentrations of CORT in male, Sprague-Dawley rats. Data are plotted as mean ± SEM. *$p<0.05$ (repeated measures 2-way ANOVA). Vehicle (n=6), MTIP (n = 9).

Figure 4. Plasma concentrations of MTIP on following 3 daily doses of 30 mg/kg, p.o. via gastric cannulae to Male, Sprague-Dawley rats. Blood sampling (100 ul) was accomplished using the Culex system and plasma MTIP concentrations determined by LC-MS using aliquots from the plasma samples assayed for CORT (Figure 3) (n=9). The dashed line indicates the plasma concentration associated with the ED_{50} extrapolated from an acute study with 10 mg/kg MTIP, p.o. The ED_{50} in that study was determined to be 1.5 mg/kg with a calculated plasma concentration of 360 ng/ml assuming dose linearity (Data on file at Eli Lilly and Company).

Figure 5. The effect of 1, 3, 10 and 30 mg/kg MTIP administered orally by gastric fistula on the basal plasma concentrations of CORT in male, Sprague-Dawley rats one hour after the third dose (n=9). Data are expressed at a percent of vehicle control CORT levels. Statistically significant reductions on CORT were observed at 10 and 30 mg/kg MTIP ($p<0.05$).

Figure 6. Effect of 3 days of MTIP (3, 10 and 30 mg/kg, p.o.) on insulin- induced (1 U/kg, i.p.) plasma glucose and CORT in male C57BL/6 mice. Insulin was dosed 1 hour following MTIP and blood was collected 1 hour following insulin. v = vehicle for MTIP, V

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= vehicle for insulin, I = 1 U/kg insulin. n=7-8, *p< 0.05 compared to v/V group, ANOVA (Tukey's post hoc) or versus v/I group.

Figure 7. Effect of MTIP on metyrapone (75 mg/kg, i.p.)-induced increases in plasma ACTH (A) and CORT (B) in mice. Intracerebroventricularly administered Astressin (1 ug/3ul) was able to partially reduce this, but MTIP (30 mg/kg, p.o.) had no effect. *p<0.05 vs veh/veh, #p<0.05 vs veh/met. ANOVA, Tukey's post hoc test, n=8.

Figure 8. Effect of a) astressin (*p<0.001 compared to veh/hc group #p<0.05 compared to veh/POS group, ANOVA, Tukey's post hoc test) or b) MTIP (*p<0.05 compared to veh/hc group, ANOVA, Tukey's post hoc test.) on predator-odor stress (POS) induced CORT in male C57BL/6 mice. n=10.

Figure 9. Effect of MTIP (30 mg/kg, p.o., 2 hr prior to caff) or Astressin (Ast, 1ug/3uL, icv, 30 min prior to caff) on caffeine induced CORT in male C57BL/6 mice. blood collection was 30 minutes after caffeine. n=7-8 (*p<0.05 compared to respective vehicle group, ANOVA, Tukey's post hoc test).

Figure 10. Effect of MTIP (30 mg/kg, p.o., 2 hr prior to yoh) or Astressin (Ast, 1ug/3ul, icv, 30 min prior to yoh) on yohimbine- induced CORT in male C57BL/6 mice. Blood collection was 30 minutes after yohimbine. n=6-9 (*P<0.01 compared to all vehicle groups, #p<0.05 compared to all yoh treated groups, ANOVA, Tukey's post hoc test)

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Figure 11. Effect of MTIP on ethanol-induced increases in plasma CORT. A. A 2 g/kg Ethanol (e) produces a significant increase in plasma CORT 30 minutes after administration in vehicle pretreated animals when compared to vehicle (V) treated animals (*$p<0.05$, ANOVA, Tukey's post-hoc, n=8). B. Pretreatment of mice with 30 mg/kg MTIP had no significant effect on Blood Alcohol Concentrations (BAC) thirty minutes after oral gavage of 2 g/kg ethanol. n = 8.

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icv CRF Induced Plasma Corticosterone Following Three Day Dosing of CRFR1 Antagonists or Astressin

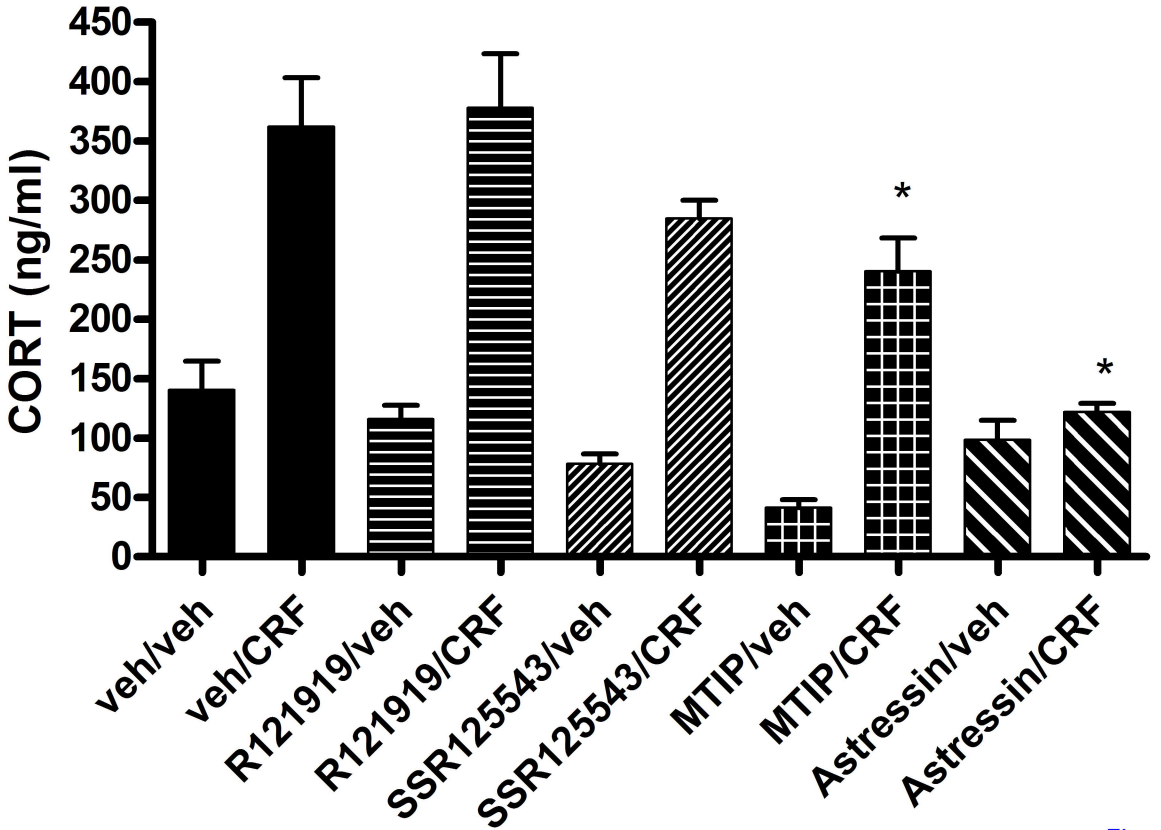


Figure 1

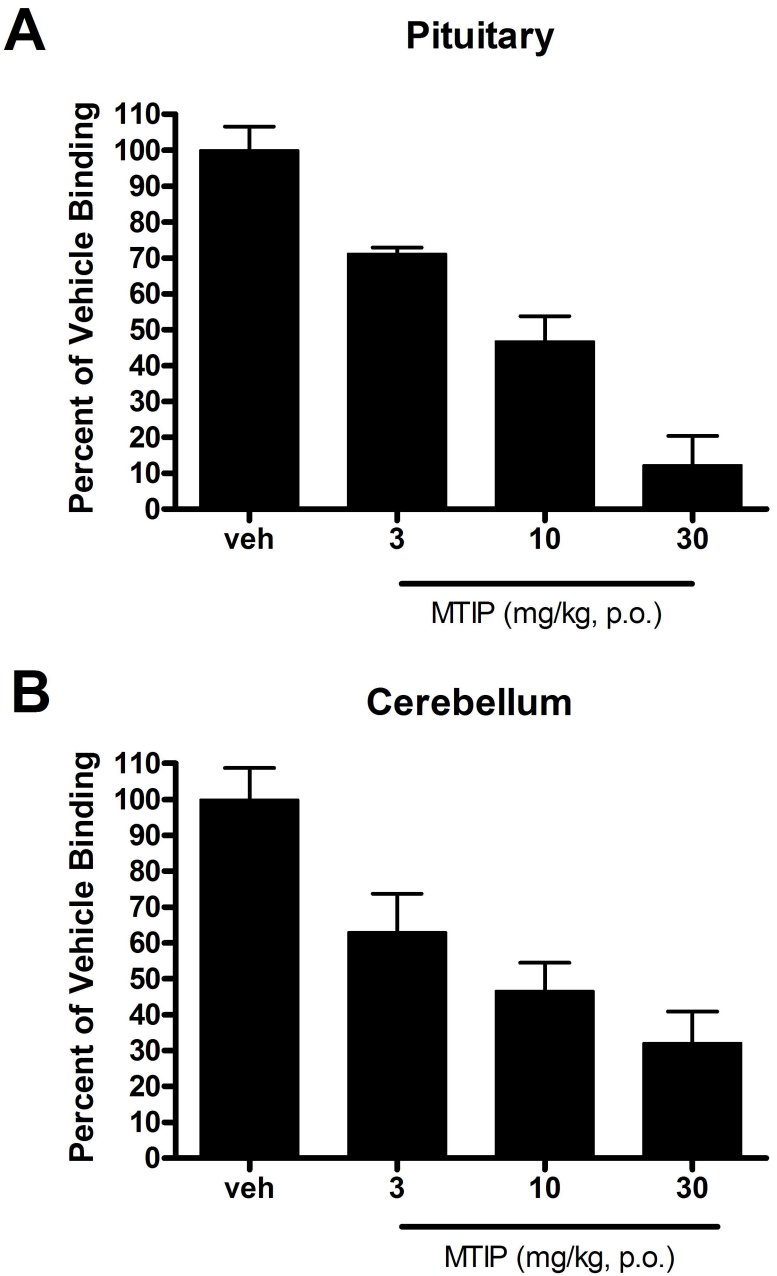

A
Pituitary
Percent of Vehicle Binding
110
100
90
80
70
60
50
40
30
20
10
0
veh
3
10
30
MTIP (mg/kg, p.o.)
B
Cerebellum
Percent of Vehicle Binding
110
100
90
80
70
60
50
40
30
20
10
0
veh
3
10
30
MTIP (mg/kg, p.o.)

Figure 2

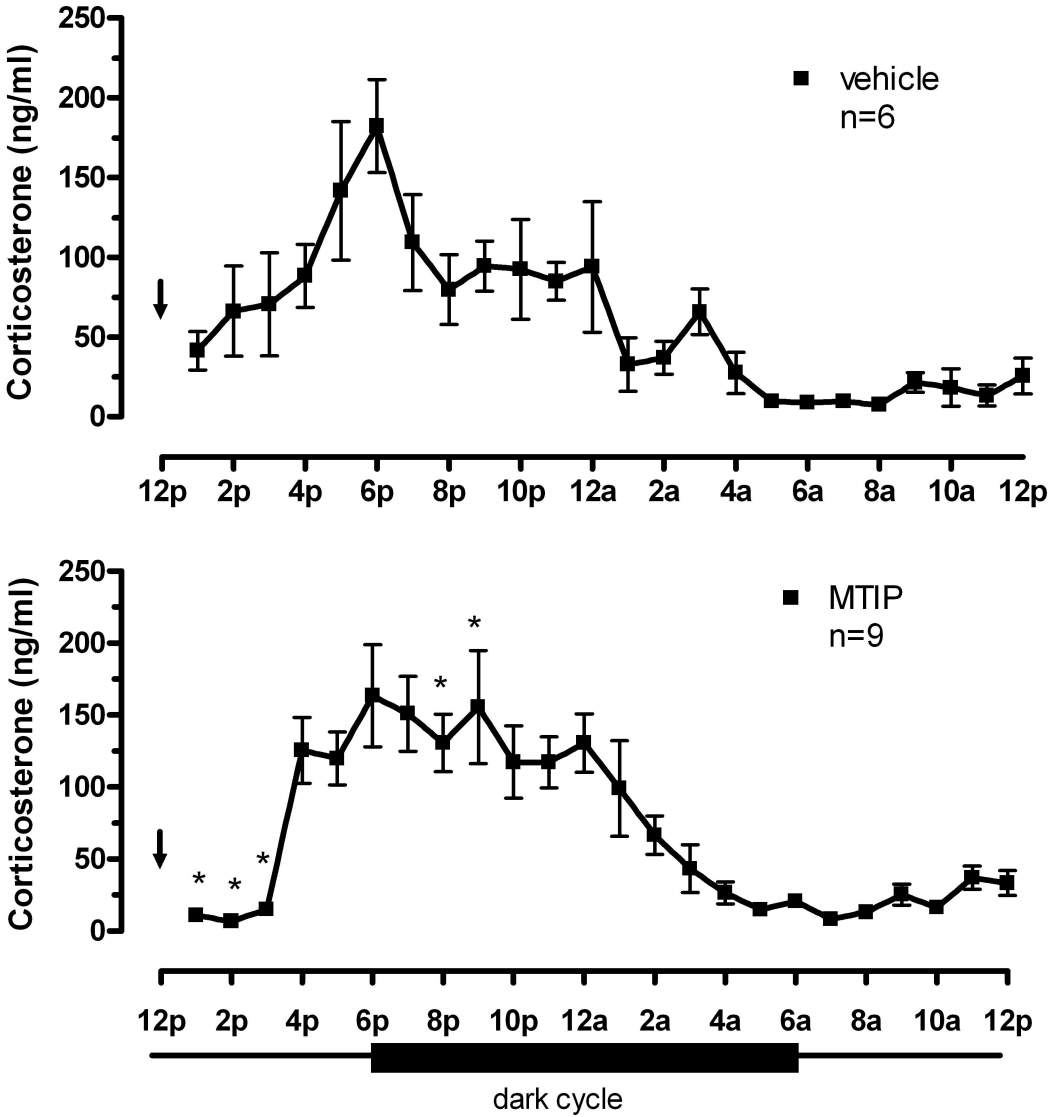

Corticosterone (ng/ml)
250
200
150
100
50
0
vehicle
n=6
12p
2p
4p
6p
8p
10p
12a
2a
4a
6a
8a
10a
12p
Corticosterone (ng/ml)
250
200
150
100
50
0
MTIP
n=9
*
*
*
*
12p
2p
4p
6p
8p
10p
12a
2a
4a
6a
8a
10a
12p
dark cycle

Figure 3

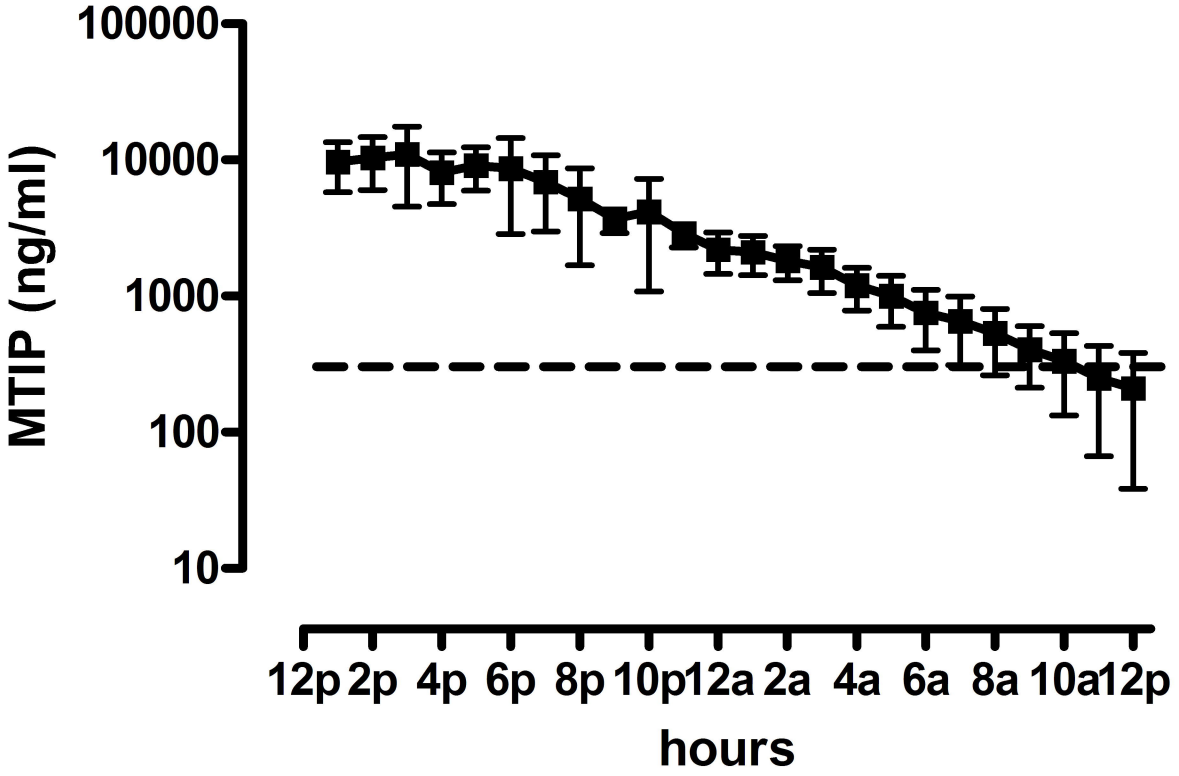

100000
10000
1000
100
10
MTIP (ng/ml)
12p 2p 4p 6p 8p 10p 12a 2a 4a 6a 8a 10a 12p
hours

Figure 4

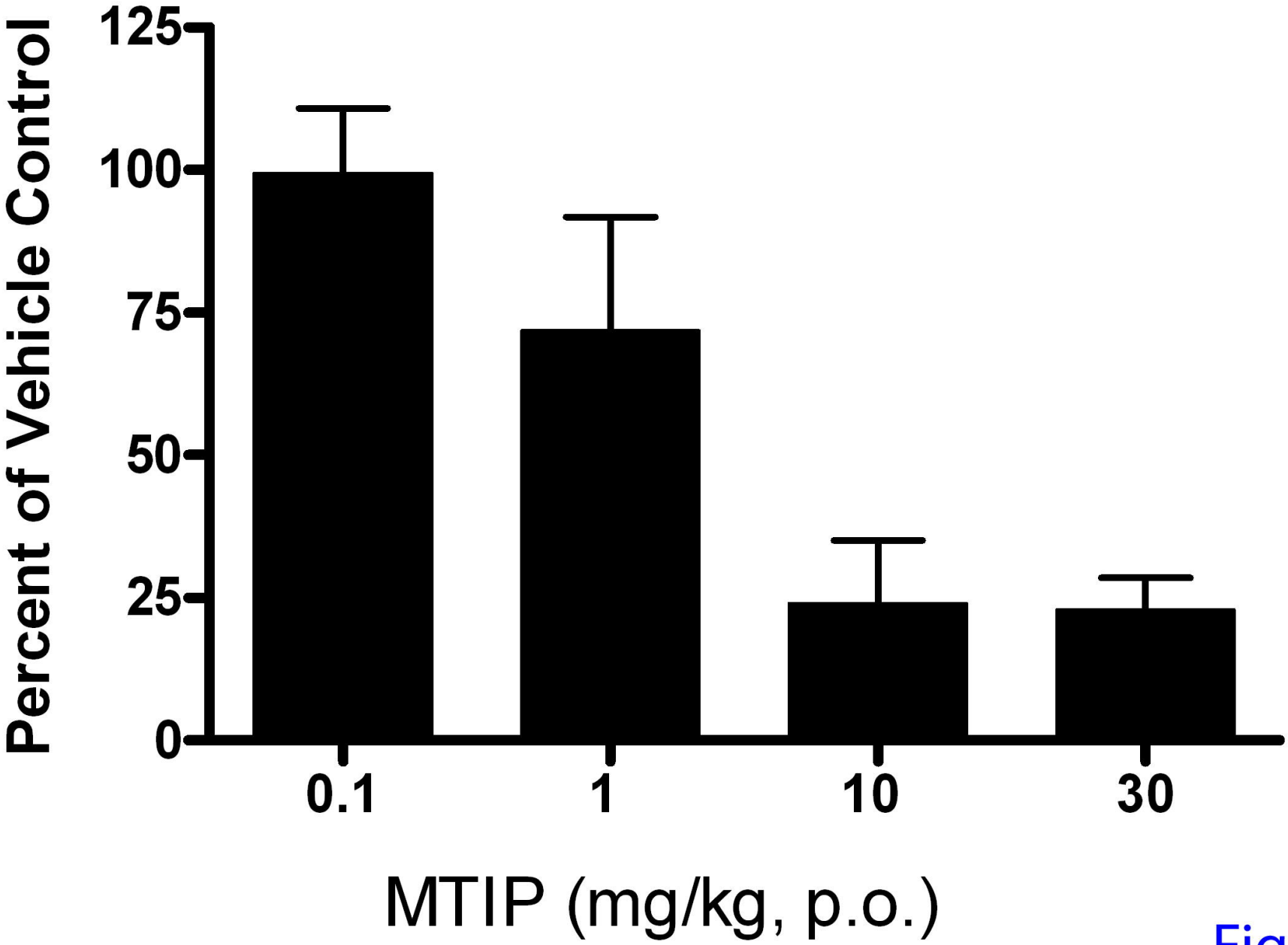
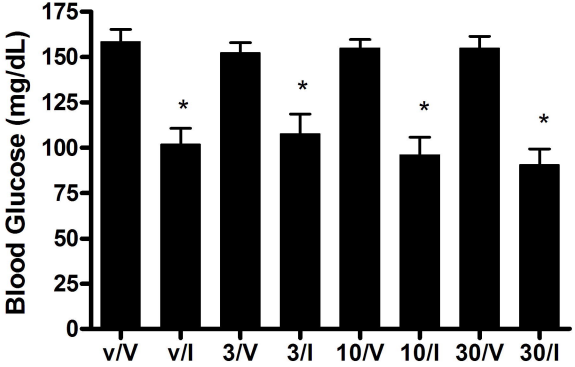

Percent of Vehicle Control
125
100
75
50
25
0
0.1
1
10
30
MTIP (mg/kg, p.o.)

Figure 5


Blood Glucose (mg/dL)
175
150
125
100
75
50
25
0
*
*
*
*
v/V
v/I
3/V
3/I
10/V
10/I
30/V
30/I

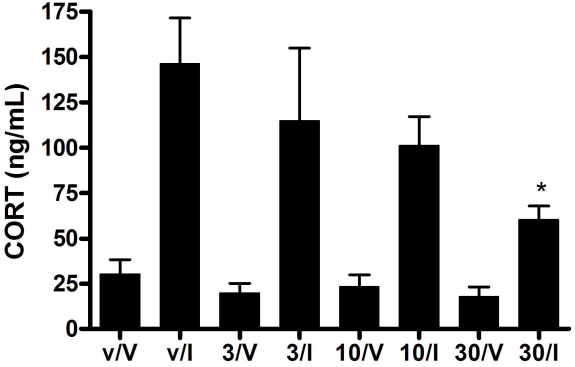

CORT (ng/mL)
175
150
125
100
75
50
25
0
*
v/V
v/I
3/V
3/I
10/V
10/I
30/V
30/I

Figure 6

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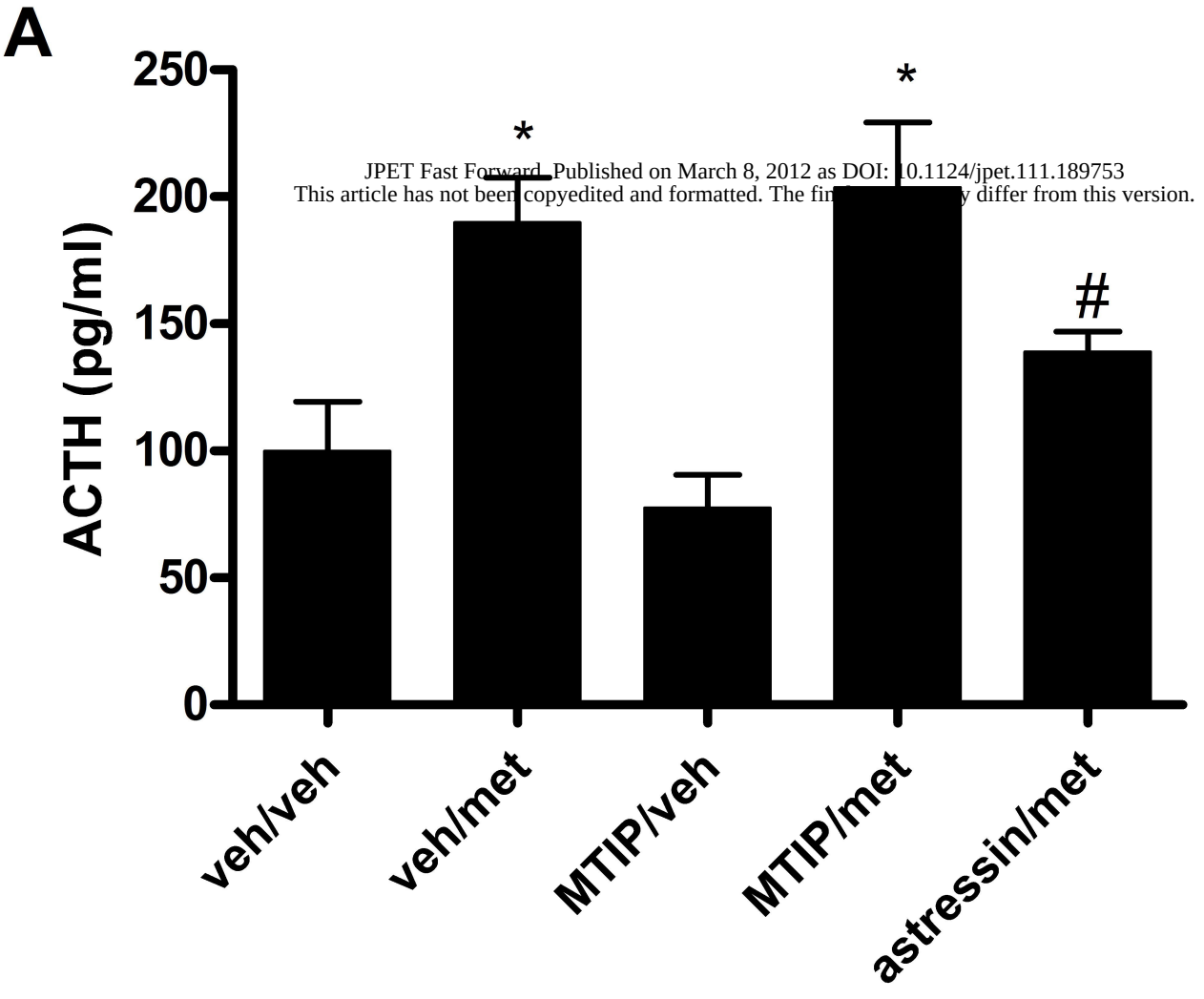

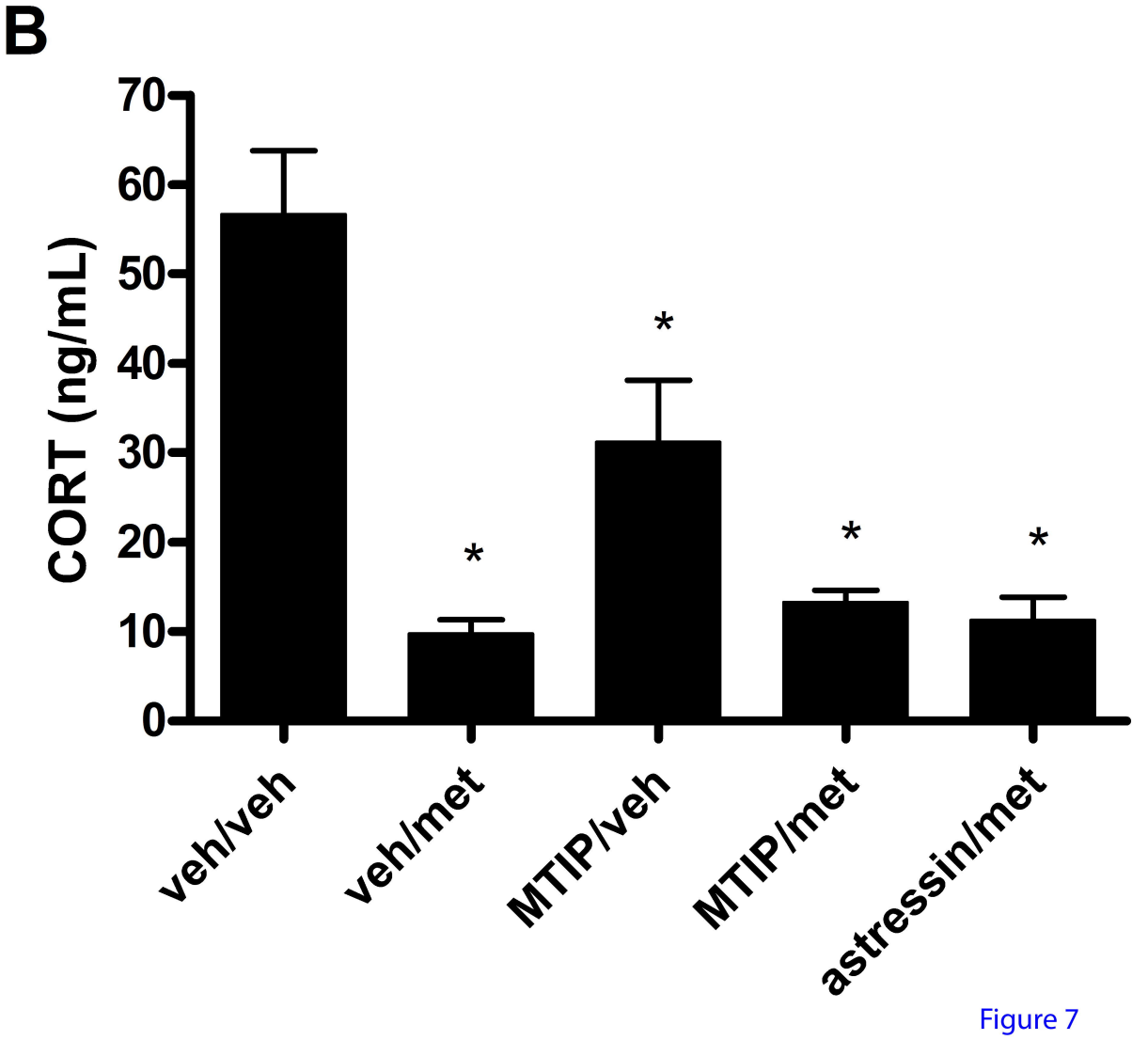

Figure 7

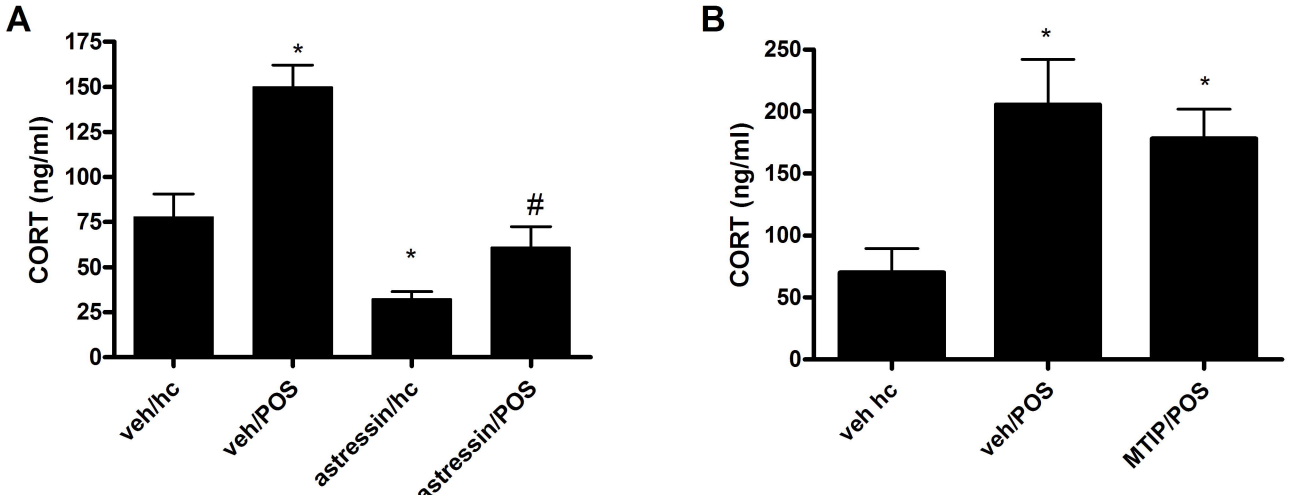
A
CORT (ng/ml)
175
150
125
100
75
50
25
0
*
*
#
veh/hc
veh/POS
astressin/hc
astressin/POS
B
CORT (ng/ml)
250
200
150
100
50
0
*
*
veh hc
veh/POS
MTIP/POS

Figure 8

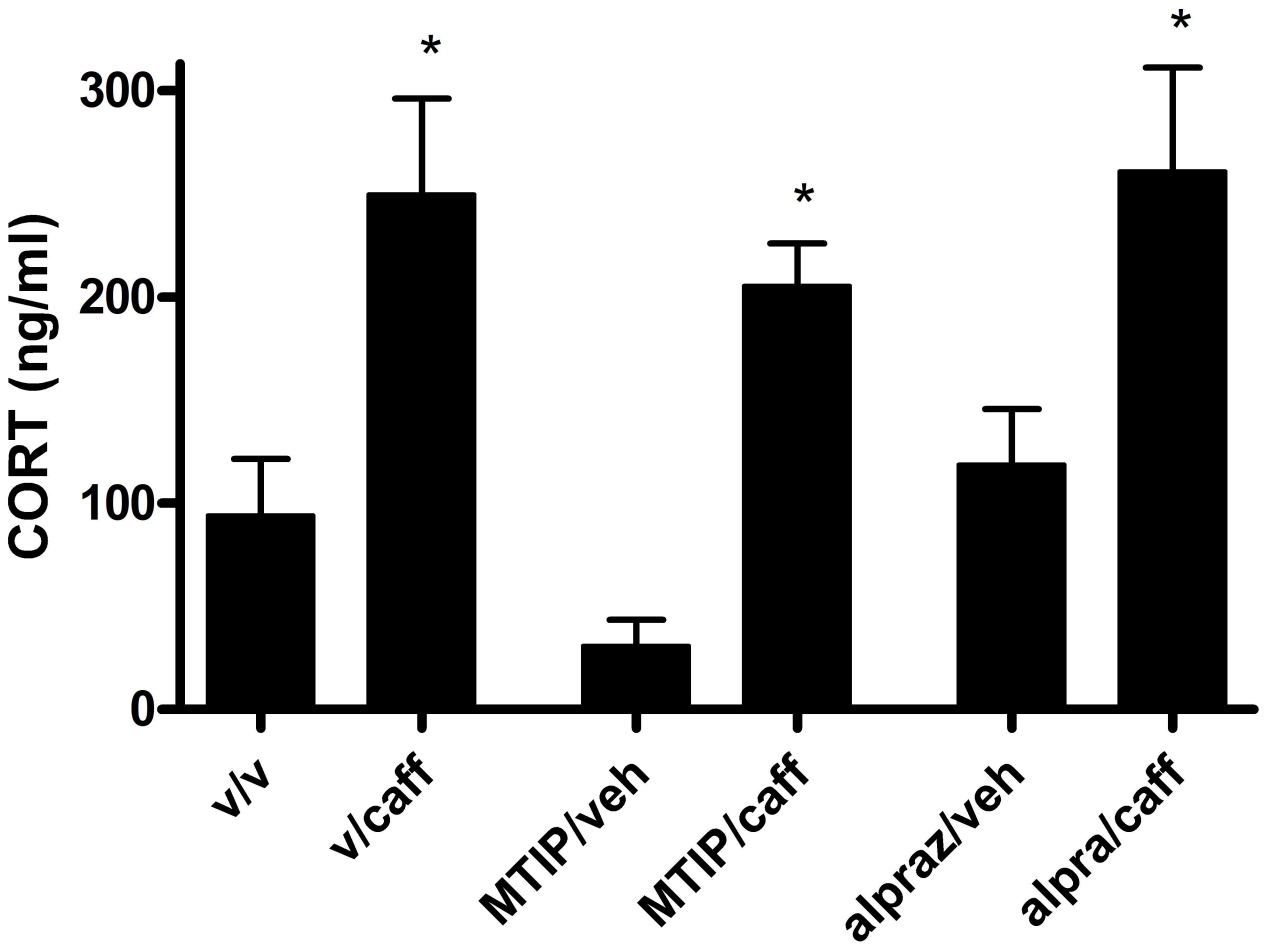
CORT (ng/ml)
300
200
100
0
*
*
*
v/v
v/caff
MTIP/veh
MTIP/caff
alpraz/veh
alpra/caff

Figure 9

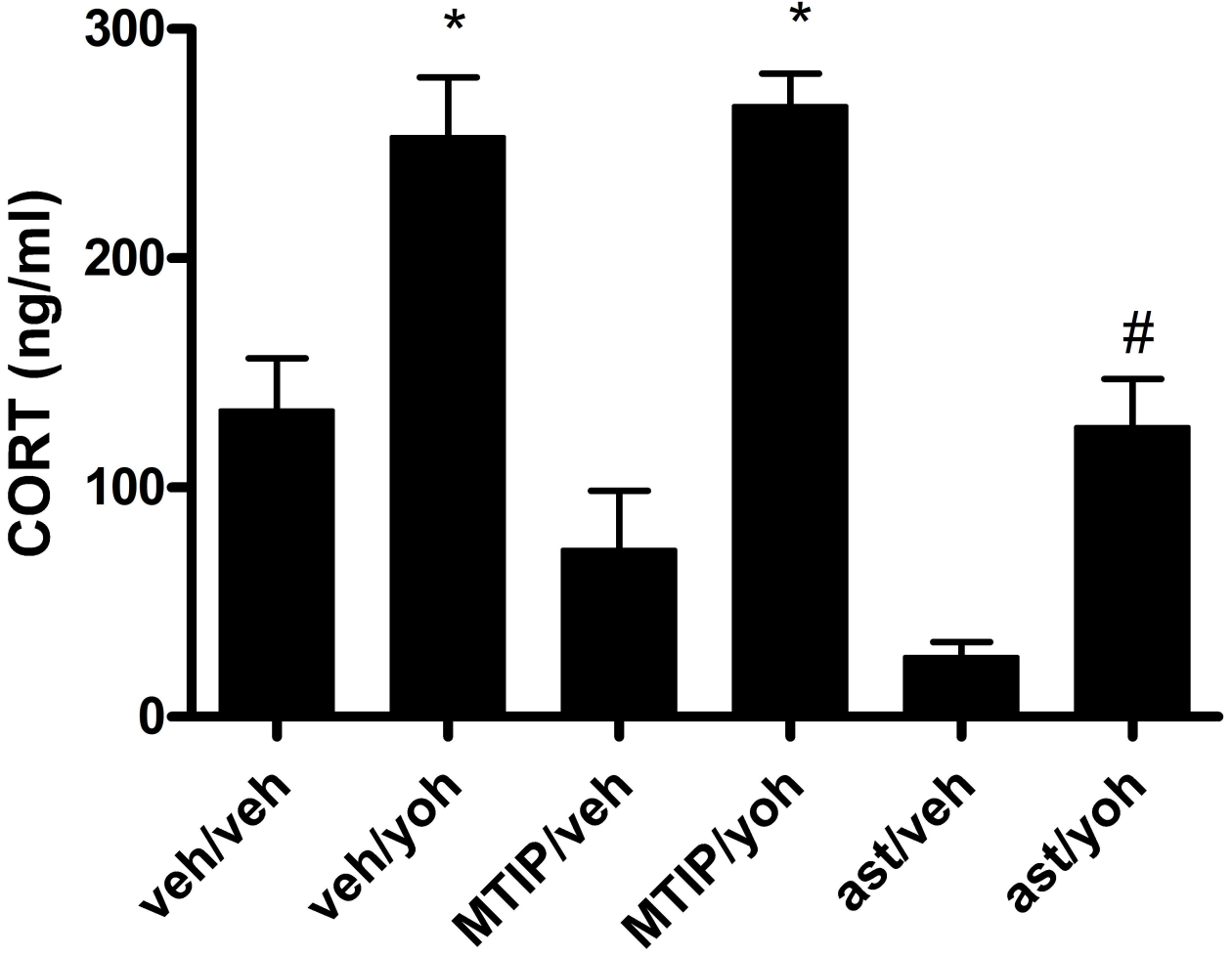
300
200
100
0
CORT (ng/ml)
*
*
#
veh/veh
veh/yoh
MTIP/veh
MTIP/yoh
ast/veh
ast/yoh

Figure 10

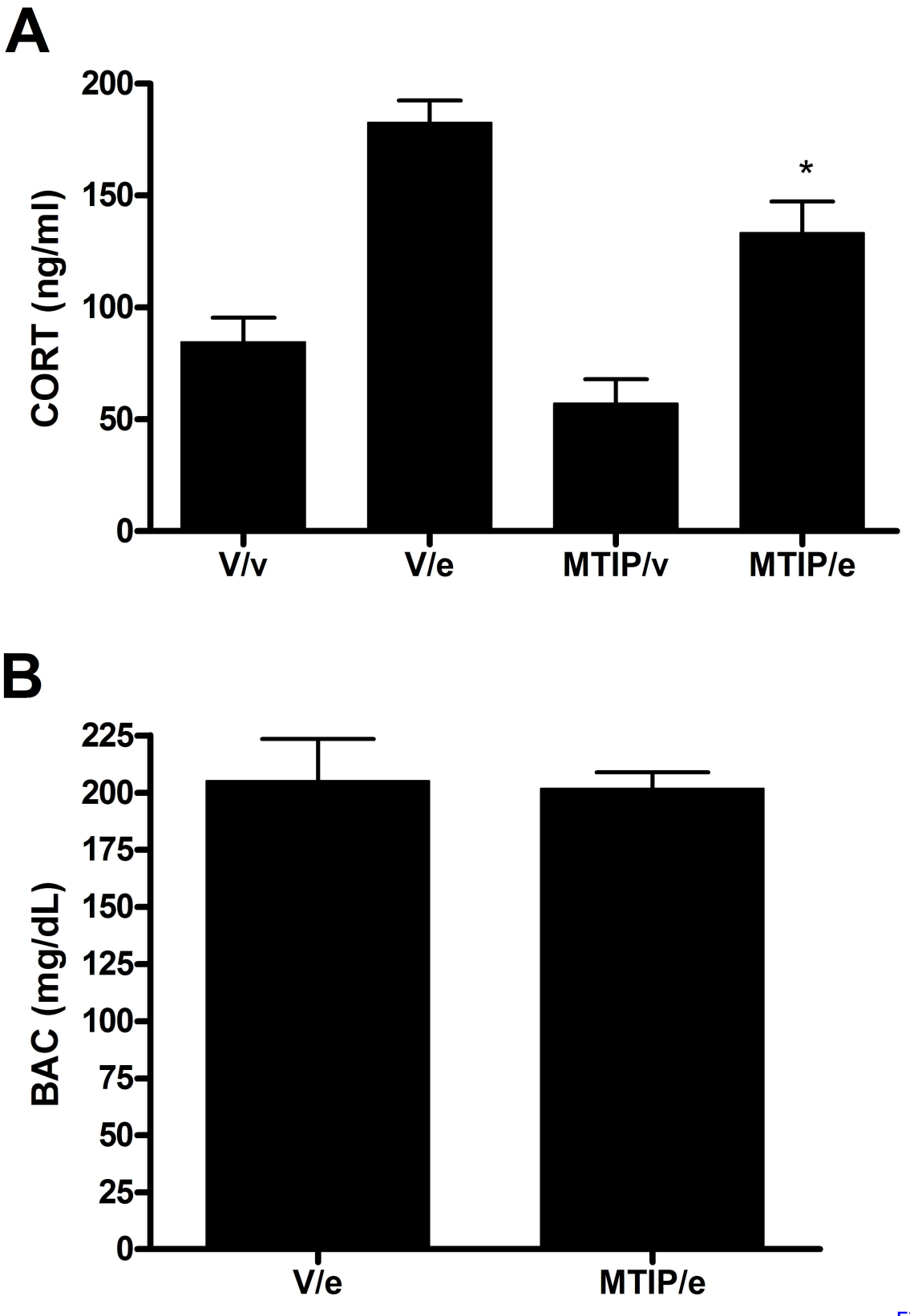
A
200
150
100
50
0
CORT (ng/ml)
*
V/v
V/e
MTIP/v
MTIP/e
B
225
200
175
150
125
100
75
50
25
0
BAC (mg/dL)
V/e
MTIP/e

Figure 11