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Receptor Occupancy of Non-Peptide CRF_1 Antagonist DMP696: Correlation with Drug Exposure and Anxiolytic Efficacy

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Abbreviations:

CRF, corticotropin releasing factor**;**

oCRF, ovine corticotropin releasing factor**;**

CRF_1, corticotropin releasing factor type-1 receptor;

DMP696, 4 (1,3-dimethoxyprop-2-ylamine)-2,7-dimethyl-8-(2,4-dichlorophenyl)-pyrazolo[1,5-a]-1,3,5-triazine

A Recommended Section Assignment: Neuropharmacology

ABSTRACT

DMP696 is a highly selective and potent, non-peptide CRF_1 antagonist. In this study, we measured *in vivo* CRF_1 receptor occupancy of DMP696 by using *ex vivo* ligand binding and quantitative autoradiography, and explored the relationship of receptor occupancy with plasma and brain exposure, and behavioral efficacy. *In vitro* affinity (IC_{50}) of DMP696 to brain CRF_1 receptors measured using the brain section binding autoradiography in this study is similar to that assessed using homogenized cell membrane assays previously. The *ex vivo* binding assay was validated by demonstrating that potential underestimation of receptor occupancy with this procedure could be minimized by identifying an appropriate *in vitro* incubation time (40 minute) based upon the dissociation kinetics of DMP696. Orally-administrated DMP696 dose-dependently occupied CRF_1 receptors in the brain with ~60% occupancy at 3 mg/kg. In the defensive withdrawal test of anxiety, this dose of DMP696 produced approximately 50% reduction in the exit latency. The time-course of plasma and brain drug levels paralleled that of receptor occupancy with peak exposure at 90 min post dosing. The plasma free concentration of DMP696 corresponding to 50% CRF_1 receptor occupancy (*in vivo* IC_{50}=1.22 nM) was similar to the *in vitro* IC_{50} (~1.0 nM). Brain concentrations of DMP696 were over 150 fold higher than the plasma free levels. In conclusion, doses of DMP696 occupying over 50% brain CRF_1 receptors are consistent with doses producing anxiolytic efficacy in the defense withdrawal test of anxiety, and the IC_{50} value estimated *in vivo* based on plasma free drug concentrations is consistent with the *in vitro* IC_{50} value.

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Corticotropin releasing factor (CRF), a 41 amino acid peptide, plays a pivotal role in the behavioral, endocrine, immune and autonomic responses of the body to stress (Owens and Nemeroff, 1991). In addition to the hypothalamic paraventricular nucleus where it was originally identified, CRF is also widely distributed across brain regions (Chalmers et al., 1996, Heinrichs and De Souza, 1999, Gilligan et al., 2000a). The physiological functions of CRF are mediated via at least two G-protein coupled receptors, CRF_1 and CRF_2 (including splice variants $CRF_{2\alpha}$, $CRF_{2\beta}$, $CRF_{2\gamma}$), both of which are linked to adenylyl cyclase activation but have distinct brain distributions. CRF_1 receptors are widespread in the cortex, limbic system, cerebellum and pituitary, whereas CRF_2 receptors are dominant in subcortical areas including the lateral septum ($CRF_{2\alpha}$), ventromedial hypotholamus ($CRF_{2\alpha}$) and choroid plexus ($CRF_{2\beta}$) (De Souza, 1987, Chalmers et al., 1995, Primus et al., 1997, Rominger et al., 1998).

Increasing evidence suggests that the CRF system is involved in pathophysiology of anxiety disorders (Heinrichs and De Souza, 1999, Gilligan et al., 2000a). Intracerebroventricular administration of CRF induces stress behaviors, whereas application of the peptide antagonist, α-helical CRF, diminishes CRF-elicited as well as stress-elicited behavioral manifestations of anxiety (Korte et al, 1994). In transgenic animals, over-expression of CRF peptide produces increased levels of anxiety, whereas knockout of CRF_1 receptors decreases stress responses (Stenzl-Poore et al., 1996, Timpl et al., 1998). Clinically, patients diagnosed with anxiety-related disorders such as post-traumatic stress disorder (Bremner et al., 1997),

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obsessive compulsive disorder (Altemus et al., 1994) and anorexia nervosa (Kaye et al, 1987) exhibit increased levels of CRF in the cerebrospinal fluid (CSF).

Evidence from recent studies on non-peptide CRF_1 antagonists supports the contention that CRF_1 antagonists may have utility in the treatment of anxiety disorders (Gilligan et al., 2000a, Takahashi, 2001). For example, the prototype CRF_1 antagonist, CP-154,526, attenuates social isolation-evoked stress responses in rat pups (Kehne et al., 2000), whereas its analog antalarmin decreases behavioral responses caused by social stress in primates (Habib et al., 2000). Another non-peptide CRF_1 antagonist, R121919, is efficacious in several stress tests including elevated plus-maze, defensive withdrawal and defensive burying (Keck et al., 2001, Heinrichs et al, 2002). In addition, the same compound showed anxiolytic and antidepressant efficacy in a small, open-labeled clinical study (Zobel et al., 2000). Likewise, several other CRF_1 antagonists including CRA1000 and CRA1001 (Okuyama et al, 1999), DPC695 (Bakthavatchalam et al., 1998, Millan et al., 2001), DMP696 (McElroy et al., 2002) and DPC904 (Gilligan et al., 2000b, Takahashi, et al, 2001) have shown anxiolytic profiles in pre-clinical animal models. For peripherally-applied non-peptide CRF_1 antagonists to be effective, these compounds should have good plasma drug exposure, blood-brain penetration and brain CRF_1 receptor occupancy. Indeed, several recent studies show that CP-154,526 and R121919 effectively cross the blood-brain barrier and occupy central CRF_1 receptors (Arborelius et al., 2000, Keck et al., 2001, Keller et al., 2002, Heinrichs et al., 2002). In the study by Heinrichs et al. (2002) a dose-dependent relationship was demonstrated between behavioral efficacy and CRF_1 receptor occupancy by R121919. A dose of R121919 that achieves minimal efficacy also occupies ~50% CRF_1 receptors in the

brain. Thus, it is of importance to test if this occupancy-efficacy relationship is applicable to other non-peptide CRF_1 antagonists. In addition, it is important to further understand the relationship between plasma and brain drug exposure and receptor occupancy and behavioral efficacy of non-peptide CRF_1 antagonists.

DMP696, 4-(1,3-dimethoxyprop-2-ylamine)-2,7-dimethyl-8-(2,4-dichlorophenyl)-pyrazolo [1,5-a]-1,3,5-triazine (see Figure 1 for chemical structure), is a potent and selective CRF_1 receptor antagonist (IC_{50}: 2-5 nM) (He et al., 2000, Zhang et al., 2001). It blocks CRF-induced adenylate cyclase activity in rat cortical membranes and inhibits ACTH release from cultured pituitary cells (He et al., 2000). In a rat defensive withdrawal test of anxiety, acute oral administration of DMP696 (3 mg/kg) reduces the latency for a rat to exit from an isolated box and reverses the stress-induced increases in plasma corticosterone levels without any effect on locomotor activity or motor coordination (He et al., 2000, McElroy et al., 2002). In addition, at a higher concentration (30 mg/kg), DMP696 attenuates the enhanced stress response caused by maternal separation in rats (Maciag et al., 2002).

The present study was designated to investigate 1) *in vivo* receptor occupancy of DMP696, in relation to the behavioral efficacy, and 2) the relationship between receptor occupancy and plasma and brain exposure of DMP696. The receptor occupancy of DMP696 was measured using *ex vivo* binding autoradiography. The anxiolytic effects were determined using the defensive withdrawal test. Plasma and brain drug exposure was measured by a liquid chromatography tandem mass spectrometric method in the same animals in which receptor

occupancy was studied. Part of this study was previously presented in an abstract form (Hill et al., 2001).

Materials and Methods

Materials

[^{125}I]sauvagine and [^{125}I]ovine CRF (oCRF) were purchased from PerkinElmer Life Sciences (Boston, MA). Urocortin II was purchased from American Peptide Co. (Sunnyvale, CA) and α-helical CRF_{9-41} was purchased from Pennisula Labs (Bemont, CA). DMP696 and CP-154,526 were synthesized by the Chemical and Physical Sciences Department, and anti-sauvagine-30 was prepared by the Applied Biotechnology Group, in Bristol-Myers Squibb Company (Wilmington, DE). For *in vitro* application, non-peptide compounds were dissolved in dimethyl sulfoxide and diluted in assay buffers. For *in vivo* application, compounds were prepared as suspensions in an aqueous vehicle of 0.25% methocel (methyl cellulose, Type AL5c, Dow Chemicals). Stock suspensions were bead-milled overnight using three layers of 4mm glass beads. Compounds were administered orally by gavage (PO) in a volume of 2 ml/kg body weight. Doses of all drugs were calculated and are expressed in terms of the free base weight.

General procedures

Male Spraque Dawley rats (200-300 grams of body weight) were purchased from Charles River Laboratories (Wilmington, MA). The rats were doubled housed in shoebox cages (except those used in defensive withdrawal test) in a colony room maintained at constant temperature (21-22^{o}C) and humidity (50±10%). The room was illuminated 12 hours per day (lights on at 0600 hr). The rats had *ad libitum* access to food and water throughout the study. For *in vivo* studies, the rats were fasted overnight before oral administration of drugs, and all

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experiments were conducted between 0600 and 1300 hours. All experimental procedures were performed according to protocols approved by the Animal Care and Use Committee of the Bristol-Myers Squibb Company and the published guidelines in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

***In vitro* binding autoradiography**

Naive rats (without treatment) were sacrificed by decapitation, and the brain and pituitary were collected, embedded in M-1 embedding matrix (Thermo Shandon, Pittsburgh, PA) and frozen in ice-chilled 2-methylbutane (Alfa Aesar, Ward Hill, MA). The brain and pituitary tissues were cut into 20-μm sections on a Cryostat and sections were mounted on superfrost slides (VWR International, Wilmington, DE) and stored at –70^{o}C until use.

Slide-mounted brain sections were brought to 22-24^{o}C, dried and pre-incubated in an assay solution containing (mM): HEPES 50, $MgCl_2$ 10 ; EGTA 2, aprotinin 100 KIU/ml, bacitracin 0.1 M, ovalbumin, 0.1% (pH 7.2) for 30 min. Aprotinin, bacitracin and ovalbumin were purchased from Sigma-Aldrich (St. Louis, MO). The sections were then incubated in the same solution containing 0.15-0.20 nM [^{125}I]sauvagine or [^{125}I]oCRF for 2 hrs at 22-24^{o}C. The adjacent sections were incubated under the same conditions in the presence of 1 μM α-helical CRF for defining non-specific binding for both CRF_1 and CRF_2 receptors . Non-specific binding for CRF_1 receptors was defined by 1 μM DMP696 or CP-154,526 and non-specific binding for CRF_2 receptors defined by 20 nM anti-sauvagine-30 or urocortin II. Concentration-related displacement of DMP696 was assessed by including the compound at a concentration of 0.01 – 1000 nM in the incubation solution. After incubation, the sections

were rinsed in phosphate buffer saline with 0.01% Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 10 min and subsequently dried under a stream of cold air. The slides of sections were then placed in cassettes against iodine-sensitive storage phosphor imaging screens (PerkinElmer Life Sciences). for 12-16 hrs and the screens were then digitally scanned with a Cyclone storage phosphor imaging system (PerkinElmer Life Sciences). Captured storage phosphor images were analyzed with OptiQuant Acquisition and Analysis software (PerkinElmer Life Sciences).

***Ex vivo* binding autoradiography**

For dose-related studies, rats were orally administrated with DMP696 at various doses (0.3, 1.0, 3.0, 10 and 30 mg/kg) or CP-154,526 (1.0, 3.0, 10 and 30 mg/kg) or vehicle and subsequently sacrificed at 90 mins post-dose. The 90 min post-dose survival time was chosen as orally-dosed DMP696 reaches the maximal concentration in plasma at the time point (see time-course study results). For time-course studies, rats were dosed with 10 mg/kg DMP696, and subsequently sacrificed at times from 10 min up to 22 hrs, and the brain and pituitary were then collected. The forebrain, the upper brainstem including the rostral portion of the cerebellum, and the pituitary were sectioned in a Cryostat (20 μM). The remaining brainstem and cerebellum tissues were used for measuring drug concentrations in the brain tissues. In most cases, the trunk blood samples were collected immediately after decapitation, and the plasma was separated by centrifugation for assessment of drug concentration.

In vitro ligand binding procedures for *ex vivo* studies were similar to the above-mentioned for *in vitro* studies except that the pre-incubation (1 min), incubation (40 min) and washing time

(4 min) were substantially shortened. The 40-min incubation time was chosen based upon dissociation time-course studies (see below). Sections from drug- and vehicle-treated rats were incubated with 0.15–0.2 nM [^{125}I]sauvagine. Non-specific binding for CRF_1 receptor sites was defined in adjacent brain sections from vehicle-treated rats by including 1 μM DMP696 in the assay solution. In order to determine the dissociation kinetics of DMP696 and to define an appropriate incubation time, two initial experiments were undertaken. In one test, brain sections from 3 rats orally dosed with either 10 mg/kg DMP696 or vehicle were incubated in 0.15-0.2 nM [^{125}I]sauvagine assay solution. In another test, brain sections from naïve rats were pre-incubated in the assay solution containing either 1 or 10 nM DMP696 for 2 hrs before incubation with 0.15-0.2 nM [^{125}I]sauvagine. For both tests, the incubation time with [^{125}I] sauvagine was varied from 10 min up to 240 min and the effect of incubation time on DMP696 inhibition of [^{125}I]sauvagine binding was examined.

Measurement of DMP696 concentrations in plasma, brain and cerebrospinal fluid

DMP696 concentrations in the plasma and brain were measured using a liquid chromatography tandem mass spectrometric method (LC/MS/MS). Briefly, the 0.1 ml of sample (plasma or homogenized brain tissue), 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 until dry under nitrogen at 60^{o}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.

Plasma protein binding of DMP696 in rats was determined *in vitro* by equilibrium dialysis using the Dianorm dialysis system. Plasma was spiked with DMP696 and equilibrated against isotonic phosphate buffer for 3 hours at 37^{o}C. Following the incubation period, plasma and buffer samples were analyzed using LC/MS/MS. DMP696 unbound fraction was calculated based on the ratio between DMP 696 buffer concentration and the plasma concentration. Free drug concentrations in plasma were determined by multiplication of total concentrations of DMP696 by the unbound fraction.

In a group of rats, DMP696 concentrations in plasma and cerebrospinal fluid (CSF) were measured and compared by using an *ex vivo* membrane binding assay. The rats were orally dosed with 10 mg/kg DMP696, and survived 1 hr or 3 hrs before they were anaesthetized with Nembutal (50 mg/kg). A small incision was then made in the back of the head and the cisterna magna was exposed and cannulated for collection of CSF (~150 µl) for 30 min. At the end of CSF collection, blood samples (2-3 ml) were collected by cardiac puncture. Measurement of DMP696 concentrations was performed using an *ex vivo* binding assay with the inhibition of 0.15 nM [^{125}I]-oCRF to membranes extracted from HEK293 cells over-expressing CRF_1 receptors.

Defensive withdrawal test

The defensive withdrawal procedure as described by McElroy et al. (2002) was employed. 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 watt 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. DMP696 and vehicle (0.25% methocel) was orally dosed 60 minutes before behavioral testing. To initiate testing, the rat was placed within the cylinder which 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.

Data analysis

Digital images were generated with a Cyclone storage phosphor imaging system and analyzed using OptiQuant Analysis software (PerkinElmer Life Sciences). Radioligand binding density in a defined brain region (according to the rat atlas by Paxinos and Waston, 1998) was measured as digital light units/mm^2. For *in vitro* studies, specific binding in a defined brain region was calculated by subtracting the value of the nonspecific binding density from that of the total binding density measured in the corresponding brain region and

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normalized using non-drug treated sections as control. For *ex vivo* ligand binding studies, percent specific binding in drug-treated rats was calculated as the following: percent specific binding = (specific binding in drug-treated minus non-specific binding in vehicle-treated)/(specific binding in vehicle-treated-non-specific binding in vehicle-treated)x100%. The percent specific binding in a drug-treated condition is inversely proportional to the percent inhibition or percent receptor occupancy by the drug.

To measure concentration-related *in vitro* effects of DMP696, the percent inhibition of binding by DMP696 in a given brain region was plotted with increasing concentrations and the concentration-effect curves of best fit were calculated by non-linear regression analysis using Prism software. From this curve, IC_{50} values (drug doses producing 50% inhibition of specific ligand binding) were estimated. Estimating an *in vivo* IC_{50}, the percent inhibition of [^{125}I]sauvagine binding sites vs. DMP696 plasma unbound (free) concentrations were fit using an inhibitory Emax model with Winnonlin program (Pharsight Corporation, Mountain View, CA) according to the following equation: $E=E_{max}*(1-(C/(C+IC_{50})))$, where E is the percent inhibition of [^{125}I]sauvagine binding sites, E_{max} is the maximum binding of [^{125}I]sauvagine, C is the unbound concentration of DMP696 in plasma, and the IC_{50} is the unbound concentration at which there is 50% inhibition of [^{125}I]sauvagine binding sites. Because 50% inhibition of [^{125}I]sauvagine binding sites is representative of 50% CRF_1 receptor occupancy according to our test conditions, the *in vivo* IC_{50} can also be viewed as the unbound concentration of DMP696 in plasma that results in 50% CRF_1 receptor occupancy in the brain.

All data are reported as the mean ± SEM, and subjected to analysis of variance (ANOVA) where appropriate, followed by individual mean comparisons using Fisher's Least Significant Difference Test or Dunnett *t* test. The significance level was set at $p<0.05$.

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RESULTS

Effects of DMP696 on [^{125}I]oCRF and [^{125}I] sauvagine binding: *in vitro* studies

The overall distribution pattern of [^{125}I]oCRF and [^{125}I]sauvagine binding corresponded well with that reported previously in the same species (De Souza et al, 1987, Aguilera et al., 1987; Primus et al., 1997; Rominger et al, 1998). CRF_1 binding sites labeled by both [^{125}I]oCRF and [^{125}I]sauvagine were dominant in the cerebral cortex, the subcortical limbic system, the cerebellar cortex and the anterior pituitary, whereas CRF_2 binding sites labeled only by [^{125}I]sauvagine were concentrated in the lateral septal nucleus, the medial nucleus of the amygdala, the ventromedial nucleus of the hypothalamus and the choroid plexus. Figure 2 illustrates [^{125}I]sauvagine and [^{125}I]oCRF binding in a representative forebrain level in the absence and presence of various blocking ligands. [^{125}I]sauvagine binding sites in the cortical regions and anterior pituitary were displaceable with 1 μM DMP696 (Fig.2B), CP-154,526 (Fig.2C), or α-helical CRF(Fig.2D), a non-selective CRF receptor antagonist, but not by 20 nM anti-sauvagine-30 (Fig.2E), a selective CRF_2 receptor antagonist, or urocortin II (Fig.2F), a selective CRF_2 agonist, indicating that these binding sites represent CRF_1 receptors. Dense [^{125}I]sauvagine binding sites in the lateral septal nucleus and choroid plexus were displaceable with α-helical CRF (Fig.2D), anti-sauvagine-30 (Fig. 2E), or urocortin II (Fig.2F), but not DPC696 (Fig.2B) or CP-154,526 (Fig.2C), indicating that they represent CRF_2 receptor binding sites. [^{125}I]oCRF binding to the cortical regions and anterior pituitary was completely displaceable with DMP696 (Fig.2GH).

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The DMP696 inhibition of [^{125}I]oCRF binding and the CRF_1 component of [^{125}I]sauvagine binding was concentration-dependent. Figure 3 shows the competitive displacement curves for [^{125}I]oCRF and [^{125}I]sauvagine in several brain regions, the anterior pituitary and the choroid plexus. Table 1 summarizes IC_{50} values of DMP696 estimated with both radioligands. DMP696 showed slightly higher potency measured with [^{125}I]sauvagine than with [^{125}I]oCRF. There was no significant difference in IC_{50} values estimated from different brain regions with either ligands. However, the IC_{50} in the anterior pituitary (2.3±0.1 nM with [^{125}I]sauvagine and 3.0±0.1 nM with [^{125}I]oCRF) was significantly higher than the averaged value in the brain (0.8±0.1 with [^{125}I]sauvagine and 1.1±0.1 nM with [^{125}I]oCRF). No significant displacement of [^{125}I]sauvagine binding was seen in the lateral septal nucleus, the ventromedial nucleus of the hypothalamus, or the choroid plexus (Fig.3C).

Effect of DPC696 on [^{125}I]sauvagine binding: *ex vivo* studies

Given the comparability between [^{125}I]sauvagine and [^{125}I]oCRF binding to CRF_1 receptor sites, we utilized [^{125}I]sauvagine exclusively for *ex vivo* studies so that *in vivo* effects of DMP696 on both CRF_1 and CRF_2 receptors could be simultaneously monitored. In order to test if *in vitro* processing (primarily incubation time) affects DMP696 receptor occupancy values, two experiments were performed to examine the dissociation kinetic profile of DMP696. In the first experiment, brain sections from rats dosed with 10 mg/kg DMP696 were incubated with [^{125}I]sauvagine for various times from 10 up to 240 minutes and the effect of the incubation time on DMP696 inhibition of [^{125}I]sauvagine binding in the parietal cortex was calculated (Fig.4A). Ten minutes after incubation, 82% of [^{125}I]sauvagine binding was inhibited. The inhibition increased to 91% at 20 minutes and maintained around 90% up

to 60 minutes after incubation. Further increasing incubation time resulted in gradual decline of the inhibition (down to 26% at 4 hrs). In the second experiment, brain sections from naïve rats were pre-incubated with DMP696 at 1 or 10nM for 2 hrs prior to incubation with [^{125}I]sauvagine from 10 up to 240 minutes. As shown in Figure 4B, between 40-60 minutes after incubation, [^{125}I]sauvagine binding was inhibited by about 50% and 90% in the sections pre-incubated with respective 1 and 10 nM DMP696. The percent inhibition are consistent with theoretically calculated values based on the IC_{50} (0.9 nM for the parietal cortex) of DMP696 at both concentrations (50% inhibition at 1 x IC_{50} and 90% inhibition at 10 x IC_{50}). The percent inhibition from incubation times shorter or longer than 40-60 minutes deviated from these values with increased variability. Based on the results from these two tests, we chose a 40 minute incubation time for all of our *ex vivo* binding studies.

We examined dose-related effects of orally administrated DMP696 on *in vitro* [^{125}I]sauvagine binding in the brain. As depicted in Figure 5, DMP696 dose-dependently inhibited the binding sites in the cortex and the anterior pituitary, but had no effect on those in the lateral septal nucleus and the choroid plexus. Figure 6 graphically illustrates the quantitative effect of DMP696 on [^{125}I]sauvagine binding in several brain regions and the anterior pituitary. In general, there was no significant difference in the effect of DMP696 at a given dose between different brain regions, and between the brain regions and the anterior pituitary. At a dose of 1 mg/kg, DMP696 produced less than 50% inhibition in the brain regions except the frontal cortex (~50%). At 3 mg/kg, DMP696 inhibition averaged 60% [^{125}I]sauvagine binding in the brain (ranging from 52% in the basolateral amygdala to 77% in the frontal cortex). The average inhibition in the brain increased to 80% at 10 mg/kg and

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90% at 30 mg/kg. For comparison, we measured the inhibitory effect of orally-dosed CP-154,526 in the parietal cortex. As shown in Figure 6F, CP-154,526 was less potent compared to DMP696. At 30 mg/kg CP-154,526 inhibited 47% of [^{125}I]sauvagine binding. This observation is consistent with a previous report (Arborelius et al., 2000).

Time-course of receptor occupancy and drug concentrations in plasma and brain

The time-course of the percent inhibition of [^{125}I]sauvagine binding by DMP696, drug plasma free concentrations, and brain concentrations of DMP696 following orally dosed 10 mg/kg of the drug were examined. Plasma protein binding of DMP696 in rats is 98.5% and, consequently, the plasma free drug concentrations were calculated by multiplying the plasma free fraction (1.5%) by the total plasma concentrations. For easy comparison, percent inhibition was expressed as receptor occupancy of CRF_1 receptors (Fig. 7). Each time point was collected from pooled data of 3-5 rats. Overall, the time-course of the receptor occupancy correlated well with that of the plasma free concentrations and the brain concentrations of DMP696 (Fig. 7). However, the drug concentrations in the brain were over 150 fold higher than the plasma free levels. At 40 minutes after dosing, the plasma free level of DMP696 reached 5 nM, total brain concentration was over 80 nM, and the receptor occupancy was over 60%. The receptor occupancy and the drug concentration in plasma and brain peaked at 90 minute post-dosing (95%, 9.8 nM (free plasma) and 1547 nM (brain), respectively), and declined afterwards. By 22 hrs after dosing, when the plasma free drug level dropped to below 1 nM, there was apparently no DMP696 occupancy in the brain.

Correlation of receptor occupancy with free plasma concentrations

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The relationship between CRF_1 receptor occupancy and free concentrations of DMP696 in the plasma was examined using pooled data from the *ex vivo* studies described above (i.e. Fig.6). Figure 8 is a plot of receptor occupancy vs. free plasma concentrations of DMP696. The data were fitted using an inhibitory Emax model with % receptor occpancy as the observed effect (Winnonlin Version 3.3, Pharmasight 2001, MountainView, CA). The kinetic-modelling analysis of the data yielded an *in vivo* IC_{50} value of 1.2 nM, which is consistent with *in vitro* IC_{50} (0.9 nM, both the *in vivo* and *in vitro* values were measured from the same brain region, parietal cortex with the same ligand, [^{125}I]sauvagine as ligand).

DMP696 concentrations in the plasma vs CSF

The purpose of this experiment was to compare drug concentrations in CSF vs plasma after an oral dose of 10 mg/kg DMP696. The data were averaged from 7 rats for each time point. One hour post-dosing, the plasma free concentration was 8.7±2.4 nM and the CSF concentration was 14±4.3 nM. Three hours after dosing, the concentration was slightly decreased in both plasma (8.0±1.4 nM) and CSF (13±2.5 nM). This result indicates the consistency of the plasma free levels of DMP696 with the CSF concentrations.

Defensive withdrawal test

In this experiment, rats were subjected to the defensive withdrawal test following varying oral doses of DMP696 (i.e. 0, 1, 3, 10, 30, and 90 mg/kg). Figure 9 shows exit latencies for each dose at 60 minutes after oral administration of DMP696. At 3 mg/kg, there was a substantial, albeit not statistically significant, decrease in the exit latency (48% from the vehicle level). At 10 mg/kg, the reduction was greater (62%) and statistically significant.

Behavioral efficacy appears to plateau at 10 mg/kg since higher doses (30 and 90 mg/kg) did not produce significantly lower exit latencies.

DISCUSSION

The present study demonstrates that DMP696 selectively and dose-dependently occupies CRF_1 receptors in the brain with no apparent regional differences. DMP696 administrated at 3 mg/kg occupied just over 50% CRF_1 receptors, a dose equivalent to that produced a minimal anxiolytic effect (~50% reduction in the exit latency). Examination of the relationship between receptor occupancy and plasma free concentrations yields an *in vivo* IC_{50} of 1.22 nM, which is similar to the *in vitro* IC_{50} (0.9 nM) of the conmpound. Furthermore, this study shows a parallel time-course of receptor occupancy, plasma free concentrations and brain tissue concentrations of DMP696.

The binding affinity of DMP696 for CRF_1 receptors measured using the brain section binding autoradigraphy is in accordance with the previously studies using homogenized cell membrane assays (He et al, 2000, Zhang et al., 2003). For example, Zhang et al (2001) revealed IC_{50} of 1.96 nM and 5.2 nM for DMP696 to inhibit CRF_1 receptors in the cortex and pituitary, respectively. These values are consistent with those observed in the present study (cortex 0.9 nM; pituitary 2.7 nM), suggesting comparability between autoradiographic and homogenized assays for assessing CRF_1 antagonist affinity. In combination with storage phosphor imaging techniques, the brain section binding assay allows evaluation of drug potency in anatomically-defined brain structures with significantly improved throughput compared with conventional film autoradiography.

There was no significant regional difference of DMP696 binding affinity to CRF_1 receptors in the brain. In contrast, DMP696 was apparently less potent in the pituitary compared to the

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brain, as described previously (Zhang et al., 2003). The functional significance of the affinity difference between the brain and pituitary is not known. However, DMP696 appears to have equal efficacy in producing anxiolytic effects in the defensive withdrawal test and in reversing stress-elicited corticosterone release (McElroy et al., 2002). In addition, our *ex vivo* binding data did not reveal any significant differences of *in vivo* receptor occupancy between the pituitary and brain structures of the compound.

Ex vivo binding autoradiography allows *in vitro* measurement of a receptor population occupied by drugs administrated *in vivo*. This method has been used for assessing *in vivo* binding profiles of a variety of drugs including CRF_1 antagonists (Arborelius et al., 2000, Keck et al., 2001, Heinrichs et al., 2002). The inherent limitation of the method is the requirement of *in vitro* processing of tissue sections, which gives rise to the probability of dissociation of receptor-bound drugs from their binding sites, causing underestimation of *in vivo* occupancy. One recent *ex vivo* binding study has demonstrated that peripherally administrated raclopride, an antipsychotic, dissociates swiftly from the binding sites during a 30 minute incubation and causes over 70% underestimation of the receptor occupancy (Kapur et al., 2001). In order to avoid potential underestimation of DMP696 occupancy, we studied the dissociation time-course of DMP696 during *in vitro* processing. In the first test on brain sections from rats dosed with 10 mg/kg DMP696, the receptor occupancy was around 90% 20-60 minutes after the beginning of radioligand incubation, and decreased significantly afterwards. In the second test, 40-60 minutes after the beginning of radioligand incubation of naïve sections which were pre-incubated with 1 or 10 nM DMP696, ~50% or ~90% receptors were occupied. The occupancy values at these two concentrations are consistent

with calculated based on the IC_{50}, e.g., 1 nM DMP696 occupying ~50% receptors and 10 nM occupying ~90%. Data from these tests suggest that a limited incubation time (40 minutes or less) is required in order to minimize underestimation of DMP696 occupancy.

Literature that suggested shortening *in vitro* incubation time "as much as possible" may not necessarily be optimal for every drug of interest (Kapur et al., 2001, Langlois et al., 2001). For DMP696, an incubation time less than 40 minutes caused decreased receptor occupancy with increased variability. Conceivably, in such a short incubation time radioligands do not have time to reach equilibrium. In this study, a 40-minute incubation may be long enough to allow radioligand binding to approach equilibrium, but not too long to cause significant dissociation of DMP696. Thus, caution should be taken in selection of an incubation time for *ex vivo* measurement of receptor occupancy. An *in vitro* dissociation time-course study of drugs of interest may be warranted before full-scale *ex vivo* measurement.

Using the *ex vivo* binding, we measured systemically receptor occupancy of DMP696 in the brain. Orally-administrated DMP696 dose-dependently occupied CRF_1, but not CRF_2 receptors, with no significant regional differences in the brain. On average, at 1 mg/kg DMP696 produced ~40% occupancy of CRF_1 receptors in the brain, and the percentage increased to ~60% at 3 mg/kg. A further dose increase to 10 and 30 mg/kg resulted in receptor occupancy over 80% and 90%, respectively. The receptor occupancy appears related to the behavioral effect. In the defensive withdrawal test, 3 mg/kg (but not 1 mg/kg DMP696) produced 48% reduction of the exit latency whereas 1 mg/kg of DMP696 was ineffective. The statistical insignificance at 3 mg/kg was likely due to a greater variability in

this study as the same dose repeatedly shows significant efficacy in the same test from our previous studies (He et al., 2000, McElroy et al., 2002). Therefore, 3 mg/kg of DMP696 appears to be the minimal oral dose for producing anxiolytic effects. At 10 mg/kg DMP696 produced a greater, but apparently saturating, effect as further increasing the dose to 30 or 90 mg/kg produced no significant increase in the efficacy, consistent with the results of previous studies (He et al., 2000, McElroy et al., 2002). Taken together, these data suggest that *in vivo* receptor occupancy of DMP696 is dose-dependent and is closely related with the anxiolytic efficacy of the compound. The dose of DMP696 that occupied ~50% CRF_1 receptors in the brain is identical to the minimally effective dose in the defensive withdrawal test. These data suggest that blockade of at least 50% of CRF_1 receptors is a requisite for anxiolytic effects of DMP696 in the defensive withdrawal model of anxiety. Further studies are necessary to confirm that this relationship holds for other models of anxiety and for potential anxiolytic effects in humans.

A close correlation of receptor occupancy with behavioral effects has also been observed for several other CRF_1 antagonists. R121919 occupied central CRF_1 receptors in a dose-dependent manner (Keck et al., 2001, Heinrichs et al., 2002). An oral dose (2.5 mg/kg) of R121919 which produced a minimal anxiolytic effect occupied 50% CRF_1 receptors (Heinrichs et al., 2002). CP-154,526 was not effective in a defensive withdrawal test until a dose over 35 mg/kg (Arborelius et al., 2000), at which the compound occupied over 50% brain CRF_1 receptor (Arborelius et al., 2000, Figure 5 of this study). In addition, we have observed the consistency of doses occupying over 50% receptors with doses effective in

behavioral tests for a number of in-house CRF_1 antagonists (Y-W. Li and J. McElroy, unpublished observations).

We explored further the relationship between drug exposure and receptor occupancy following DMP696 dosing. An understanding of the relationship is important in that it could potentially serve as a guide for the selection of a dose regime in clinical studies. The parallel decline of unbound (free) plasma concentrations and total brain concentrations of DMP696 in relation to receptor occupancy from our *in vivo* time-course study (Fig. 7) suggests that DMP696 rapidly equilibrates with CRF_1 receptors in the brain.

For DMP696, a highly lipophilic compound (ClogP 4.97), passive diffusion is likely to be the main route of its entry into the brain (Pardridge, 1998). Unbound drug concentrations in plasma is an important factor which governs the extent of brain distribution of compounds that enter via passive diffusion (Sawchuck and Yang, 1999). Thus, unbound rather than total plasma concentrations of DMP696 were used to examine the relationship between receptor occupancy and plasma exposure. Analysis of the brain receptor occupancy and the free plasma concentrations yielded an estimated *in vivo* IC_{50} value of 1.22 nM. Interestingly, the *in vivo* IC_{50} value is remarkably close to the *in vitro* IC_{50} of DMP696 observed throughout *in vitro* binding assays from this and a previous study (Zhang et al., 2003). This suggests that the availability of DMP696 for CRF_1 receptors in the brain is similar to free concentrations observed in plasma. We have found that free plasma levels of CRF_1 antagonists are an important factor in the availability of these compounds to the receptors in the brain. Previously examined CRF_1 antagonists with plasma protein binding >99.8% showed no

appreciable receptor occupancy despite having excellent plasma exposures of total drug (H. Wong and Y-W. Li, unpublished observations). Compared to the plasma, brain had remarkably high concentrations of DMP696. The peak concentration in the brain at 90 minute post-dosing was 1547 nM, over 150-fold higher than the unbound plasma level. The accumulation of DMP696 in the brain is likely the consequence of the lipid-enriched brain tissue functioning as a 'sink' for the highly lipophilic compound. It is therefore conceivable that brain concentrations of compounds like DMP696 may not necessarily be meaningful in predicting their occupancy of targeted receptors.

In conclusion, the results from this study supports the hypothesis that the anxiolytic effect of DMP696 is mediated by acting on brain CRF_1 receptors and suggests that at least 50% receptor occupancy is needed for efficacy. The similarity in the *in vivo* and *in vitro* IC_{50} values for DMP696 suggests that plasma free concentrations of DMP696 are important for the entry of the compound into the brain and binding to CRF_1 receptors.

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REFERENCES

Aguilera G, Millan MA, Hauger RL, and Catt KJ (1987) Corticotropin-releasing factor receptors: distribution and regulation in brain, pituitary, and peripheral tissues. *Ann N Y Acad Sci*. **512**:48-66.

Altemus M, Swedo SE, Leonard HL, Richter D, Rubinow DR, Potter WZ, and Rapoport JL (1994) Changes in cerebrospinal fluid neurochemistry during treatment of obsessive-compulsive disorder with clomipramine. *Arch Gen Psychiatry* **51**:794-803.

Arborelius L, Skelton KH, Thrivikraman KV, Plotsky PM, Schulz DW, and Owens MJ (2000) Chronic administration of the selective corticotropin-releasing factor 1 receptor antagonist CP-154,526: behavioral, endocrine and neurochemical effects in the rat. *J Pharmacol Exp Ther* **294**:588-97.

Bakthavatchalam R, Arvanitis AG, Gilligan PJ, Olson RE, Robertson DW, Trainor G Smith SC, Fitzgerald LW, Zaczek R, Shen H and Christ DW (1998) The discovery of DMP695: an orally active corticotropin-releasing hormone (CRF1) receptor antagonist. *ACS National Meeting, Boston*, MA, **Aug** 23-27; MEDI 134.

Bremner JD, Licinio J, Darnell A, Krystal JH, Owens MJ, Southwick SM, Nemeroff CB, and Charney DS (1997) Elevated CSF corticotropin-releasing factor concentrations in posttraumatic stress disorder. *Am J Psychiatry*. **154**:624-9.

Chalmers DT, Lovenberg TW, and De Souza EB (1995) Localization of novel corticotropin-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF1 receptor mRNA expression. *J Neuroscience* **10**:6340-50.

Chalmers DT, Lovenberg TW, Grigoriadis DE, Behan DP, and De Souza EB (1996) Corticotrophin-releasing factor receptors: from molecular biology to drug design. *Trends Pharmacol Sci* **174**:166-72.

De Souza EB (1987) Corticotropin-releasing factor receptors in the rat central nervous system: characterization and regional distribution. *J Neuroscience* **1**:88-100.

Gilligan PJ, Robertson DW, and Zaczek R (2000a) Corticotropin releasing factor (CRF) receptor modulators: progress and opportunities for new therapeutic agents. *J Med Chem* **43**:1641-60.

Gilligan PJ, Baldauf C, Cocuzza A, Chidester D, Zaczek R, Fitzgerald LW, McElroy J, Smith MA, Shen HS, Saye JA, Christ D, Trainor G, Robertson DW, and Hartig P (2000b) The discovery of 4-(3-pentylamino)-2,7-dimethyl-8-(2-methyl-4-methoxyphenyl)-pyrazolo-[1 ,5-a]-pyrimidine: a corticotropin-releasing factor (hCRF1) antagonist. *Bioorg Med Chem* **1**:181-9.

Habib KE, Weld KP, Rice KC, Pushkas J, Champoux M, Listwak S, Webster EL, Atkinson AJ, Schulkin J, Contoreggi C, Chrousos GP, McCann SM, Suomi SJ, Higley JD, and Gold PW (2000) Oral administration of a corticotropin-releasing hormone receptor antagonist significantly attenuates behavioral, neuroendocrine, and autonomic responses to stress in primates. *Proc Natl Acad Sci U S A* **97**:6079-84.

He L, Gilligan PJ, Zaczek R, Fitzgerald LW, McElroy J, Shen HS, Saye JA, Kalin NH, Shelton S, Christ D, Trainor G, and Hartig P (2000) 4-(1,3-Dimethoxyprop-2-ylamino)-2,7-dimethyl-8-(2, 4-dichlorophenyl)pyrazolo[1,5-a]-1,3,5-triazine: a potent, orally bioavailable CRF(1) receptor antagonist. *J Med Chem* **43**:449-56.

Heinrichs SC, and De Souza EB (1999) Corticotropin-releasing factor antagonists, binding-protein and receptors: implications for central nervous system disorders. *Baillieres Best Pract Res Clin Endocrinol Metab* **4**:541-54.

Heinrichs SC, De Souza EB, Schulteis G, Lapsansky JL, and Grigoriadis DE (2002) Brain penetrance, receptor occupancy and antistress in vivo efficacy of a small molecule corticotropin releasing factor type I receptor selective antagonist. *Neuropsychopharmacology* **2**:194-202.

Hill G, Zaczek R, and Li Y-W (2001) Corticotropin releasing factor type-1 receptor occupancy of DMP696 in rat brain: ex vivo binding autoradiography. *Soc Neurosci Abs* **27.**

Kapur S, Barlow K, VanderSpek SC, Javanmard M, and Nobrega JN (2001) Drug-induced receptor occupancy: substantial differences in measurements made in vivo vs ex vivo. *Psychopharmacology (Berl)* **2**:168-71.

Kaye WH, Berrettini WH, Gwirtsman HE, Chretien M, Gold PW, George DT, Jimerson DC, and Ebert MH (1987) Reduced cerebrospinal fluid levels of immunoreactive pro-opiomelanocortin related peptides (including beta-endorphin) in anorexia nervosa. *Life Sci* **41**:2147-55.

Keck ME, Welt T, Wigger A, Renner U, Engelmann M, Holsboer F, and Landgraf R (2001) The anxiolytic effect of the CRH(1) receptor antagonist R121919 depends on innate emotionality in rats. *Eur J Neuroscience* **2**:373-80.

Kehne JH, Coverdale S, McCloskey TC, Hoffman DC, and Cassella JV (2000) Effects of the CRF(1) receptor antagonist, CP 154,526, in the separation-induced vocalization anxiolytic test in rat pups. *Neuropharmacol*ogy **8**:1357-67.

Keller C, Bruelisauer A, Lemaire M, and Enz A. (2002) Brain pharmacokinetics of a nonpeptidic corticotropin-releasing factor receptor antagonist. *Drug Metab Dispos* **30**:173-76.

Korte SM, Korte-Bouws GA, Bohus B, and Koob GF (1994) Effect of corticotropin-releasing factor antagonist on behavioral and neuroendocrine responses during exposure to defensive burying paradigm in rats. *Physiol Behav* **1**:115-20.

Langlois X, te Riele P, Wintmolders C, Leysen JE, and Jurzak M (2001) Use of the beta-imager for rapid ex vivo autoradiography exemplified with central nervous system penetrating neurokinin 3 antagonists. *J Pharmacol Exp Ther* **299**:712-7.

Maciag CM, Dent G, Gilligan P, He L, Dowling K, Ko T, Levine S, and Smith MA (2002) Effects of a non-peptide CRF antagonist (DMP696) on the behavioral and endocrine sequelae of maternal separation. *Neuropsychopharmacology* **5**:574-82.

McElroy JF, Ward KA, Zeller KL, Jones KW, Gilligan PJ, He L, Lelas S (2002) The CRF(1) receptor antagonist DMP696 produces anxiolytic effects and inhibits the stress-induced hypothalamic-pituitary-adrenal axis activation without sedation or ataxia in rats. *Pyschopharmacology (Berl)* **165**:86-92.

Millan MJ, Brocco M, Gobert A, Dorey G, Casara P, and Dekeyne A (2001) Anxiolytic properties of the selective, non-peptidergic CRF(1) antagonists, CP154,526 and DMP695: a comparison to other classes of anxiolytic agent. *Neuropsychopharmacology* **4**:585-600.

Okuyama S, Chaki S, Kawashima N, Suzuki Y, Ogawa S, Nakazato A, Kumagai T, Okubo T, and Tomisawa K (1999) Receptor binding, behavioral, and electrophysiological profiles of nonpeptide corticotropin-releasing factor subtype 1 receptor antagonists CRA1000 and CRA1001. *J Pharmacol Exp Ther* **2**:926-35.

Owens MJ, and Nemeroff CB. (1991) Physiology and pharmacology of corticotropin-releasing factor. *Pharmacol Rev* **43**:425-73

Pardridge WM (1998) CNS drug design based on principles of blood-brain barrier transport. *J Neurochem* **5**:1781-92.

Paxinos G, and Watson C (1998) *The Rat Brain in Stereotaxic Coordinates*, 4th ed, Academic Press, Inc., San Diego, CA

Primus RJ, Yevich E, Baltazar C, and Gallager DW (1997) Autoradiographic localization of CRF1 and CRF2 binding sites in adult rat brain. *Neuropsychopharmacology* **5**:308-16.

Rominger DH, Rominger CM, Fitzgerald LW, Grzanna R, Largent BL, and Zaczek R (1998) Characterization of [125I]sauvagine binding to CRH2 receptors: membrane homogenate and autoradiographic studies. *J Pharmacol Exp Ther* **1**:459-68.

Sawchuk RJ, and Yang Z (1999) Investigation of distribution, transport and uptake of anti-HIV drugs to the central nervous system. *Adv Drug Deliv Rev* **39**:5-31.

Stenzel-Poore MP, Duncan JE, Rittenberg MB, Bakke AC, and Heinrichs SC (1996) CRH overproduction in transgenic mice: behavioral and immune system modulation. *Ann N Y Acad Sci*. **780**:36-48.

Takahashi LK (2001) Role of CRF(1) and CRF(2) receptors in fear and anxiety. *Neurosci Biobehav Rev*. **25**:627-36.

Takahashi LK, Ho SP, Livanov V, Graciani N, and Arneric SP (2001) Antagonism of CRF(2) receptors produces anxiolytic behavior in animal models of anxiety. *Brain Res* **902**:135-42.

Timpl P, Spanagel R, Sillaber I, Kresse A, Reul JM, Stalla GK, Blanquet V, Steckler T, Holsboer F, and Wurst W(1998) Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. *Nat Genet* **19**:162-6.

Zhang G, Huang N, Li Y-W., Qi X., Marshall AP, Yan XX, Hill G, Rominger C, Prakash SR, Bakthavatchalam R, Rominger DH, Gilligan PJ and Zaczek R (2003) Pharmacological characterization of a novel non-peptide antagonist radioligand, (+/-)-N-[2-methyl-4-methoxyphenyl]-1-(1-(methoxymethyl) propyl)-6-methyl-1H-1, 2, 3 -triazolo[4,5-c] pyridin-4-amine ([3H]SN003) for corticotropin-releasing factor1 (CRF1) receptors. *J Pharmacol Exp Ther in press*.

Zobel AW, Nickel T, Kunzel HE, Ackl N, Sonntag A, Ising M, and Holsboer F (2000) Effects of the high-affinity corticotropin-releasing hormone receptor 1 antagonist R121919 in major depression: the first 20 patients treated. *J Psychiatr Res* **34**:171-81.

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LEGENDS FOR FIGURES

Figure 1. Chemical structure of DMP696 (4-(1,3-dimethoxyprop-2-ylamine)-2,7-dimethyl-8-(2,4-dichlorophenyl)-pyrazolo[1,5-a]-1,3,5-triazine.

Figure 2. Representative autoradiograms of coronal forebrain sections showing [^{125}I]-sauvagine binding in the absence (A) and presence of 1 μM DMP696 (B), 1 μM CP-154,526 (C), 1 μM α-helical CRF (D), 30 nM anti-sauvagine-30 (E) or 30 nM urocortin II (F), and [^{125}I]-oCRF binding in the absence (G) and presence (H) of 1 μM DMP696. Abbreviations: FC, frontal cortex, PC, parietal cortex, LS, lateral septum, AP, anterior pituitary, ChP, choroid plexus, CPu, caudate-putamen. Scale bar = 2 mm.

Figure 3. Comparison of concentration-dependent effects of DMP696 on [^{125}I]oCRF and [^{125}I]sauvagine binding in several brain regions, anterior pituitary and choroid plexus. Data are the mean ± SEM of percent specific binding (n=4). A: [^{125}I]oCRF binding. B and C: [^{125}I]sauvagine binding.

Figure 4. Effects of [^{125}I]sauvagine incubation time on DMP696 inhibition of specific binding. A. *Ex vivo* binding: brain sections collected from rats (n=3) orally dosed with 10 mg/kg DMP696. B. *In vitro* binding: naïve rat (n=3) brain sections pre-incubated with 1 or 10 nM DMP696 for 2 hrs before incubation with [^{125}I]sauvagine. Data are the mean ± SEM for 3 binding tests.

Figure 5. Representative autoradiograms of coronal forebrain sections showing dose-dependent inhibition of [^{125}I]sauvagine binding by orally dosed DMP696. Note: DMP696 did not affect [^{125}I]sauvagine binding in the choroid plexus. FC, frontal cortex, PC, parietal cortex, LS, lateral septum, AP, anterior pituitary. Scale bar = 2 mm.

Figure 6. Orally dosed DMP696 (A, B, C, D, E) and CP-154,526 (F) produced dose-dependent inhibition of [^{125}I]sauvagine binding in several brain regions and anterior pituitary.

Figure 7. Time course of CRF_1 receptor occupancy, drug plasma free concentrations and brain tissue concentrations following a single oral dose of DMP696 (10 mg/kg). CRF_1 receptor occupancy was defined by percent inhibition of specific [^{125}I]sauvagine in the parietal cortex. The data represent the mean ± SEM for 4 rats at each time point.

Figure 8. Relationship between CRF_1 receptor occupancy in the parietal cortex and DMP696 plasma free concentrations. The data were fitted using an inhibitory Emax model with a predicted *in vivo* IC_{50} of 1.2 nM.

Figure 9. Effect of orally dosed DMP696 (1-90 mg/kg) on the exit latency in a defense withdrawal test of anxiety. Exit latency is defined as the time taken for an animal to emerge from a darkened chamber into a novel environment. Total test time is 900 seconds. Data presented are the mean ± SEM for 8 animals per group; * $p < 0.05$ (compared with the vehicle (0.25% methocel) group).

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Table I. *In vitro* IC_{50} values (nM) of DMP696 in a number of brain regions and anterior pituitary measured with [^{125}I]sauvagine and [^{125}I]oCRF as radioligands

	[^{125}I]sauvagine	[^{125}I]oCRF
frontal cortex	0.63± 0.10	0.96± 0.05
parietal cortex	0.94 ± 0.09	1.2± 0.04
prefrontal cortex	1.01 ± 0.12	1.45 ± 0.15
basolateral amygdala	0.76 ± 0.13	1.0 ± 0.08
cerebellar cortex	0.75 ± 0.12	0.89 ± 0.07
anterior pituitary	2.33 ± 0.10	3.02 ± 0.07

Data are the mean ± SEM (n=4).

Figure 1

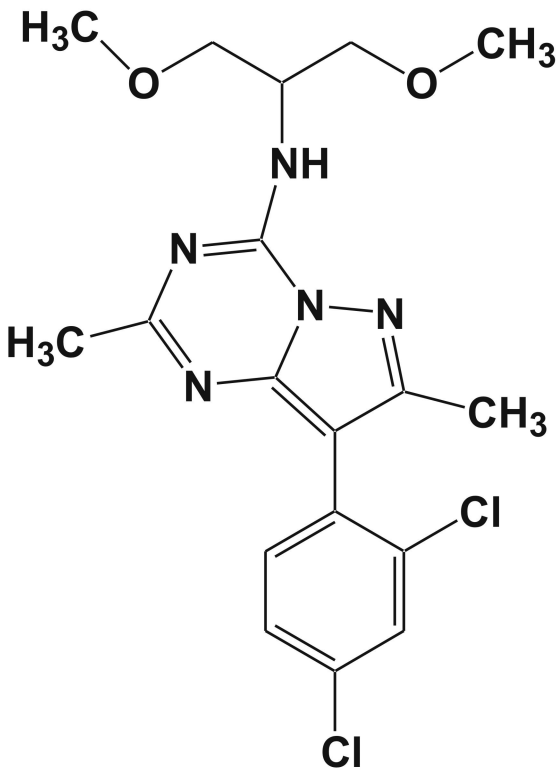


Figure 2

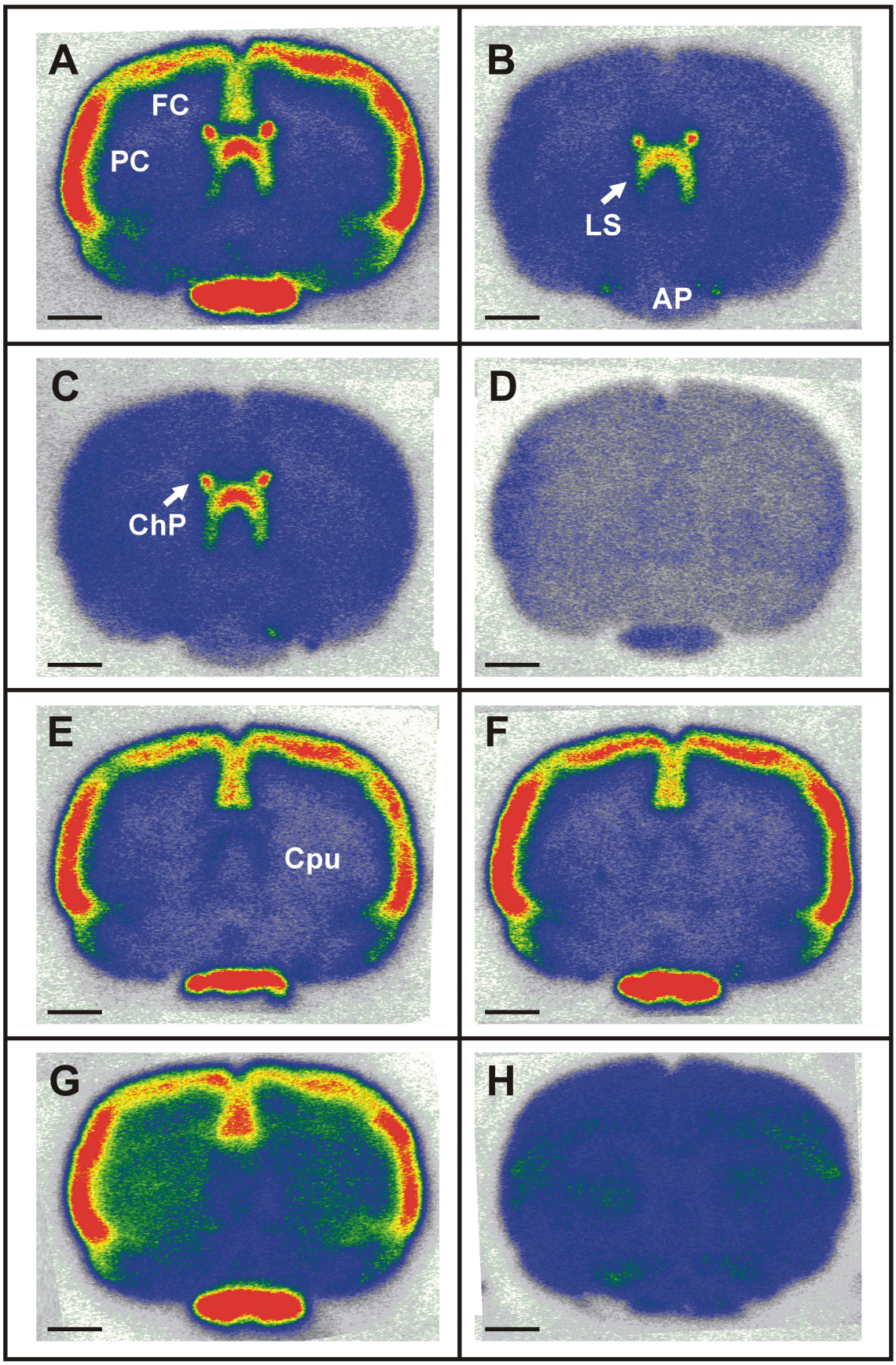


Figure 3

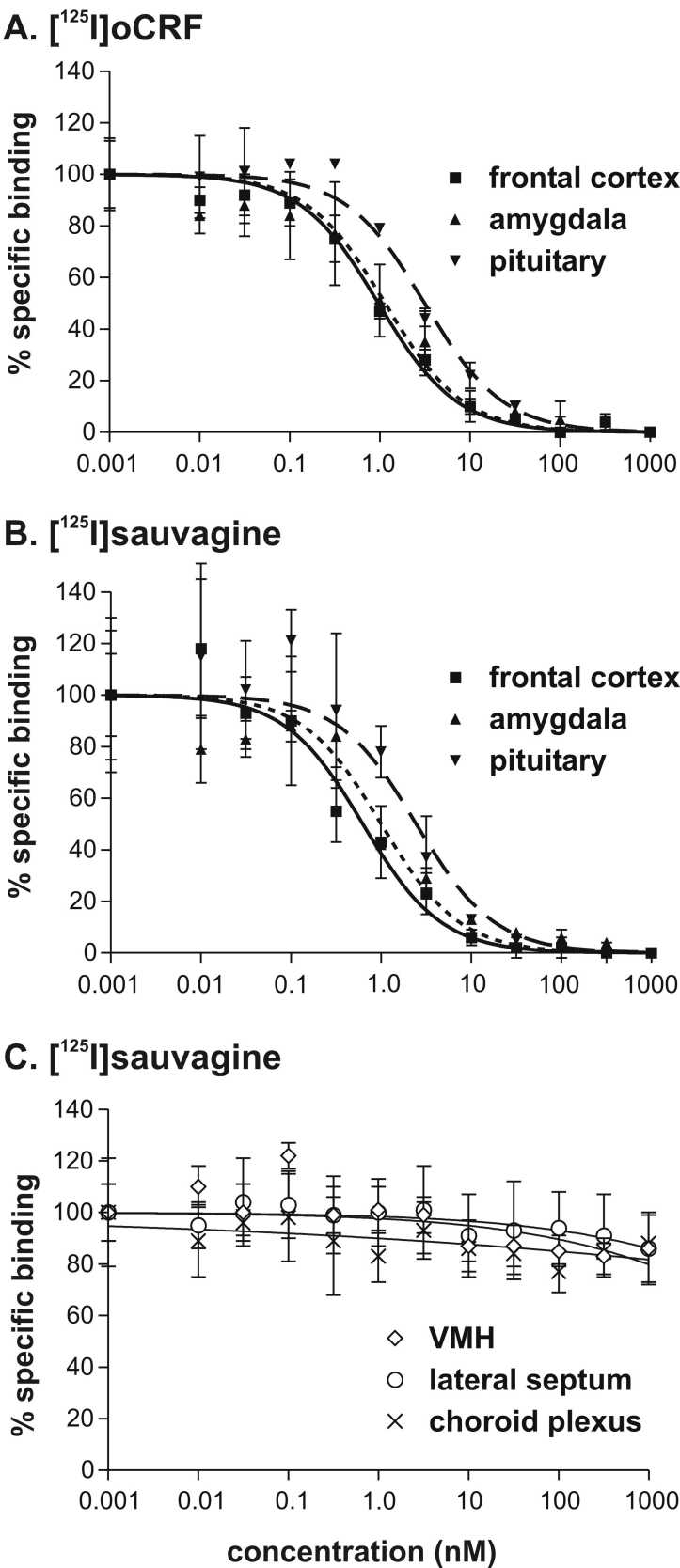

A. [125I]oCRF
% specific binding
140
120
100
80
60
40
20
0
0.001
0.01
0.1
1.0
10
100
1000
frontal cortex
amygdala
pituitary
B. [125I]sauvagine
% specific binding
frontal cortex
amygdala
pituitary
C. [125I]sauvagine
% specific binding
VMH
lateral septum
choroid plexus
concentration (nM)

Figure 4

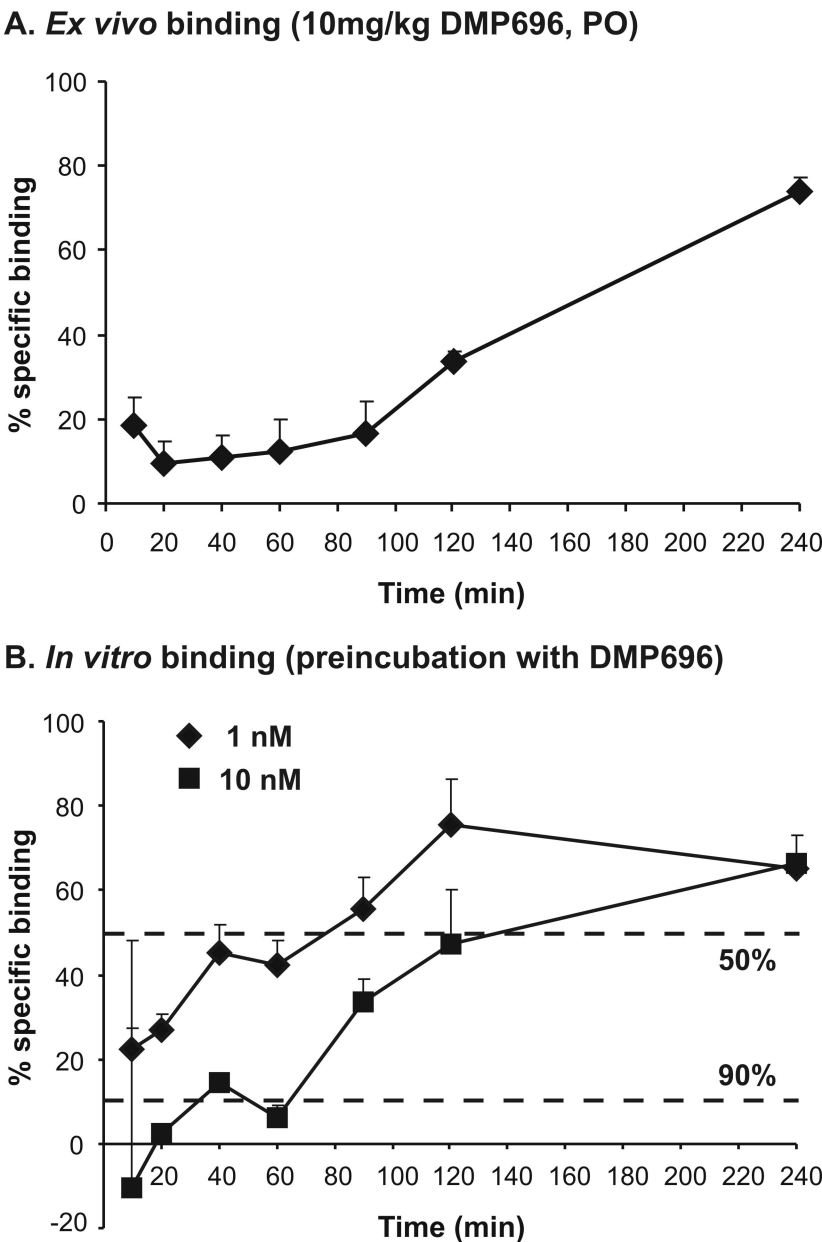

A. *Ex vivo* binding (10mg/kg DMP696, PO)
% specific binding
100
80
60
40
20
0
0 20 40 60 80 100 120 140 160 180 200 220 240
Time (min)
B. *In vitro* binding (preincubation with DMP696)
1 nM
10 nM
% specific binding
100
80
60
40
20
0
-20
50%
90%
20 40 60 80 100 120 140 160 180 200 220 240
Time (min)

Figure 5

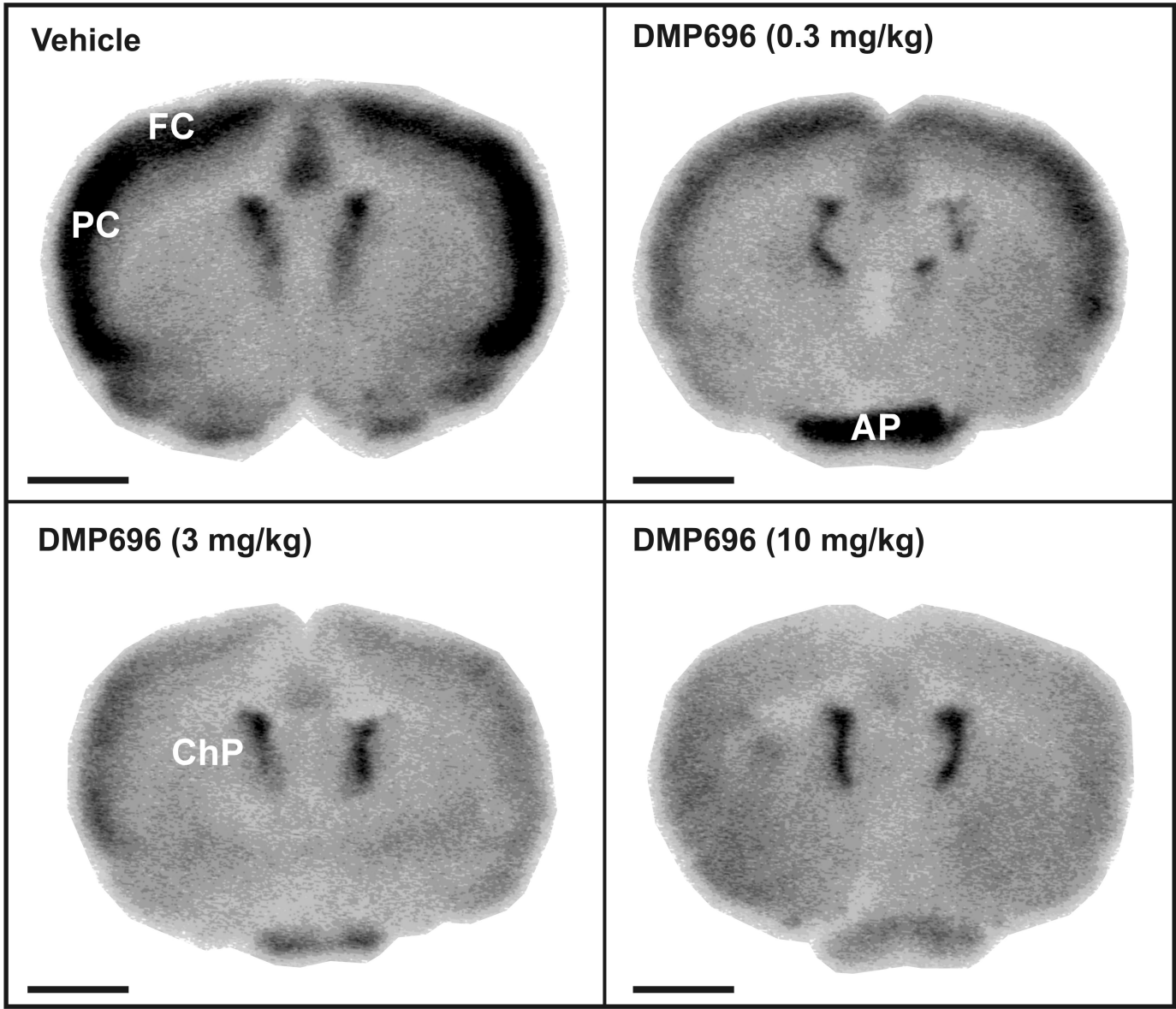

Vehicle
FC
PC
DMP696 (0.3 mg/kg)
AP
DMP696 (3 mg/kg)
ChP
DMP696 (10 mg/kg)

Figure 6

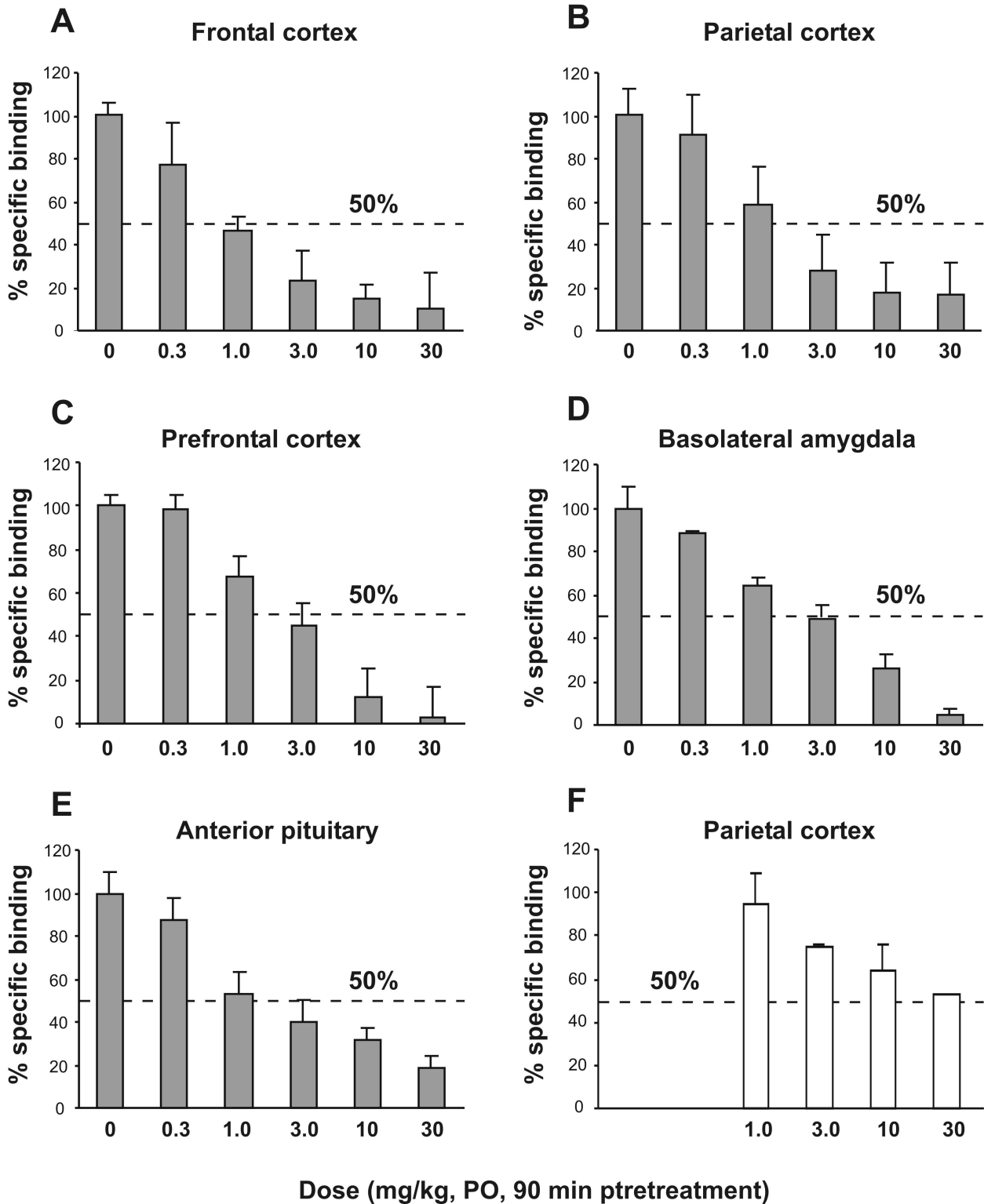

A
Frontal cortex
% specific binding
120
100
80
60
40
20
0
50%
0
0.3
1.0
3.0
10
30
B
Parietal cortex
% specific binding
120
100
80
60
40
20
0
50%
0
0.3
1.0
3.0
10
30
C
Prefrontal cortex
% specific binding
120
100
80
60
40
20
0
50%
0
0.3
1.0
3.0
10
30
D
Basolateral amygdala
% specific binding
120
100
80
60
40
20
0
50%
0
0.3
1.0
3.0
10
30
E
Anterior pituitary
% specific binding
120
100
80
60
40
20
0
50%
0
0.3
1.0
3.0
10
30
F
Parietal cortex
% specific binding
120
100
80
60
40
20
0
50%
1.0
3.0
10
30
Dose (mg/kg, PO, 90 min ptretreatment)

Figure 7

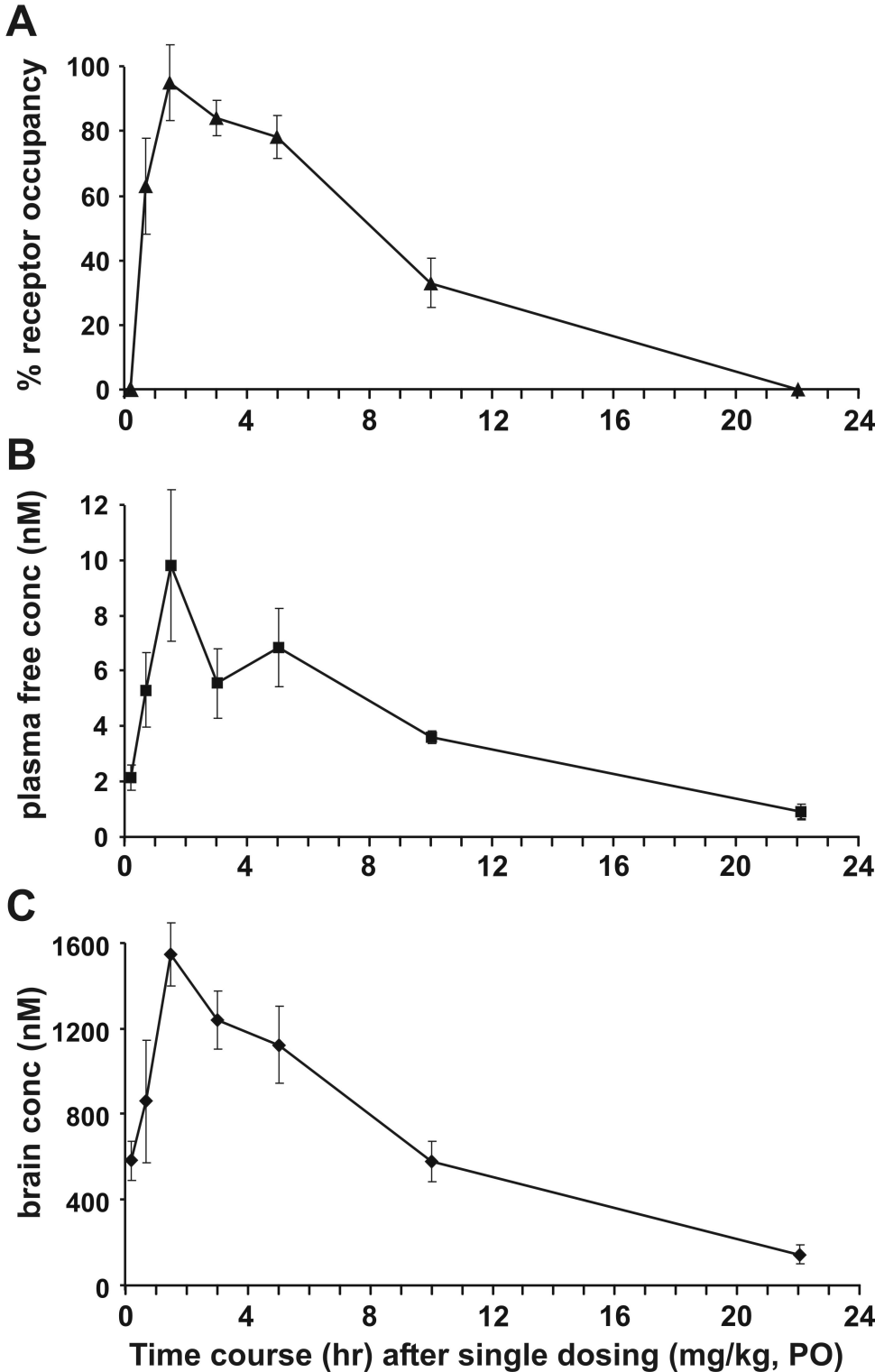

A
% receptor occupancy
100
80
60
40
20
0
0
4
8
12
16
20
24
B
plasma free conc (nM)
12
10
8
6
4
2
0
0
4
8
12
16
20
24
C
brain conc (nM)
1600
1200
800
400
0
0
4
8
12
16
20
24
Time course (hr) after single dosing (mg/kg, PO)

Figure 8

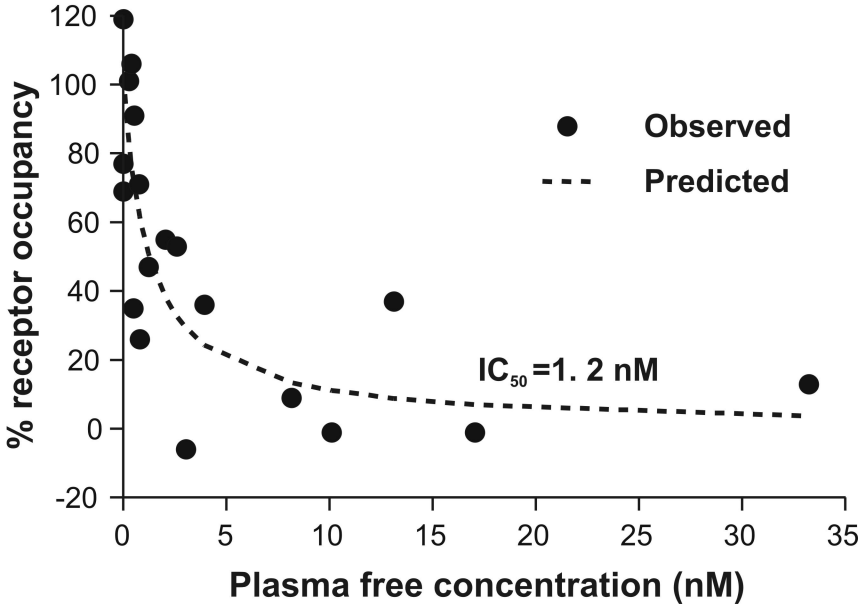
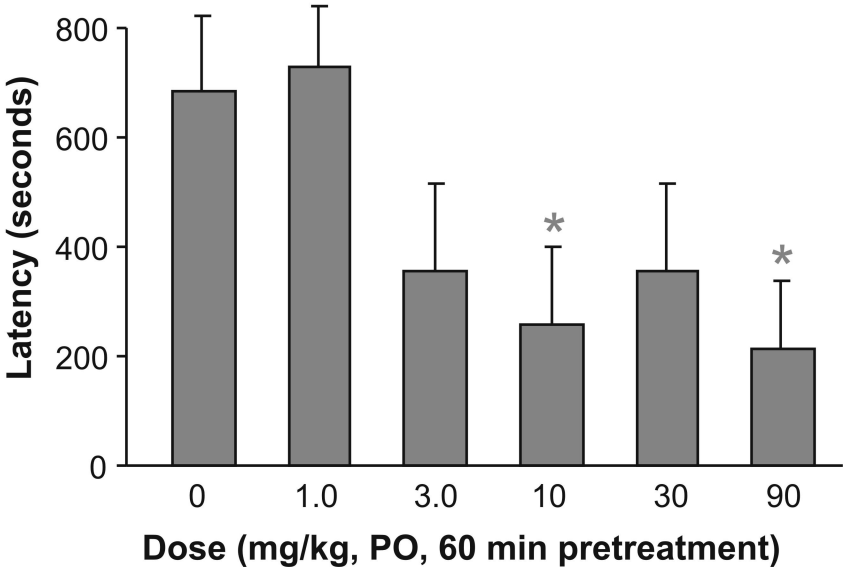

120
100
80
60
40
20
0
-20
% receptor occupancy
Observed
Predicted
IC50=1. 2 nM
0
5
10
15
20
25
30
35
Plasma free concentration (nM)

Figure 9


800
600
400
200
0
Latency (seconds)
*
*
0
1.0
3.0
10
30
90
Dose (mg/kg, PO, 60 min pretreatment)