JPET Fast Forward. Published on January 23, 2004 as DOI: 10.1124/jpet.103.058784

Anxiolytic-like Effects of the CRF_1 Antagonist DMP904 Administered Acutely or Chronically at Doses Occupying Central CRF_1 Receptors in Rats

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RUNNING TITLE: Behavioral and Central CRF_1 Receptor Effects of DMP904

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Number of text pages: 33

Number of tables: 0

Number of figures: 10

Number of references: 21

Number of words in the Abstract: 253

Number of words in the Introduction: 802

Number of words in the Discussion: 1413

List of Abbreviations: CRF, corticotropin-releasing factor; HPA, hypothalamic-pituitary-adrenal; CP-154,526, butyl-ethyl-[2,5-dimethyl-7-(2,4,6,-trimethyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl]-amine; DMP696, 4-(1,3-dimethoxyprop-2-ylamino)-2,7-dimethyl-8-(2,4-dichlorophenyl)-pyrazolo[1,5-*a*]-1,3,5-triazine; DMP904, 4-(3-pentylamino)-2,7-dimethyl-8-(2-methyl-4-methoxyphenyl)-pyrazolo-[1,5-*a*]-pyrimidine; R121919, 3-[6-(dimethylamino)-4-methyl-pyrid-3-yl]-2,5-dimethyl-*N*,*N*-dipropyl-pyrazolo[2,3-*a*]pyrimidin-7-amine; SSR125543A, 4-(2-chloro-4-methoxy-5-methylphenyl)-*N*-[(1S)-2-cyclopropyl-1-(3-fluoro-4-methylphenyl)ethyl]5-methyl-*N*-(2-propynyl)-1,3-thiazol-2-amine hydrochloride; FR, fixed ratio; CDP, chlordiazepoxide; LC/MS/MS, liquid chromatography/tandem mass spectometry

Recommended section assignment: Behavioral Pharmacology

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ABSTRACT

CRF_1 antagonists may be effective in the treatment of anxiety disorders while having fewer side effects compared with classical benzodiazepines. The behavioral effects of DMP904 and its effects on HPA axis were related to its levels in plasma and estimated occupancy of central CRF_1 receptors. DMP904 (10-30 mg/kg, PO) and alprazolam (10 mg/kg, PO) increased time spent in open arms of an elevated-plus maze. In addition, acutely or chronically (14 days) administered DMP904 (1.0-30 mg/kg, PO) and acute alprazolam (1.0-3.0 mg/kg, PO) significantly reduced exit latency in the defensive withdrawal model of anxiety in rats, suggesting that tolerance may not develop to the anxiolytic-like effects of DMP904 in this model of anxiety. Acutely, DMP904 reversed the stress-induced increase in plasma corticosterone levels in defensive withdrawal at doses of 3.0 mg/kg and higher. These doses also resulted in levels of DMP904 in plasma similar to (for anxiolytic-like effects) or 4-fold higher (for effects on HPA axis) than the in vitro IC_{50} value for binding affinity at CRF_1 receptors and greater than 50% occupancy of CRF_1 receptors. Unlike alprazolam, DMP904 did not produce sedation, ataxia, or chlordiazepoxide-like subjective effects (as measured by locomotor activity, rotorod performance, and chlordiazepoxide discrimination assays, respectively) at doses at least 3-fold higher than anxiolytic-like doses. In conclusion, anxiolytic-like effects and effects on stress-activated HPA axis of DMP904 in the defensive withdrawal model of anxiety required 50% or greater occupancy of central CRF_1 receptors. This level of CRF_1 receptor occupancy resulted in fewer motoric side effects compared with classical benzodiazepines.

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Corticotropin-releasing factor $(CRF)_1$ antagonists are a new class of compounds that may have anxiolytic-like effects in non-human animals without motoric side effects associated with classical anxiolytic agents (for a review, see Takahashi, 2001). CP-154,526 reversed separation-induced increase in ultrasonic vocalization in rat pups (Kehne et al., 2000) and reduced expression of conditioned fear in rats (Hikichi et al., 2000). In addition, this compound also increased time spent in open arms of an elevated-plus maze in rats (Lundkvist et al., 1996), although this result was not repeated in another study (Griebel et al., 1998a). Furthermore, SSR125543A increased punished responding in a conflict test in rats (Griebel et al., 2002), although CP-154,526 was not effective in this kind of anxiety model (Griebel et al., 1998b). Finally, DMP696 decreased exit latency in the defensive withdrawal model of anxiety in rats (McElroy et al., 2002). In non-human primates, antalarmin reversed behavioral signs of anxiety produced by social stress (Habib et al., 2000). In human subjects, R121919 reduced anxiety scores in a small, open-labeled clinical trial (Zobel et al., 2000). Collectively, the majority of preclinical evidence suggests that CRF_1 antagonists may be effective anxiolytic agents in humans.

The extent to which anxiolytic-like effects of CRF_1 antagonists are independent of their effects on the hypothalamic-pituitary-adrenal (HPA) axis is at present unclear. CRF is the major factor regulating basal and stress-induced activation of the HPA axis. Thus, CRF_1 antagonists may exert their anxiolytic-like effects through blockade of central CRF_1 receptors, through reduction of CRF-mediated activation of the HPA axis in response to stress, or through a combination of both effects. DMP696 reversed the stress-induced increase in plasma corticosterone in animals exposed to the novel environment in the defensive withdrawal model

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of anxiety at doses that had anxiolytic-like effects in the same animals (McElroy et al., 2002). Higher doses of DMP696 showed a further decrease in plasma corticosterone levels to the level of non-stressed animals. These results are in contrast to the effects of the benzodiazepine chlordiazepoxide (CDP), which at higher doses enhanced the stress-induced increase in plasma corticosterone (McElroy et al., 2002). The latter data indicate that anxiolytic-like effects can be obtained despite increases in plasma corticosterone levels. In contrast to these findings with benzodiazepines, studies on CRF_1 antagonists to date suggest that the anxiolytic-like effects of CRF_1 antagonists cannot be dissociated from their effects on stress-induced HPA axis activation.

Benzodiazepines, such as alprazolam used in the present study, produce a number of undesirable side effects such as sedation, ataxia, amnesia, rebound anxiety on withdrawal, and development of tolerance and dependence with chronic treatment (Costa and Guidotti, 1996). In a previous study, DMP696, unlike CDP, did not produce sedation or ataxia at doses 3- to 10-fold higher than the lowest effective anxiolytic-like doses (McElroy et al., 2002). Similarly, CP-154,526 showed anxiolytic-like effects in rats without sedation (Hikichi et al., 2000) or ataxia (Kehne et al., 2000). In addition, DMP696 did not substitute for the discriminative stimulus effects of CDP and could not be established as a discriminative stimulus in rats at doses 6-fold higher than the lowest anxiolytic-like dose, indicating that it may not share subjective effects with benzodiazepines (Lelas et al., 2003). If the efficacy of CRF_1 antagonists and the absence of the side effects in preclinical studies is confirmed in studies with human subjects, these findings could establish CRF_1 antagonists as superior anxiolytic agents compared with benzodiazepines.

The purpose of the present study was to examine the relationship between acute behavioral effects of a CRF_1 antagonist DMP904, its effects on stress-induced activation of the HPA axis, its estimated occupancy of central CRF_1 receptors, and plasma free concentration of

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DMP904 in rats. DMP904, 4-(3-pentylamino)-2,7-dimethyl-8-(2-methyl-4-methoxyphenyl)-pyrazolo-[1,5-*a*]-pyrimidine (Figure 1), is a small-molecule antagonist highly selective for the CRF_1 receptor subtype (Gilligan et al., 2000; Zhang et al., 2003). DMP904 binds with high affinity to human CRF_1 receptors (1.16 nM), has no affinity for human CRF_2 receptors or CRF-binding protein, and inhibits CRF-induced adenylate cyclase activity in HEK293 cells (IC_{50} = 10 nM). In the present study, DMP904 was evaluated in the elevated-plus maze model after acute administration and defensive withdrawal model of anxiety after acute and chronic administration in rats. In the latter model, rats are placed in a dark cylinder in an illuminated open field, and the latency to exit the cylinder and explore the novel environment is used as an index of anxiolytic-like effects. The exposure to the novel environment results in an increase in plasma corticosterone concentration and thus, effects of CRF_1 antagonists on stress-induced HPA axis activation can be evaluated in the same animals. Finally, plasma free levels of DMP904 and its estimated occupancy of central CRF_1 receptors are determined in the same animals. In addition, to determine whether DMP904 has a more favorable side effect profile compared with benzodiazepines, its effects were compared directly with those of alprazolam.

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METHODS

Subjects

Male Sprague-Dawley rats weighing 180-300 g were purchased from Charles River Laboratories (Wilmington, Mass.). The rats were housed individually in suspended wire cages (for defensive withdrawal and drug discrimination studies) or three to a plastic cage (elevated-plus maze, rotorod, and locomotor activity studies) in colony rooms maintained at constant temperature (21 ± 2^{o}C) and humidity (50 ± 10%). The rooms were illuminated 12 hours per day (lights on at 0600 h). The rats had *ad libitum* access to food and water throughout the studies, except in the drug discrimination study in which they were restricted to 12 g of laboratory chow (Bio-Serv, Frenchtown, N.J.) per day. Behavioral studies were conducted between 0600 and 1300 h. Animals were maintained in accordance with the guidelines of the Committee on Animals of the Bristol-Myers Squibb Company, the "Guide for Care and Use of Laboratory Animals" (Institute of Animal Laboratory Resources, 1996), and the guidelines published in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Research protocols were approved by the Bristol-Myers Squibb Company Institutional Animal Care and Use Committee.

Elevated-Plus Maze

The elevated-plus maze, constructed of black, opaque Plexiglas, consisted of four arms at right angles to each other, and elevated 30 cm off the floor. Two of the arms were enclosed and two arms had no walls (open arms). The illumination was 60 lux in both closed and open arms. Rats were placed individually in the center of the maze facing one of the open arms. Behavior was assessed for 5 minutes by a trained observer (unaware of treatment assignment) via a video

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monitor. The time in open arms was recorded (in seconds) and data are expressed as percent time in open arms. Entry into an open or a closed arm was defined as placement of all four paws in the open or closed arm. The maze was cleaned with 1.0 % glacial acetic acid between animals to prevent olfactory cues from influencing the behavior of subsequently tested animals. DMP904 (3.0, 10, 18, or 30 mg/kg) or alprazolam (1.0, 3.0, or 10 mg/kg) was administered acutely PO one hour prior to behavioral testing.

Defensive Withdrawal

The defensive withdrawal procedure was used as described by McElroy et al. (2002). Briefly, the testing apparatus consisted of an opaque plexiglass open field (106 cm length x 92 cm width x 50 cm height), containing a cylindrical galvanized chamber (14 cm length, 10 cm diameter) that was positioned lengthwise against one wall, with the open end 40 cm from the corner. The open field was illuminated by a 60-W incandescent bulb and illumination was titrated by a powerstat transformer to a 23-lux reading at the entrance to the cylinder. Rats were habituated to handling by gently stroking their dorsal surface for approximately one minute daily for 5-6 consecutive days before testing. To initiate testing, each rat was placed within the cylinder that was then secured to the floor. Behavior was assessed for 15 minutes by a trained observer (unaware of treatment assignment) via a video monitor in an adjacent room. The latency to exit the chamber, defined by the placement of all four paws into the open field was recorded (in seconds). The plexiglass chamber and the cylinder were cleaned with 1.0 % glacial acetic acid between animals to prevent olfactory cues from influencing the behavior of subsequently tested animals. Two studies of acute effects of DMP904 were performed. In the first study, DMP904 (0.1, 0.3, 1.0, 3.0, 10, or 30 mg/kg) or alprazolam (0.3, 1.0, or 3.0) was administered acutely PO

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one hour prior to behavioral testing. Animals treated with DMP904 or vehicle were then decapitated and tissues collected for subsequent determination of drug and corticosterone concentrations in plasma, and estimated occupancy of central CRF_1 receptors. In the second study, effects of acute and chronic administration of DMP904 were compared directly. DMP904 (0.3, 1.0, or 3.0 mg/kg) or vehicle were administered PO once a day for 14 days. Animals treated chronically with DMP904 were tested on day 14 one hour following administration of the last dose of DMP904. Animals chronically treated with vehicle were given a dose of DMP904 acutely (0.3, 1.0, or 3.0 mg/kg) on day 14, one hour before testing.

Ex Vivo Binding and Quantitative Autoradiography

The procedure described by Li et al. (2003) was used. Briefly, following testing in the defensive withdrawal procedure (75 minutes following oral administration of DMP904), animals were decapitated and brain tissue was excised, frozen, and sectioned. Slide-mounted brain sections were pre-incubated for 2 min at room temperature (22°C) in an assay solution containing: 50 mM HEPES, 10 mM $MgCl_2$, 2 mM EGTA, 100 KIU/ml aprotinin, 0.1 M bacitracin, and 0.1% ovalbumin (pH 7.2). Sections were then incubated for 40 min in the same assay solution containing 0.15 nM [^{125}I-Tyr^0]-sauvagine (specific activity, 2200 Ci/mmol, PerkinElmer Life Sciences, Boston, Mass.) at room temperature. Non-specific binding was defined by incubation of adjacent sections under identical conditions in the presence of 1 μM DMP696, a small molecule CRF_1 antagonist (He et al., 2000). The sections were exposed to phosphorescent imaging screens (PerkinElmer Life Sciences, Boston, Mass.) and processed with Cyclone Storage Phosphor Imaging System (PerkinElmer Life Sciences, Boston, Mass.). Specific binding was calculated as follows: percent specific binding = (specific binding in vehicle-treated

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tissues – specific binding in drug-treated tissues) / specific binding in vehicle-treated tissues x 100. Percent CRF_1 receptor occupancy was estimated by subtracting the value obtained for specific binding from 100 for each animal, then averaging across animals for each dose of DMP904.

Plasma DMP904 Determination

The procedure described by Li et al. (2003) was used to determine systemic DMP904 exposure levels immediately after behavioral testing (75 min after oral administration). DMP904 concentration in the plasma was measured using a liquid chromatography tandem mass spectrometric method (LC/MS/MS). Briefly, 0.1 ml of plasma, 50 µl of 200 nM internal standard solution, and 0.1 ml of 0.1 M Na_2CO_3 were mixed followed by the addition of 1.0 ml of 1:1 MTBE:EtOAc. Samples were vortexed, centrifuged, and the organic layer was transferred and evaporated to dryness under nitrogen at 60°C. Residues were reconstituted with 0.1 ml of $H_2O/CH_3CN/HCOOH$: 50/50/0.1 (v/v/v). HPLC separation was achieved using an acetonitrile (0.1% formic acid) / water (0.1% formic acid) gradient on a Zorbax, SB-C18 column (2 x 50 mm, 5µm), at a flow rate of 200 µl/min with an analysis time of 5 min. Detection was performed in positive, MRM mode using a Quattro Ultima with an EI source as the LC/MS/MS interface. Levels of DMP904 in plasma are expressed as plasma free drug concentration, calculated by multiplying total plasma concentration by unbound fraction (0.64%). Plasma protein binding of DMP904 in rats was determined in vitro by equilibrium dialysis using the Dianorm (Munich, Germany) dialysis system. Plasma was spiked with DMP904 and equilibrated against isotonic phosphate buffer for 3 hours at 37°C. Following the incubation period, plasma and buffer samples were analyzed using LC/MS/MS as described above.

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DMP904 unbound fraction was calculated based on the ratio between DMP904 buffer concentration and the plasma concentration.

Plasma Corticosterone Determination

A second aliquot of plasma was used to determine the levels of corticosterone in plasma (15 minutes after initial exposure to the defensive withdrawal apparatus and 75 minutes after drug administration). These data were compared with baseline corticosterone values obtained from rats not exposed to the defensive withdrawal procedure, but otherwise housed and handled in an identical manner. Trunk blood was collected in chilled tubes containing EDTA and allowed to clot. Plasma was separated and stored at -80^{O}C for subsequent analysis of corticosterone content by radioimmunoassay (ICN Biomedicals, Costa Mesa, Calif.). The intra-assay variability was 4.4%, and the detection limit was 25 ng/ml.

Spontaneous Locomotor Activity

The testing apparatus consisted of plexiglass chambers (42 x 42 x 30 cm) equipped with Digiscan activity monitors (Omnitech Electronics, Columbus, Ohio) that detect interruptions of 16 photobeams spaced 2.5 cm apart and 2.5 cm above the floor. DMP904 (0.3, 3.0, or 30 mg/kg) or alprazolam (0.3, 1.0, 3.0, 10, or 30 mg/kg) was administered PO, one hour prior to behavioral testing. Horizontal activity was recorded in 5-minute bins for a total of 15 minutes (corresponding to the 15-minute exposure to the defensive withdrawal test) and expressed as total distance traveled (cm).

Motor Coordination

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The degree of motor coordination or balance was determined using a standard accelerating rotorod treadmill (Ugo Basile, Comerio-Varese, Italy) that was 6 cm in diameter and 24 cm above the base. The speed was increased gradually from 2 rpm to a maximum speed of 20 rpm. The time each animal remained on the rotating rod was automatically recorded, up to a maximum of 5 min. Three acclimation trials were conducted for each animal prior to administration of drugs. The time on rotorod from the third trial was used to counterbalance animals for subsequent drug testing. DMP904 (0.3, 3.0, or 30 mg/kg) or alprazolam (1.0, 3.0, or 10 mg/kg) was administered PO, one hour prior to behavioral testing.

Chlordiazepoxide Discrimination

Twelve model E10-10 Coulbourn operant chambers (28 x 26 x 31 cm) were housed in lightproof, sound-attenuated, and fan-ventilated chambers. Each operant chamber was equipped with two non-retractable levers, requiring a downward force equivalent to 15 g (0.15 N), that were mounted 3 cm from the side wall, 3 cm above the metal grid floor, and 5 cm from a centrally placed food tray that delivered one 45-mg food pellet (Dustless Precision Pellets, Bio-Serv, Frenchtown, N.J.). The experimental chambers were connected to a Micro PDP11/73 computer using a LAB LINC interface. A SKED-11 operating system (State System, Kalamazoo, Mich.) was used to record and control behavior.

After habituation to the operant chamber, rats were trained to alternate daily between response levers on a Fixed Ratio 1 (FR 1) schedule of food reinforcement. Once lever pressing was well established, the reinforcement contingency was increased incrementally to an FR 10 schedule of food reinforcement, while maintaining the lever alternation. Rats were then trained to discriminate between drug (5.0 mg/kg CDP, IP) and vehicle (0.25% methocel) administered

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30 minutes prior to the start of the session. Ten consecutive responses on the lever designated correct by the injection administered 30 minutes before start of the session resulted in food delivery. Responses on the incorrect lever did not result in pellet delivery. Following an injection of CDP, responding on the right lever was reinforced for half the rats and responding on the left lever was reinforced for the other rats. In each 2-week period, there were 5 drug days and 5 vehicle days, with the constraint that there not be more than 3 consecutive drug or vehicle days.

Drug test sessions were conducted once a week with training sessions scheduled on intervening days. The criterion for testing was achieved if no more than three incorrect responses occurred prior to the first reinforcer for at least 9 out of 10 consecutive sessions. During test sessions, the lever on which the rat first made 10 consecutive responses resulting in delivery of the first food pellet was designated the “selected “ lever and subsequent pellet delivery was made contingent on responding on this selected lever on an FR10 schedule. The selected lever, the number of responses prior to the first food pellet delivery, and the total number of responses in the session were recorded for each rat. The duration of test sessions was 10 minutes. CDP (0.5, 2.0, or 5.0 mg/kg, IP) was administered 30 minutes prior to session. DMP904 (0.1, 1.0, 10, or 30 mg/kg, PO) or alprazolam (0.1, 1.0, or 3.0 mg/kg, PO) was administered one hour prior to session.

Drugs

Alprazolam and CDP hydrochloride were purchased from Sigma (St. Louis, Mo.). DMP904 was prepared by BMS chemist Paul J. Gilligan. Compounds were prepared as suspensions in an aqueous vehicle of 0.25% methocel (methyl cellulose, Type A15c, Dow Chemicals). Stock

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suspensions were bead-milled overnight to ensure even suspension using three layers of 4-mm glass beads. Dilutions were made using the 0.25% methocel vehicle. DMP904 and alprazolam were administered orally by gavage (PO) 60 minutes before behavioral testing in a volume of 2 ml/kg body weight. For the chronic defensive withdrawal study, DMP904 was administered PO once a day for 14 days. In the discrimination study, CDP was administered IP 30 minutes before behavioral testing in a volume of 1 ml/kg. Doses of all drugs were calculated and are expressed in terms of the free base weight.

Data Analysis

Results for percent time in open arms, exit latency, percent estimated CRF_1 receptor occupancy, plasma corticosterone concentration, distance traveled in the locomotor activity assay, and time on rotorod are expressed as the mean ± SEM. The percent time in open arms, percent estimated CRF_1 receptor occupancy, plasma corticosterone concentration, and distance traveled in the locomotor activity assay were subjected to analysis of variance (ANOVA) followed by individual mean comparisons using Fisher's Least Significant Difference Test (Kirk, 1968) or Dunnett's t test (receptor binding) where appropriate. The significance level was set at $p<0.05$. The exit latency and time on rotorod data were subjected to the Kruskal-Wallis test, followed by individual comparisons using the Kolmogorov-Smirnov test. The significance level was set at $p<0.05$. Results for drug discrimination are expressed as percent of animals selecting the CDP-appropriate lever.

Levels of DMP904 in plasma are expressed as plasma free drug concentration, calculated by multiplying the total plasma concentration by the plasma free fraction (0.64%). The percent specific binding (i.e., percent inhibition of [^{125}I] sauvagine binding) was plotted as a function of

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plasma free concentration. The data were fitted using an inhibitory E_{max} model using Winnonlin (Pharsight Corporation, Mountain View, Calif.) according to the following equation: $E = E_{max} * (1-(C/(C+IC_{50})))$, where E is the percent inhibition of [^{125}I] sauvagine binding, E_{max} is the maximum binding of [^{125}I] sauvagine binding, C is the plasma free concentration of DMP904 in plasma, and the IC_{50} is the plasma free concentration that results in 50% inhibition of [^{125}I] sauvagine binding. In effect, this estimated *in vivo* IC_{50} is the plasma free concentration that results in 50% estimated CRF_1 receptor occupancy. Correlation coefficients were calculated for exit latency and plasma corticosterone levels when plotted as a function of percent estimated CRF_1 receptor occupancy.

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RESULTS

Elevated-Plus Maze

Vehicle-treated animals spent less than 10% of the test time in the open arms of the elevated-plus maze (Figure 2, left panel). The mean percent time in open arms were 5.2 ± 1.4 and 9.4 ± 3 in the DMP904 and alprazolam studies, respectively.

Pretreatment with DMP904 increased percent time in open arms [$F(4,20)=2.85$, $p=0.05$], with the lowest effective dose of 10 mg/kg ($p=0.04$; Figure 2, left panel). The two higher doses of DMP904 (18 and 30 mg/kg) also significantly increased percent time in open arms ($p=0.01$ and $p=0.02$, respectively). Alprazolam also increased percent time spent in open arms [$F(3,35)=8.78$, $p=0.0002$] with the lowest effective dose of 10 mg/kg ($p<0.0001$; Figure 2, left panel). The maximal effect of alprazolam (39% of time spent in the open arms at 10 mg/kg) was higher ($p=0.10$) than the maximal effect of DMP904 (19% of time spent in the open arms at 18 mg/kg).

Defensive Withdrawal

Vehicle-treated animals showed long latencies to exit the dark chamber and explore the open field (Figure 2, right panel). The mean exit latencies were 789 ± 77 and 797 ± 103 s in the DMP904 and alprazolam studies, respectively [88 and 89% of the total test duration (900 s) in the DMP904 and alprazolam studies, respectively].

Pretreatment with DMP904 decreased exit latency [$H(6)=27.27$, $p=0.0001$], with the lowest effective dose of 1.0 mg/kg decreasing exit latency by 72% relative to vehicle-treated animals (Figure 2). A lower dose of DMP904 (0.3 mg/kg) decreased exit latency by 33%

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(p=0.27). Higher doses of DMP904 (3.0, 10, and 30 mg/kg) decreased exit latency by 69, 86, and 84%, respectively. Alprazolam similarly decreased exit latency [H(3)=18.97, p=0.0003] with the lowest effective dose of 1.0 mg/kg reducing exit latency by 81% relative to vehicle-treated animals (Figure 2). The highest dose of alprazolam tested (3.0 mg/kg) produced a similar reduction in exit latency (86%).

In the chronic study, the vehicle-treated animals from the acute and chronic arms did not differ in exit latency (p>0.05) and thus, those two groups were combined. When administered chronically for 14 days or acutely, DMP904 decreased exit latency [H(6)=24.75, p=0.0004; Figure 3]. The lowest effective dose following chronic treatment with DMP904 was 0.3 mg/kg which reduced exit latency by 56% relative to vehicle-treated animals. A higher dose of DMP904 (1.0 mg/kg) administered chronically similarly decreased exit latency (55% relative to vehicle-treated controls). Thus, the same doses of DMP904, whether administered acutely or chronically, produced similar decreases in exit latency (p>0.05). This study also confirmed that acutely administered DMP904 is effective in reducing exit latency, with the lowest effective dose of 0.3 mg/kg reducing exit latency by 63% and a higher dose (1.0 mg/kg) reducing latency by 55% relative to vehicle-treated controls.

Estimation of *Ex Vivo* CRF_1 Receptor Occupancy and Plasma DMP904 Concentration

The receptor occupancy of DMP904 in the rat brain from animals tested in the defensive withdrawal test was estimated using *ex vivo* radioligand binding and quantitative autoradiography (Figure 4, upper left panel). Doses of 0.1 and 0.3 mg/kg of DMP904 resulted in 32 and 48% estimated CRF_1 receptor occupancy, respectively, in the rat fronto-parietal cortex 75 minutes after oral administration. The lowest effective dose of DMP904 (1.0 mg/kg) in the

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defensive withdrawal test produced 66% estimated CRF_1 receptor occupancy. Higher doses of DMP904 (3.0, 10, and 30 mg/kg), all effective in reducing exit latency, resulted in 75, 78, and 80% estimated CRF_1 receptor occupancy, respectively [$F(6,35)=15.48$, $p<0.0001$]. Dose-dependent displacement of [^{125}I] sauvagine binding by DMP904 in the cortex is shown in Figure 5.

When administered acutely PO to animals tested in the defensive withdrawal test, plasma free concentration of DMP904 increased dose-dependently [$F(5,37)=547$, $p<0.0001$; Figure 4, upper right panel). At doses ineffective in reducing exit latency (0.1 and 0.3 mg/kg), the mean free plasma concentration of DMP904 was 0.02 nM. The free plasma concentration at the lowest effective dose of DMP904 (1.0 mg/kg) was 1 nM, a concentration comparable to the IC_{50} value obtained from rat brain homogenate (1.16 nM). Compared with the 1.0 mg/kg dose of DMP904, higher doses (3.0, 10, and 30 mg/kg), all effective in reducing exit latency in the defensive withdrawal test, produced 4-, 25-, and 102-fold higher concentrations of DMP904, respectively.

The relationship between estimated CRF_1 receptor occupancy and plasma free concentration of DMP904 is shown in the bottom panel of Figure 4. The data were fitted using an inhibitory E_{max} model. The analysis of the data yielded an in vivo IC_{50} value of 1.74 nM, which is similar to the in vitro IC_{50} value of 1.16 nM.

When mean exit latency produced by different doses of DMP904 is plotted as a function of percent estimated CRF_1 receptor occupancy (Figure 6), the correlation coefficient obtained is 0.96 ($p=0.0005$), indicating that the efficacy of DMP904 in reducing exit latency is highly dependent on CRF_1 receptor occupancy.

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Plasma Corticosterone Concentration

Exposure of vehicle-treated rats to the defensive withdrawal apparatus produced an increase in plasma corticosterone concentration compared with control animals not exposed to the novel environment (home-cage controls). The mean plasma corticosterone concentration in control animals was 33 ± 20 ng/ml. Fifteen-minute exposure to the mildly stressful environment of the defensive withdrawal test produced a 10-fold increase in plasma corticosterone concentration (mean 346 ± 60 ng/ml).

DMP904 dose-dependently reversed the stress-induced increase in plasma corticosterone levels [$F(7,57)=9.1$, $p<0.0001$] in animals exposed to the defensive withdrawal test (Figure 7; left panel). However, the lowest effective anxiolytic-like dose of DMP904 in the initial dose-response study (1.0 mg/kg; Figure 2, left panel) did not significantly reduce the stress-induced increase in plasma corticosterone level. The 3.0 mg/kg dose was the lowest dose that significantly reduced plasma corticosterone levels (57% reduction relative to vehicle-treated animals). The two higher doses of DMP904, 10 and 30 mg/kg, further decreased plasma corticosterone levels by 93 and 92%, respectively, relative to vehicle-treated animals. These two doses (10 and 30 mg/kg) also resulted in levels of plasma corticosterone that did not differ from non-stressed, home-cage animals ($p>0.05$). The correlation coefficient obtained when plasma corticosterone levels following DMP904 administration are plotted as a function of percent estimated CRF_1 receptor occupancy (Figure 7, right panel) is 0.76 ($p=0.04$), indicating that the efficacy of DMP904 in reversing the stress-induced increases in corticosterone levels is highly dependent on CRF_1 receptor occupancy.

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Spontaneous Locomotor Activity

DMP904 did not alter spontaneous locomotor activity recorded 60-75 minutes after acute oral administration (corresponding to the 15-min test duration in the defensive withdrawal test, one hour after drug administration) at doses up to and including 30 mg/kg [$F(3,28)=0.33$, $p=0.8$; Figure 8, left panel]. In contrast, in the same observation period, alprazolam produced a dose-dependent decrease in spontaneous locomotor activity [$F(5,42)=20.93$, $p<0.0001$; Figure 8, left panel]. The lowest effective dose (3.0 mg/kg) produced a 45% decrease in locomotor activity relative to vehicle-treated animals. Higher doses of alprazolam, 10 and 30 mg/kg, further decreased locomotor activity by 62 and 75%, respectively, relative to vehicle-treated animals.

Motor Coordination

DMP904 did not alter rotorod performance after acute oral administration 60 minutes prior to testing at doses up to and including 30 mg/kg [$H(3)=2.87$, $p=0.41$; Figure 8, right panel]. In contrast, alprazolam produced a dose-dependent reduction in time on rotorod [$H(3)=8.39$, $p=0.04$], with the lowest effective dose of 10 mg/kg producing a 54% decrease in time on rotorod relative to vehicle-treated animals (Figure 8, right panel).

Chlordiazepoxide Discrimination

Discrimination acquisition required 25 ± 2 training sessions (including the 10 criterion sessions) to meet the performance criterion of no more than three incorrect responses prior to the first reinforcer for at least 9 out of 10 consecutive sessions. Following administration of the training dose of CDP (5.0 mg/kg), all animals responded on the CDP-appropriate lever (Figure 10, upper panel). A 10-fold lower dose of CDP (0.5 mg/kg) occasioned no CDP-lever

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responding and an intermediate dose (2.0 mg/kg) occasioned CDP-lever responding in 64% of the animals. The ED_{50} value for CDP was 1.7 mg/kg. All three doses of CDP significantly increased response rates relative to vehicle control rates (Figure 10, lower panel). CDP doses of 0.5, 2.0, and 5.0 mg/kg increased response rates to 120 ± 4 [t(10)=5.2], 127 ± 9 [t(10)=3.3], and 143 ± 11% [t(10)=4.5] of control, respectively (all p values < 0.05).

DMP904 did not occasion CDP-appropriate responding up to and including the dose of 30 mg/kg (Figure 10, upper panel). The percent of animals selecting the CDP lever after 0.1, 1.0, 10, and 30 mg/kg of DMP904 was 0, 9, 18, and 0%, respectively. The doses of 0.1, 1.0, and 30 mg/kg of DMP904 did not significantly alter rates of responding (Figure 10, lower panel). Mean response rates, expressed as percent of control, for 0.1, 1.0, and 30 mg/kg of DMP904 were 103 ± 2.8, 105 ± 3.3%, and 108 ± 2.1%, respectively (p > 0.05 for all doses). The dose of 10 mg/kg of DMP904 tested resulted in a decrease in response rates to 88 ± 4.7% of control [t(10)=2.4, p=0.04].

In contrast to DMP904, alprazolam resulted in a dose-dependent increase in the number of animals selecting the CDP lever, with the doses of 1.0 and 3.0 mg/kg resulting in 57 and 80% of animals selecting the CDP lever, respectively (Figure 10, upper panel). The ED_{50} value for alprazolam was 0.89 mg/kg. Alprazolam did not significantly alter rates of responding at the doses tested (Figure 10, lower panel). Mean response rates, expressed as percent of control, were 90 ± 8, 113 ± 20, and 141 ± 19% for doses 0.1, 1.0, and 3.0 mg/kg, respectively (p > 0.05 for all doses).

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DISCUSSION

The present study demonstrates that a high-affinity, selective CRF_1 antagonist DMP904 (Gilligan et al., 2000; Zhang et al., 2003) increased time in open arms in an elevated-plus maze and decreased latency to exit the dark chamber and explore the open field in the defensive withdrawal model in rats, indices of anxiolytic-like activity in rodents. In the present study, DMP904 was found to be equipotent with the benzodiazepine alprazolam, which also significantly increased time in open arms at 10 mg/kg (PO) and significantly reduced exit latency at 1.0 mg/kg (PO) under identical dosing conditions. These results are consistent with other reports on anxiolytic-like effects of CRF_1 antagonists (McElroy et al., 2002; Lundkvist et al., 1996; Griebel et al., 1998b, 2002). A difference between alprazolam and DMP904 emerged in the elevated-plus maze test, in which the maximal effects of alprazolam were higher than the maximal effects of DMP904 in increasing the time in open arms of the maze. The reason for this difference is not clear at present. In addition, the potency difference between the elevated-plus maze and the defensive withdrawal tests appears to be approximately 10-fold. One possible explanation for this difference may be different levels of stress engendered by the two tests. However, as the plasma corticosterone levels were not measured in the elevated-plus maze test, the present data do not allow testing of that hypothesis.

The potency of DMP904 to produce anxiolytic-like effects in the defensive withdrawal model in rats was not altered when the drug was administered PO once a day for 14 days. These data are in agreement with an earlier study using DMP696, which showed that 10 mg/kg of DMP696 (PO) was similarly effective and potent in reducing exit latency in this model after acute or chronic administration (Lelas et al., 2003). These data suggest that tolerance may not

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develop to the anxiolytic-like effects of CRF_1 antagonists, as measured by the defensive withdrawal model of anxiety. Although tolerance to the anxiolytic-like effects of benzodiazepines has been reported, it is generally accepted that the tolerance to the sedative/ataxic effects (e.g., File, 1984; Rosenberg et al., 1991) is more pronounced than to the anxiolytic-like effects of benzodiazepines (Soderpalm et al., 1989). Our own unpublished data suggest that CDP is equally effective in reducing exit latency whether it is administered acutely or chronically (J.F. McElroy, unpublished observations). Although further testing is necessary in additional models of anxiety, the present data suggest that CRF_1 antagonists such as DMP904 and DMP696 may be similar to the benzodiazepines in that tolerance may not develop to their anxiolytic-like effects, an important characteristic of any potentially clinically useful anxiolytic given the chronic nature of treatment of anxiety disorders in humans.

In addition to reducing exit latency in the defensive withdrawal model, DMP904 reversed the stress-induced increase in plasma corticosterone levels in these same animals at doses 3-fold greater than the lowest anxiolytic-like dose. Similar data were obtained with DMP696 which reduced exit latency at a dose 3-fold lower than the lowest dose that significantly reversed the stress-induced increases in plasma corticosterone (McElroy et al., 2002). These data are in contrast to the effects of benzodiazepines which at higher doses exacerbate the effect of stress on plasma corticosterone, although at lower doses they do decrease stress-induced elevations in plasma corticosterone (McElroy et al., 1987, 2002). These latter data indicate that anxiolytic-like effects can be obtained despite increases in plasma corticosterone, at least in the case of benzodiazepines. In the case of CRF_1 antagonists, the difference between the lowest effective dose that produced anxiolytic-like effects and the lowest effective dose that reversed stress-induced increase in plasma corticosterone was only 3-fold, which is not sufficient to firmly

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conclude that anxiolytic-like effects of CRF_1 antagonists are independent of their effects on the HPA axis.

Anxiolytic-like effects of DMP904, as measured by the defensive withdrawal test, occurred at doses that resulted in greater than 1.0 nM plasma free levels of DMP904 and greater than 50% estimated occupancy of CRF_1 receptors in the fronto-parietal cortex (1.0 mg/kg or higher). However, doses reversing the stress-induced activation of the HPA axis required a 4-fold greater free level of plasma DMP904 and greater than 75% estimated occupancy of central CRF_1 receptors. Although these correlations do not establish a causal link between plasma free levels, receptor occupancy, and anxiolytic-like effects and effects on the HPA axis of DMP904, some preliminary conclusions can be drawn. First, 1.0 nM plasma free concentration (similar to the in vitro IC_{50} value) is necessary for greater than 50% CRF_1 receptor occupancy with DMP904, and for anxiolytic-like effects in the defensive withdrawal test in rats (1.0 mg/kg administered PO). This finding is consistent with studies with other CRF_1 antagonists in this (DMP696, Li et al., 2003; R121919, Heinrichs et al., 2002) and other models of anxiety (Kehne et al., 2000; Hikichi et al., 2000; Habib et al., 2000). Plasma free levels of 4.2 nM or higher do not produce a significantly greater level of estimated CRF_1 receptor occupancy or a significantly higher reduction in exit latency. Second, greater than 4.2 nM plasma free levels and greater than 75% estimated CRF_1 receptor occupancy appear to be necessary for reversal of stress-induced increases in plasma corticosterone. Finally, whereas higher doses of DMP904 (3.0, 10, and 30 mg/kg) do not produce a more robust anxiolytic-like effect compared with the lowest effective dose (1.0 mg/kg) in the defensive withdrawal model, these higher doses are more effective than the 1.0 mg/kg dose in reversing the stress-induced elevations in corticosterone. These data suggest that the effect of DMP904 on the HPA axis is more closely related to its concentration in

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plasma than its occupancy of central CRF_1 receptors. Although occupancy of CRF_1 receptors in the pituitary by DMP904 has not been estimated in the present study, data from other studies suggest that occupancy of these receptors is closely related to the occupancy of the CRF_1 receptors in the cortex (Y.-W. Li, personal communication). Additional studies are necessary to determine if these preliminary conclusions are supported by data obtained with other CRF_1 antagonists.

Finally, the effects of DMP904 and alprazolam on locomotor activity and motor coordination were compared using identical route of administration and pretreatment time as those employed in the anxiety models. Alprazolam both decreased locomotor activity and impaired rotorod performance at doses similar to anxiolytic-like doses (as estimated by elevated-plus maze) or only 3-fold (locomotor activity) and 10-fold (rotorod) higher than anxiolytic-like doses (as estimated by defensive withdrawal). In marked contrast, DMP904 had no effect on locomotor activity or rotorod performance at doses 3- to 30-fold higher than the lowest effective dose in the elevated-plus maze or defensive withdrawal models, respectively. These results are in agreement with previous studies with other CRF_1 antagonists (e.g., McElroy et al., 2002), and suggest that CRF_1 antagonists may result in fewer or less severe motoric side effects compared with benzodiazepines.

In addition, like DMP696 (Lelas et al., 2003) but in contrast to alprazolam, DMP904 did not substitute for the CDP discriminative stimulus up to and including doses 3- to 30-fold higher than the lowest effective anxiolytic-like dose in the two models of anxiety. Drug discrimination is frequently used to assess similarities between subjective effects of a novel drug and the training drug. Thus, the present data suggest that DMP904 may not share subjective effects with CDP at the doses studied. While self-administration studies are necessary to determine abuse

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potential of any new drug, the present data suggest that DMP904 may have lower abuse potential compared with benzodiazepines.

In summary, DMP904 produced anxiolytic-like effects and reversed stress-induced elevations in plasma corticosterone in the elevated-plus maze and defensive withdrawal models in rats. In the defensive withdrawal model, these effects occurred at doses that resulted in free levels of DMP904 in plasma similar to or 4-fold higher the in vitro IC_{50} value, respectively, and occupied greater than 50% of CRF_1 receptors in the fronto-parietal cortex. In addition, the effectiveness and potency of DMP904 to produce anxiolytic-like effects was not altered by chronic administration of DMP904, suggesting that tolerance may not develop to this effect of DMP904, as assessed in the defensive withdrawal model. Finally, these doses of DMP904 did not result in sedation or ataxia, nor did they share discriminative stimulus effects with CDP. Alprazolam similarly produced anxiolytic-like effects in the elevated-plus maze and defensive withdrawal model, but, unlike DMP904, also produced sedation and ataxia. Additional studies are needed on the effects of CRF_1 antagonists on memory, their potential for interaction with ethanol, and for development of dependence. While only clinical studies will show whether CRF_1 antagonists are superior to the currently available agents, preclinical studies showing equal potency in producing anxiolytic-like effects and lack of adverse effects suggest that CRF_1 antagonists may replace benzodiazepines as the treatment of choice for anxiety disorders.

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ACKNOWLEDGEMENTS

We thank Drs. Mark Lindner and Tanya Wallace for comments on the manuscript.

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LEGENDS FOR FIGURES

Figure 1. Chemical structure of DMP904.

Figure 2. Anxiolytic-like effects of DMP904 and alprazolam in the elevated-plus maze (left panel) and defensive withdrawal tests (right panel) in rats. Abscissae: dose (mg/kg). Left panel ordinate: mean (± SEM) percent time in open arms for n=5-10 animals per dose. Right panel ordinate: mean (± SEM) latency to exit the dark chamber (in seconds) for n=8 animals per dose. Maximum latency is 900 s. DMP904 and alprazolam were administered in 0.25% methocel PO, 60 minutes prior to testing. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 3. Anxiolytic-like effects of DMP904 following acute (left panel) and chronic (right panel) administration in the defensive withdrawal test in rats. In the acute study, DMP904 was administered in 0.25% methocel PO, 60 minutes prior to testing. In the chronic study, DMP904 was administered in 0.25% methocel PO for 14 days, with the testing on the last day (day 14) 60 minutes after administration of the last dose of DMP904. * $p<0.05$, ** $p<0.01$. For other details, see Figure 2.

Figure 4. CRF_1 receptor occupancy produced by DMP904 estimated through inhibition of [^{125}I-Tyr^0]-sauvagine binding (left panel) and plasma free levels (right panel) of DMP904 following oral administration in the defensive withdrawal model. Upper panel abscissae: dose (mg/kg). Upper left panel ordinate: mean (± SEM) percent CRF_1 receptor occupancy for n=6 animals per dose. Upper right panel ordinate: mean plasma free concentration of DMP904 for n=6 animals per dose. Animals were sacrificed immediately following testing in the defensive withdrawal

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test and CRF_1 receptor occupancy and plasma levels of DMP904 were estimated 75 minutes following PO administration (in 0.25% methocel). Bottom panel: Relationship between percent specific binding (i.e., percent inhibition of [^{125}I] sauvagine binding; ordinate) and DMP904 plasma free concentration (abscissa). The data were fitted using an inhibitory E_{max} model with a predicted in vivo IC_{50} of 1.74 nM.

Figure 5. Representative autoradiograms of coronal forebrain sections showing dose-dependent inhibition of [^{125}I]sauvagine binding by orally administered DMP904. LV, left ventricle; Ctx, cortex.

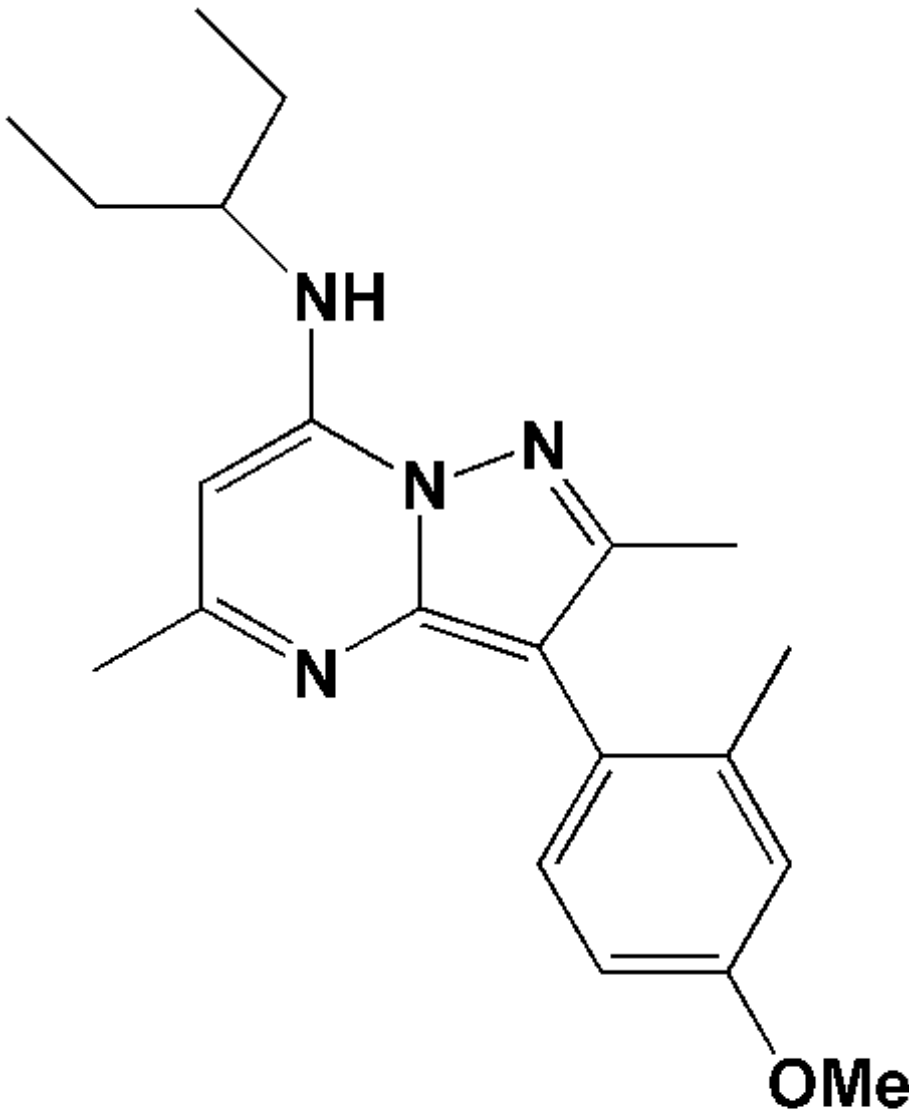
Figure 6. Relationship between estimated CRF_1 receptor occupancy by DMP904 and exit latency in the defensive withdrawal model. Abscissa: percent estimated CRF_1 receptor occupancy for n=6 per dose. Ordinate: latency to exit the dark chamber (in seconds) for n=8 animals per dose.

Figure 7. Effect of DMP696 on plasma corticosterone concentration in the defensive withdrawal test in rats (left panel) and relationship between plasma corticosterone concentration and estimated CRF_1 receptor occupancy (right panel) for DMP904. Left panel abscissa: dose (mg/kg), NSC = non-stressed controls. Right panel abscissa: percent estimated CRF_1 receptor occupancy. Ordinate: mean (± SEM) plasma corticosterone concentration (in ng/ml) for n=8 animals per dose. Animals were sacrificed immediately following testing in the defensive withdrawal test and plasma corticosterone levels were assessed 75 minutes following PO administration of DMP904 (in 0.25% methocel). * $p<0.05$, *** $p<0.001$ vs. vehicle-treated

animals exposed to the novel environment in the defensive withdrawal test.

Figure 8. Effect of DMP904 and alprazolam on spontaneous locomotor activity (left panel) and rotorod (right panel) in rats. Abscissae: dose (mg/kg). Left panel ordinate: mean (± SEM) distance traveled (in cm) for n=8 animals per dose. Right panel ordinate: mean (± SEM) time on rotorod (in seconds) for n=8 animals per dose. DMP904 and alprazolam were administered in 0.25% methocel PO, 60 minutes prior to behavioral testing. * $p<0.05$, *** $p<0.001$.

Figure 9. Discriminative stimulus and rate-decreasing effects of CDP, DMP904, and alprazolam in rats (n=5-12) discriminating between CDP and vehicle. Abscissae: dose (mg/kg). Upper panel ordinate: percent of rats selecting the CDP lever. Lowe panel ordinate: mean response rates (± SEM) expressed as percent control rates (vehicle training days). * $p<0.05$. The dashed line in the upper panel refers to 75% of animals selecting the drug lever (criterion for substitution). CDP was administered in 0.25% methocel IP, 30 minutes before session. DMP904 and alprazolam were administered in 0.25% methocel PO, 60 minutes prior to behavioral testing.



DMP 904

Figure 2

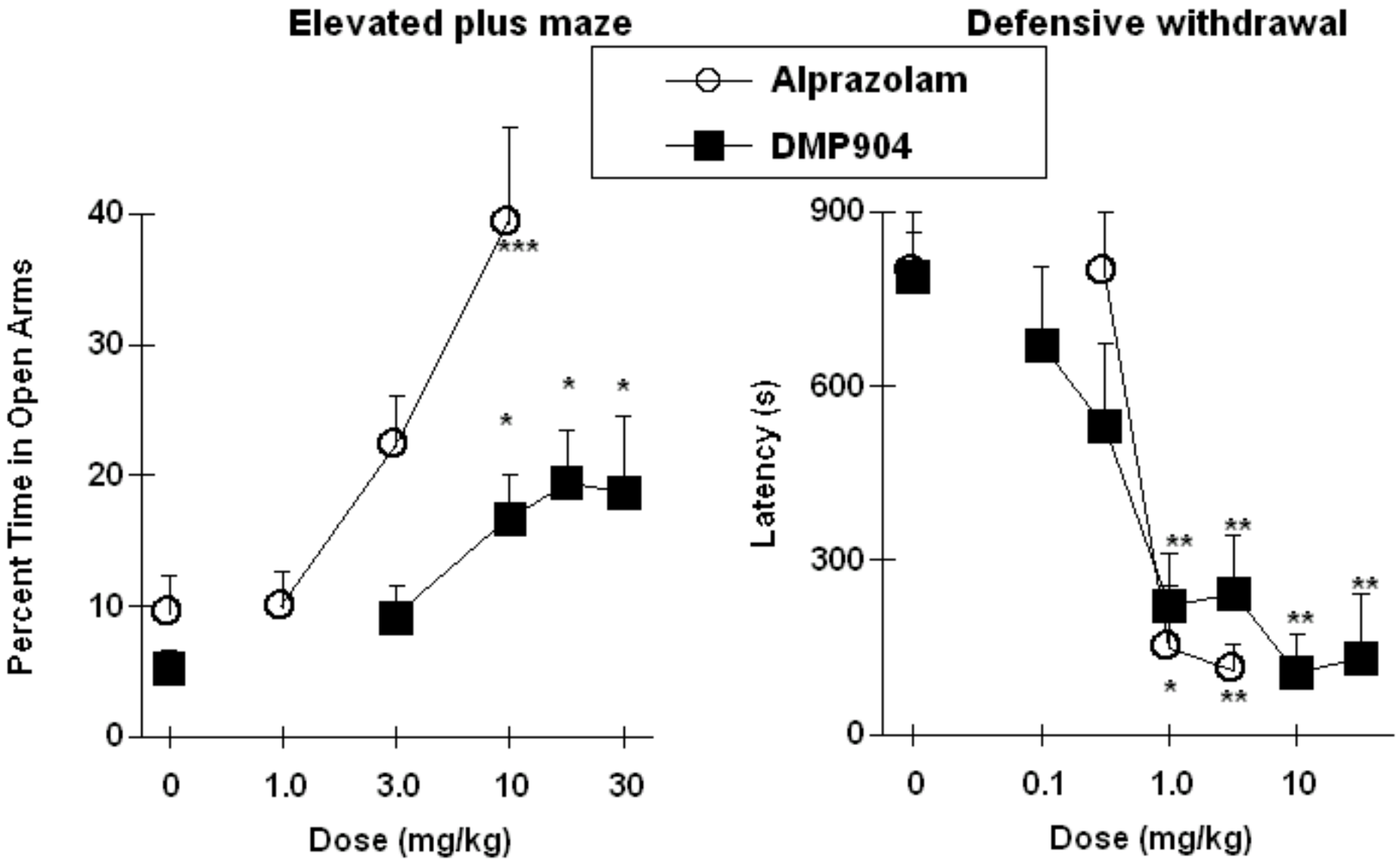


Figure 3

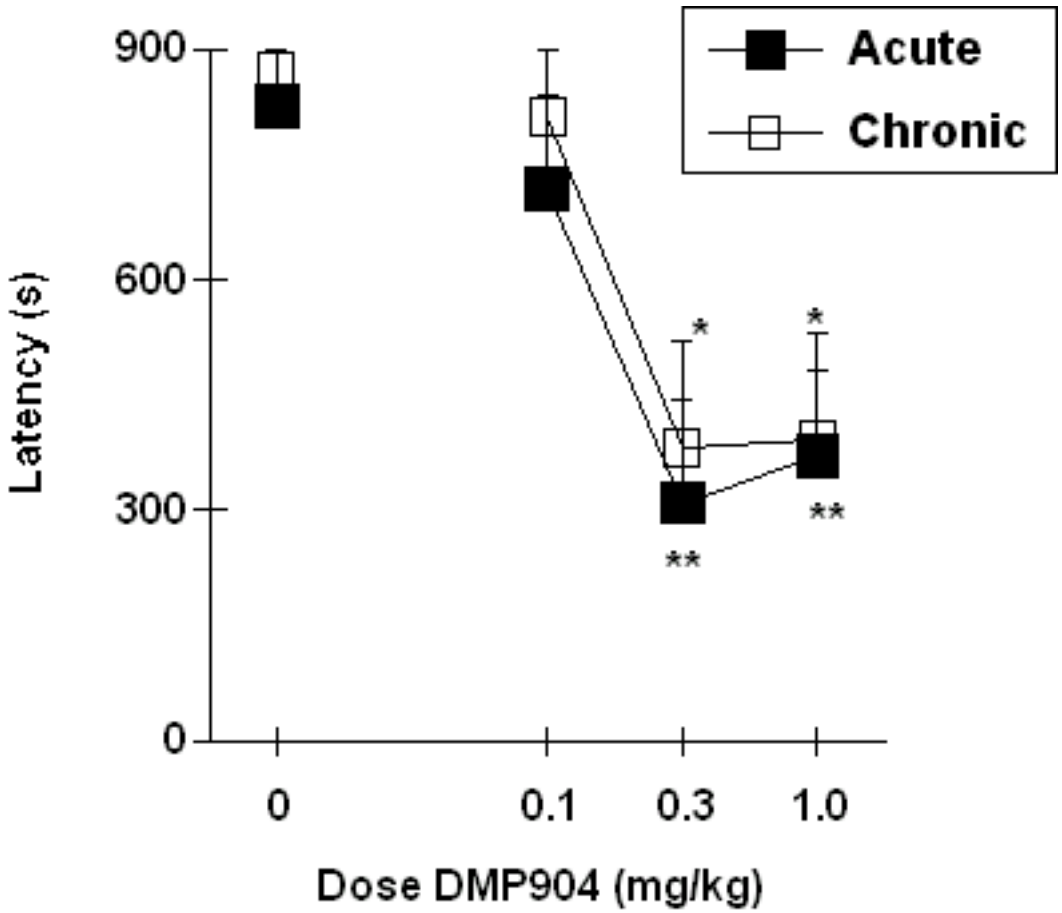
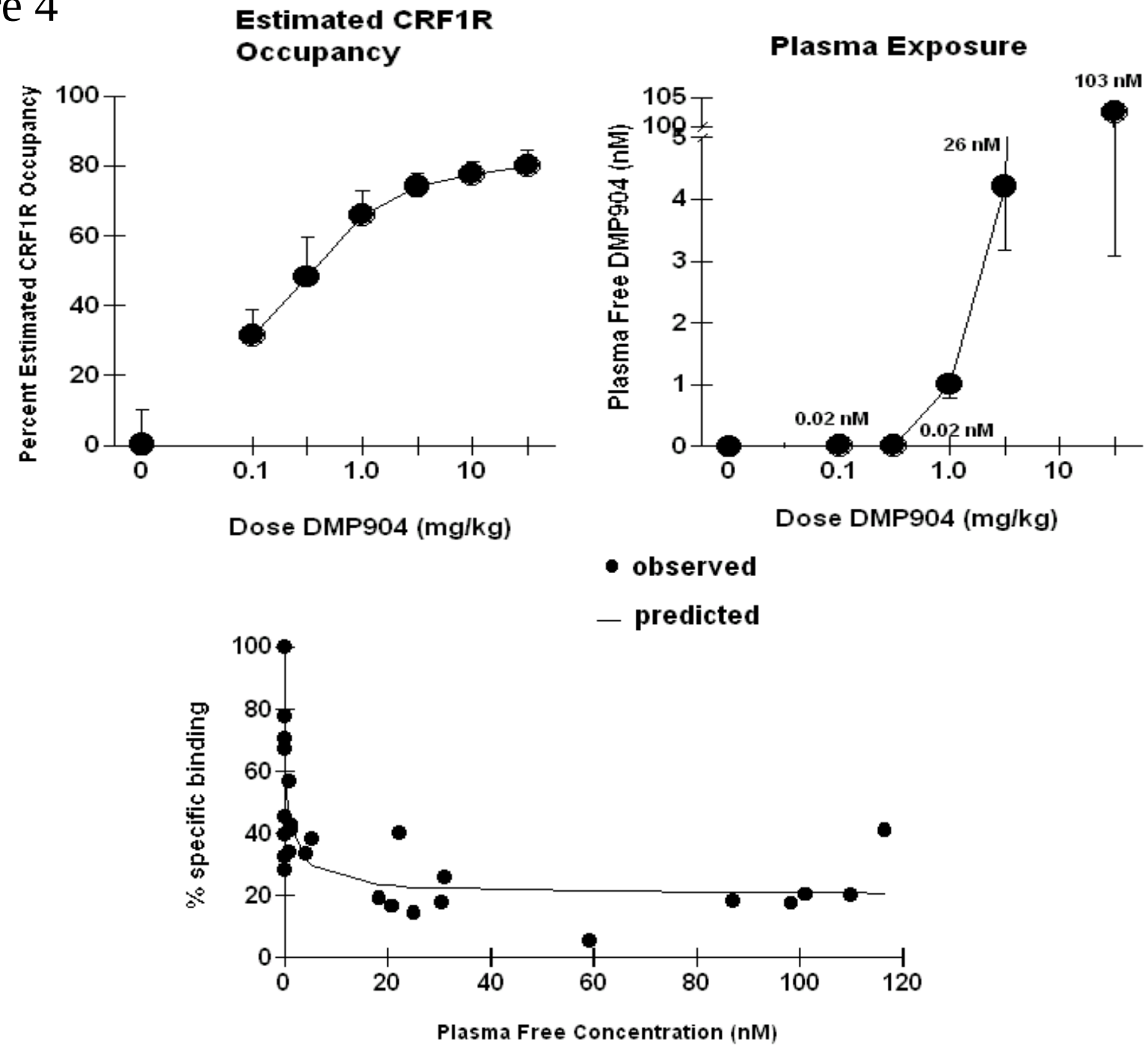


Figure 4



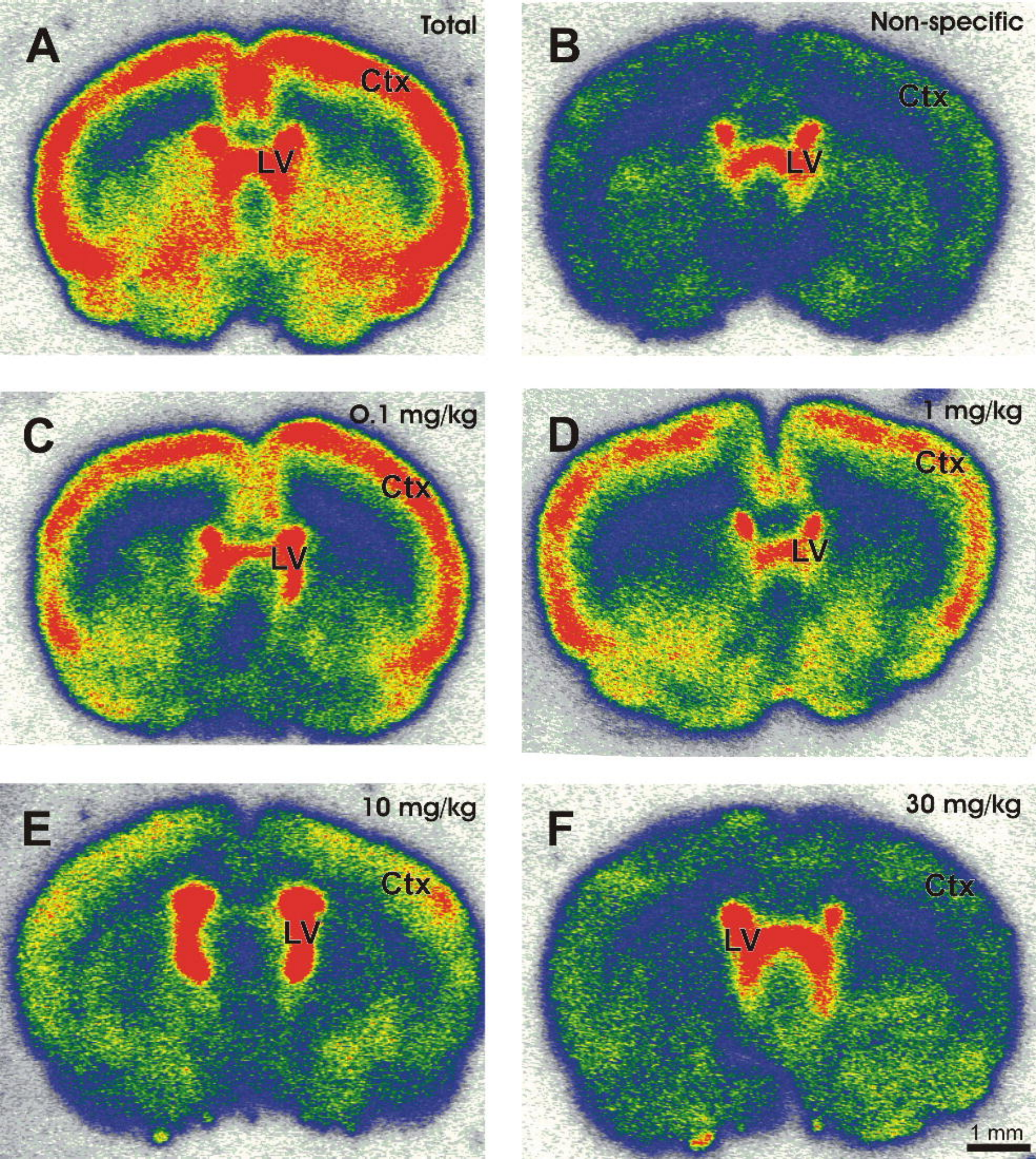

A
Total
Ctx
LV
B
Non-specific
Ctx
LV
C
0.1 mg/kg
Ctx
LV
D
1 mg/kg
Ctx
LV
E
10 mg/kg
Ctx
LV
F
30 mg/kg
Ctx
LV
1 mm

Figure 6

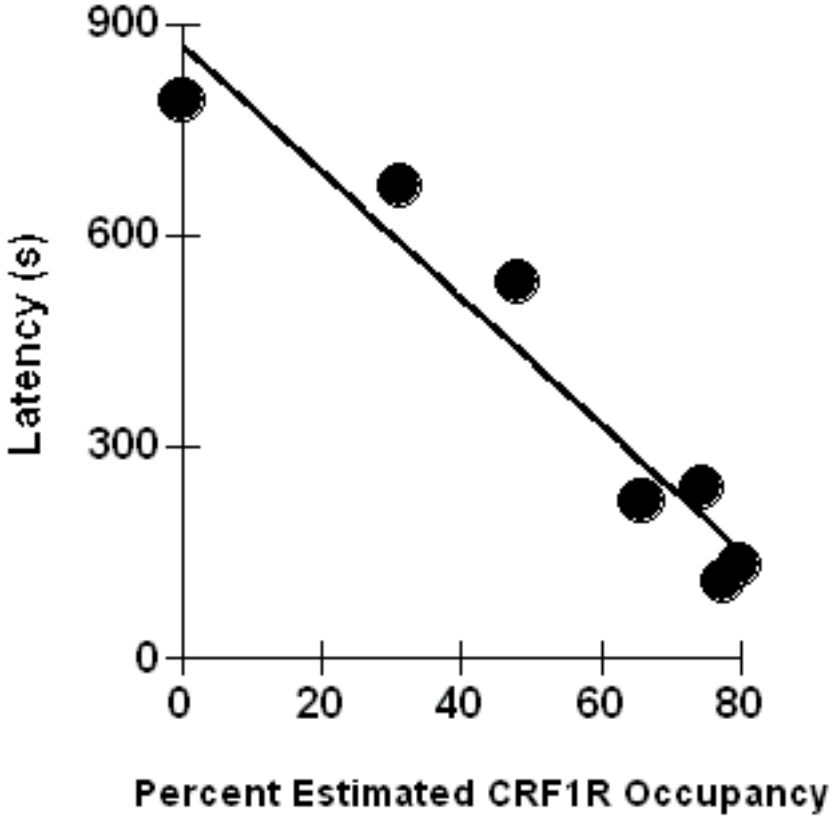


Figure 7

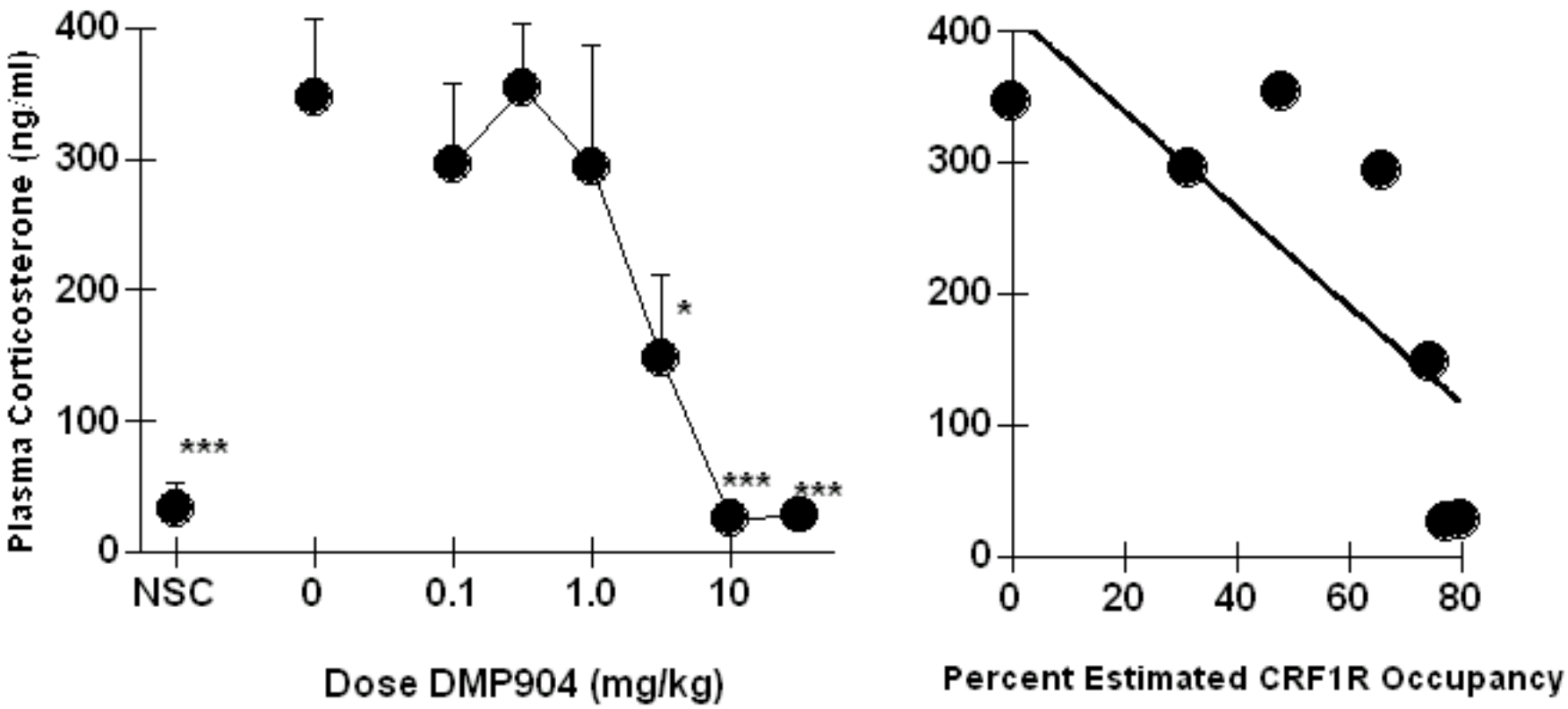
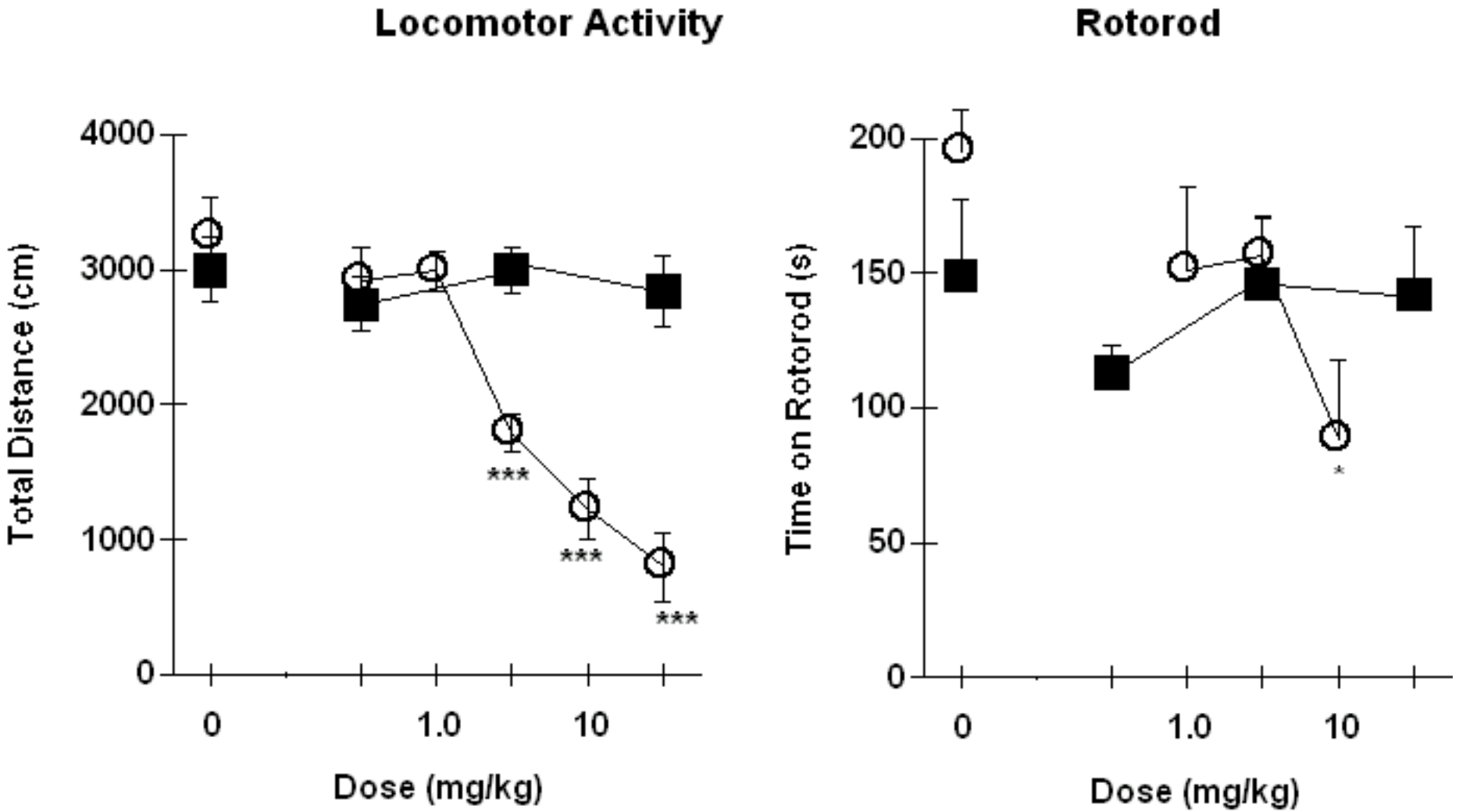


Figure 8



JPET Fast Forward. Published on January 23, 2004 as DOI: 10.1124/jpet.103.058784
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Figure 9