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A chimeric human/murine anti-cocaine monoclonal antibody inhibits the distribution of cocaine to the brain in mice.

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Abbreviations: mAb, monoclonal antibody; BE, benzoylecgonine; EME, ecgoninemethylester; ELISA, enzyme- linked immunosorbent assay; H, heavy chain; L, light chain; Vdss, volume of distribution at steady state; $t_{1/2}$, elimination half-life in a non-compartmental pharmacokinetic model; $t_{1/2\alpha}$, distribution half-life in a two-compartment pharmacokinetic model; $t_{1/2\beta}$, terminal elimination half-life in a two-compartment pharmacokinetic model; AUC, area under the drug

concentration-time curve; MSTFA, trimethylsilyl-trifluoroacetamide; GC/MS, gas chromatography/mass spectrometry; BSA, bovine serum albumin.

Section: Neuropharmacology

Abstract

The predominantly human sequence, high-affinity anti-cocaine monoclonal antibody (mAb) 2E2, was cleared slowly from mouse blood by a first order process with an elimination half-life ($t_{1/2}$) of 8.1 days. Infused 2E2 also produced a dramatic dose-dependent increase in plasma cocaine concentrations and a concomitant decrease in the brain cocaine concentrations produced by an i.v. injection of cocaine HCl (0.56 mg/kg). At the highest dose of 2E2 tested (3:1, mAb:drug), cocaine was not detectable in the brain. Pharmacokinetic studies showed that the normal disappearance of cocaine from plasma was described by a 2-compartment pharmacokinetic model with distribution $t_{1/2\alpha}$ and terminal elimination $t_{1/2\,\beta}$ values of 1.9 and 26.1 min, respectively. In the presence of an equimolar dose of mAb 2E2 there was a 26-fold increase in the area under the plasma cocaine concentration-time curve (AUC) relative to the AUC in the absence of 2E2. Consequently, 2E2 decreased cocaine's volume of distribution from 6.0 l/kg to 0.20 l/kg, which approximated that of 2E2 (0.28 l/kg). However, cocaine was still rapidly cleared from plasma and its elimination was now described by a single compartment model with an elimination $t_{1/2}$ of 17 min. Importantly, 2E2 also produced a 4.5-fold (78%) decrease in the cocaine AUC in the brain. Therefore, the effect of 2E2 on plasma and brain cocaine concentrations was predominantly due to a change in the distribution of cocaine with negligible effects on its rate of clearance. These data support the concept of immunotherapy for drug abuse.

Introduction

Despite decades of basic and clinical research there is still no approved pharmacotherapy for the prevention of relapse in cocaine abusers (Vocci and Ling, 2005). The drug-induced reinstatement (priming) of drug self-administration behavior represents an animal model of relapse (DeWitt and Stewart, 1981) with the concentration of cocaine in the body a critical determinant of the probability of reinstating cocaine self-administration (Norman et al., 1999, 2002). As the site of action of cocaine is presumably in the brain, decreasing the concentrations reaching the brain would be expected to decrease the probability of relapse. Antibodies with high affinity and specificity for cocaine would be expected to act as pharmacokinetic antagonists by sequestering cocaine in the peripheral circulation and preventing its entry to the brain. Indeed, active immunization of animals with hapten-carrier conjugates can elicit the production of polyclonal anti-cocaine antibodies with sufficient levels and affinity for cocaine that they can reduce the amount of cocaine entering the brain (Fox et al., 1996). Active immunization has also been shown to attenuate the behavioral effects (Carrera et al., 1995; Fox et al., 1996; Ettinger et al., 1997) and the priming effect (Carrera et al., 2000) of systemically administered cocaine in rats. Furthermore, the ability of active immunization to produce levels of polyclonal anti-cocaine antibodies in humans (Kosten et al., 2002) that were associated with a decrease in use of cocaine (Martell et al., 2005) demonstrates the potential efficacy of immunotherapy for cocaine abuse.

Passive immunization using a monoclonal antibody (mAb) with a defined affinity, specificity and dose may be even more efficacious. Indeed, passive immunization with murine anti-cocaine mAbs attenuates the behavioral effects of cocaine (Fox et al., 1996; Mets et al., 1998; Carrera et

al., 2000) and therefore represents a potential alternative or adjunct to active immunization (Kosten and Owens, 2005). Unfortunately, murine sequence anti-cocaine mAbs would be expected to elicit an immune response in humans similar to that elicited by the murine mAb OKT-3 (Abramowicz et al., 1996) used for immunosuppression for organ transplant procedures with the potential to decrease or neutralize the long-term efficacy of an immunotherapeutic agent. A predominantly human sequence antibody is likely to decrease the probability of inducing such a neutralizing immune system response. Recently, we reported the generation and characterization of an anti-cocaine mAb, designated 2E2 (Paula et al., 2004) that was generated in transgenic mice that produce human sequence mAbs (Lonberg et al., 2005). It has now been established that mAb 2E2 has a human sequence γ_1 heavy and a murine λ light chain (see: Methods). In addition to 2E2, Redwan et al. (2003) have generated, characterized and "humanized" the murine anti-cocaine mAb, GNC92H2, the murine version of which has been demonstrated to have *in vivo* efficacy in rat models of cocaine addiction (Carrera et al, 2001). Also, two catalytic murine anti-cocaine mAbs that are designed to reduce blood cocaine levels through its hydrolysis have been generated and characterized (Larsen et al., 2004; Matsushita et al., 2001). However, the murine catalytic mAbs are likely to be immunogenic in humans. Furthermore, the antibody affinity for cocaine should also be a major determinant of clinical efficacy. Unfortunately, the affinities of the catalytic mAbs for cocaine were reported to be approximately 220 μM (Larsen et al., 2004) and 55 – 5,240 μM (Matsushita et al., 2001), while the affinity of the anti-cocaine mAb GNC92H2 was reported to be 200 nM (Larsen et al., 2001). In contrast, the affinity of 2E2 for cocaine is approximately 4 nM (Paula et al., 2004), which is considerably higher than that of the other anti-cocaine mAbs currently under study. Additionally, 2E2 has high specificity for cocaine over the major metabolites of cocaine.

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Therefore, 2E2 has important physicochemical properties that may be expected to confer efficacy as a passive immunotherapeutic agent.

Surprisingly, despite the demonstrated ability of anti-cocaine mAbs to antagonize the behavioral effects of cocaine, there have been no detailed studies of the effects of anti-cocaine mAbs on the pharmacokinetics or disposition of cocaine *in vivo*. Thus far, the pharmacokinetic antagonism by mAbs of drugs of abuse has been measured only for phencyclidine (PCP) (Valentine and Owens, 1996), methamphetamine (Laurenzana et al., 2003) and nicotine (Keyler at al., 2005). We have now manufactured and purified sufficient mAb 2E2 to test its effects on cocaine pharmacokinetics *in vivo*. We report herein our initial demonstration that the anti-cocaine mAb, 2E2, binds to and significantly alters the distribution of cocaine *in vivo* and thereby substantially decreases the brain concentrations of cocaine without decreasing its rate of elimination.

Methods:

Animals. Jugular vein catheterized male Swiss-Webster mice (22 – 28 g at the start of the studies) were purchased from Taconic Farms (Taconic, NY). Mice were housed individually with free access to food and water and kept on a 12 h light/dark cycle,. These studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals under a protocol approved by the Institutional Animal Care and Use Committee at the College of Medicine, University of Cincinnati.

Cocaine pharmacokinetic studies. Prior to the start of the studies the patency of the venous catheters was verified by demonstrating the ability to withdraw blood or inject normal saline via the catheter. The antibody (3-5 mg/ml) in phosphate-buffered saline (PBS) or vehicle (PBS) was infused at a rate of approximately 0.35 ml/min for up to two min, depending on the antibody concentration and the body weight of the animal, with the animal held under mild restraint. One hour after completion of the infusion of mAb, cocaine HCl (0.56 mg/kg) plus heparin (400 units/kg) was injected i.v. through the same catheter at a volume of 4.0 ml/kg body weight. At most sampling times, sodium pentobarbital (45 mg/kg, i.p) was administered three min prior to sacrificing the animal. For the 0.75 min time point the cocaine was injected into anesthetized mice. At the designated times after the injection of cocaine anesthetized mice were sacrificed by decapitation and trunk blood (typically 0.8 - 1.2 ml) was collected in a 1.5 ml polypropylene microcentrifuge tube containing 11.2 µl heparin (1.0 unit/µl) and NaF (16 mg/0.8 ml of blood) to inhibit, respectively, blood coagulation and enzymatic hydrolysis of cocaine (Warner and Norman, 2000). The blood samples were centrifuged at 5,000 x g for 3 min, then the plasma

(typically 0.4 - 0.8 ml) was carefully separated from packed red blood cells, placed into sterile 1.5 ml Eppendorf microcentrifuge tubes, rapidly frozen on dry ice and then stored at -80º C until analysis.

At the same time a separate sample of blood (approximately 100 µl) was collected from each mouse and rapidly frozen on dry ice then stored at –80º C. The concentration of hemoglobin and, where appropriate 2E2, was measured in these samples.

The whole brain was quickly removed from the decapitated mice, surface blood was blotted away, and the brain was placed in a polypropylene tube, rapidly frozen on dry ice and then stored at -80º C until analysis. For analysis, brains were weighed and cold deionized, distilled water added to produce a total volume of 1 ml, then homogenized and centrifuged at 13,000 rpm for 45 min at 4º C. The resulting supernatants (0.4 - 0.6 ml) were collected into sterile polypropylene microcentrifuge tubes and an aliquot (0.05- 0.40 ml) was processed for cocaine/metabolite analysis by GC/MS and hemoglobin content. Any remaining sample was stored at -80º C.

Determination of blood and brain hemoglobin concentrations. The hemoglobin contents of brain and blood were quantified spectroscopically by combining the method reported by Choudhri et al. (1997) and a protocol provided by Pointe Scientific, Inc. (MI). In this procedure, 10 µl aliquots of blood or 50 µl aliquots of brain homogenate supernatants were diluted with 90 µl hemoglobin reagent (0.6 mM $K_3Fe(CN)_6$, 0.7 mM KCN) in glass test tubes. The reaction was allowed to proceed at room temperature for 15 min with gentle mixing. When the reaction was complete aliquots from the standards and samples were all transferred into PVC microtiter plate

wells and the absorbance was measured at a wavelength of 490 nm for the measurement of cyanmethemoglobin formation. For the similarly prepared hemoglobin standards the absorbance was directly proportional to the hemoglobin concentration over the range used (0.3 – 12 g/dl). The standard curve was verified using control standards and the hemoglobin concentration in each sample was determined by comparison with the standard curve. The mean ± SEM concentration of hemoglobin in whole blood and brain were determined to be 8.90 ± 0.32 g/dl and 0.22 ± 0.04 g/dl, respectively. The average hemoglobin content in brain tissue relative to that present in whole blood was, therefore, approximately 2.5 %.

2E2 *in vivo* pharmacokinetic studies: Sample preparation. Mice, while under mild restraint, were administered mAb 2E2 (120 mg/kg, at 4.2 mg/ml in PBS) via an intravenous infusion over a 2 min period. Then at varying times, to obtain blood samples for mAb quantification, the mice were anesthetized using isoflurane and a sterile 27-gauage hypodermic needle or, alternatively, a sterile scalpel blade was used to puncture or make a small cut in a tail vein and 10 µl of blood was collected using a heparinized capillary pipette tip. The blood was immediately placed in a 1.5 ml polypropylene microcentrifuge tube containing 40 µl of 0.1 M sodium citrate/0.1% sodium azide pH 4.75. These samples were then rapidly placed on ice and then stored at 4º C until use. A blood sample was taken immediately prior to the infusion of 2E2 and then at 3, 15 and 30 min, 1, 2 , 4 and 8 hr, 1 day and periodically up to 49 days as shown in the results.

mAb 2E2 quantification: ELISA. The *in vivo* concentrations of 2E2 were determined using an enzyme-linked immunosorbent assay (ELISA) described previously (Paula et al., 2004) that compared the quantity of mAb in varying dilutions of the mouse blood samples to that quantified

in a standard curve generated using known dilutions of purified 2E2 or human IgG. Briefly, the conjugate benzoylecgonine-ovalbumin (3µg/ml, 100 µl/ well) in 1 mM EGTA pH 7.4 was adsorbed onto PVC 96-well microtiter plates for 1 hr. The plates were then washed 3 times with and all wells exposed for 10 min to 0.5% BSA in TBS (10 mM Tris, 140 mM NaCl and 0.02% NaN_3, pH 6.9) in order to block non-specific protein binding. The second layer added then was 100 µl/well of the blood samples diluted (1:500) into BSA-TBS and incubated for 2 hr. Serving as quantitation standards, duplicate 100 µl/well samples of human IgG or 2E2 diluted over a range of concentrations from 0.003 - 3.0 µg/ml were also similarly plated and incubated. The plates were washed with a Solution A, containing 0.5% BSA, 10 mM sodium phosphate, 145 mM NaCl, 1.5 mM $MgCl_2$, 0.05% triton X-100 and 0.02% NaN_3 , pH 7.2. Then 50 µl/well of affinity-purified biotinylated goat anti-human IgG diluted 1:500 in Solution A was added and incubated for 1 hr. After washing, 50 µl/well of streptavidin-alkaline phosphatase, diluted (1:200) in Solution A, was added, incubated for 1 hr and removed. Then 50µl/well of the colorimetric reaction mixture, comprised of the substrate para-nitrophenylphosphate (1 mg/ml) in substrate buffer (50 mM Na_2CO_3, 50 mM $NaHCO_3$ 1 mM $MgCl_2$ at pH 9.8), was added. After 6-8 min the reaction was stopped with 1M sodium hydroxide (50 µl/well). All steps were performed at room temperature. The reaction endpoint was measured with an ELISA reader (Molecular Devices) at a wavelength of 405 nm. Each determination was done in duplicate.

Antibodies: The hybridoma cell line secreting mAb 2E2 was generated using standard hybridoma technology by fusing spleenocyctes obtained from a transgenic mouse, strain HCo7/Ko5, following its immunization with benzoylecgonine (BE) coupled to 1,4-butanediamine-derivatized keyhole limpet hemocyanin (KLH) with the mouse cell line P3X63-

Ag8.653, as previously described (Paula et al., 2004). Production of mAb 2E2 was accomplished by growing hybridomas in severe combined immunodeficient (SCID) mice (at BioDesign International, Inc., Saco, ME) and collecting the ascites fluid. The hybridoma-secreted mAb was purified from ascites by sodium sulfate precipitation and a several step protein A-Sepharose column chromatography procedure adapted from that previously described (Ball, et al., 1999). Identification of the full length amino acid sequences of the polyacrylamide gel separated heavy and light chains of the 2E2 molecule was accomplished using liquid chromatography/mass spectroscopy (LC/MS/MS) analysis of their tryptic fragments. The heavy (H) chain was identified as a γ_1 protein of the human VH3 family gene DP-50. The light (L) chain was identified as a mouse λ VL2. The MS sequencing was consistent with and confirmed results obtained previously from Edman degradation NH_2-terminal sequencing of the Western blotted H and L chains as well as the sequencing of mRNA-dependent cDNA representing the 2E2, V_H and V_L chain regions (unpublished results, Dr. Amelia Black, Medarex, Inc., Nutley, NJ). The γ1 human H chain NH_2-terminal sequence was: EVQLVESGGGLVKPGGSLRL-, while the mouse λ chain NH_2-terminal sequence was: QAVVT/IQESALTTSPGGTV-. Although the 2E2 hybridoma has been shown to contain the recombined DNA sequence for a human κ L6 light chain (see Paula, et al., 2004) and this is consistent with the human κ chains of anti-digoxin antibodies generated from these transgenic mice in previous work (Ball et al., 1999), the L chain for the mAb expressed and used in these studies was a murine λ. These results are consistent with a recent report (Lonberg et al., 2005) that hybridomas from the HCo7/Ko5 strain of transgenic mouse can generate mixed-chain, human H, mouse L mAbs in addition to human sequence mAbs. Overall, 2E2 has an 87% sequence identity/homology with human IgG(λ)1 immunoglobulins.

The murine anti-cocaine mAb 3P1A6 obtained from BioDesign International, Inc., (Saco, ME) has previously been reported to have a high affinity (K_d = 0.2 nM) for cocaine and approximately 12-fold and 1,500-fold lower affinities for the inactive metabolites benzoylecgonine (BE) and ecgoninemethylester (EME), respectively (Paula et al., 2003). The murine anti-cocaine mAb B4E10 was purchased from The Binding Site (San Diego, CA) and has been determined to have a moderate affinity for cocaine (K_d = 40 nM) and approximately 30-fold and 50,000-fold lower affinities for BE and EME, respectively (unpublished data). Therefore, the murine mAbs and 2E2 have similar specificities for cocaine over its major metabolites, but an approximately 200-fold range in affinity. As an additional control, to test for non-specific *in vivo* effects resulting from infusion of mAb, non-specific human polyclonal IgG immunoglobulin (Sigma, St. Louis, MO) was administered to mice. These latter immunoglobulins had no measurable affinity for cocaine or its major metabolites (data not shown).

Solid Phase Extraction of cocaine and metabolites from plasma and brain. In order to determine *in vivo* concentrations of cocaine and its metabolites BE and EME, following the i.v. injection of cocaine, 100 - 400 µl samples of heparinized/NaF treated plasma and 400 µl samples of brain homogenates obtained from cocaine-treated animals were added to 2 ml of 0.1 M Na phosphate buffer, pH 6.0. This was followed by the addition of 5% trichloroacetic acid at a volume equal to that of the experimental sample (100 - 400 µl). These mixtures were shaken for 20 minutes and then centrifuged for 15 min at 7000 rpm, all at room temperature, in order to precipitate the denatured protein. The supernatants were collected and adjusted to pH 5.4 with 10 M NaOH. Then to serve as internal standards for establishing the identification of cocaine

and its metabolites as well as for normalization of the recovery of cocaine/metabolites from the mouse samples, 50 µl of a sample containing deuterated (D_3) cocaine-D_3, BE-D_3 and EME-D_3 (each at 1µg/ml) was added to all of the experimental and the standard control samples before their under going solid-phase extraction/column elution. Duplicate, standard control tubes (2 ml) were also prepared containing; 0.1 M Na phosphate buffer, 50 µl of the internal standards D_3 cocaine/BE/EME (1 µg/ml, each), 200 µl of normal mouse plasma and varying amounts of cocaine (1-500 ng) and used to generate the standard cocaine concentration curves. Similarly, standard concentration curves were also generated for BE and EME. Also 10 µl of the stock solution of cocaine HCl (0.139 mg/ml) that was infused into the mice was also mixed with the phosphate buffer and the cocaine-D_3/metabolite-D_3 standards for quantification of the cocaine administered to the animals. Thus, the cocaine/metabolite levels were determined relative to that of standard samples undergoing the same column extraction, elution and derivatization procedures. The procedure of Varian (Harbor City, CA) was used to extract and recover cocaine/metabolites from the plasma and brain samples and standards. First, Bond Elut Certify columns with the non-polar C8 sorbent, set in a Varian vacuum manifold were conditioned by washing with 2 ml methanol, followed by 2 ml of 0.1 M Na phosphate buffer, pH 6.0. Next, the prepared plasma and brain homogenate samples (2 ml) were loaded onto the Bond Elut columns. The columns were then washed with 6 ml of deionized water, 3 ml of 0.1 M HCl, and 9 ml of methanol. The column-bound analytes were then eluted with 2-3 ml of a freshly prepared solution of dichloromethane:2-propanol:ammonium hydroxide (mixed: 78:20:2, v/v/v). These extracts were then evaporated to dryness under nitrogen at 45º C for 15 min. The residue samples were derivatized with 25 µl N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) mixed with 25 µl ethyl acetate. These samples were vortexed and incubated at 65º C for 30 min.

After cooling, the trimethylsilyl-derivatized samples were transferred to glass autosampler vials for analysis by GC/MS. The GC/MS analysis of analytes was typically completed within 1 – 2 hours of sample derivatization. Analyses carried out more than eight hours after derivatization were discarded.

Gas chromatography/mass spectrometry. The gas chromatograph/mass spectrometer (GC/MS) consisted of a Shimadzu GC 17A series GC, interfaced with a Shimadzu QP-5050A quadrupole MS (Shimadzu, Columbia, MD) fixed in an electron impact ionization mode with selective ion monitoring. The GC/MS was operated with a transfer line temperature of 280º C and a source temperature of 280º C. The MS was calibrated on a daily basis using perfluorotributylamine. The electron multiplier voltage was set at 1.2 kV. Chromatographic separation was achieved using a Restek Rtx-5MS cross linked 5% diphenyl-, 95% dimethylsiloxane capillary column (30 m x 0.25 mm i.d, 0.25 µm film thickness). Helium was the carrier gas and used at a flow rate of 1.0 ml/min. A Shimadzu AOCs autosampler was used to inject 2 µl of extract sample into the GC/MS. The GC, equipped with split/splitless injection port, was operated at 280º C in the splitless mode with a high pressure injection set at 150 kPa for 0.75 min. The oven temperature profile was established as follows: the initial temperature was 100º C and it was held for 1 min, then increased at a rate of 20º C/min up to 320º C. This temperature was held for 8 min resulting in a total run time of 20 min. The lower limits of cocaine/BE/EME detection ranged from 1–5 ng/ml and the linear dynamic range for most analytes was 1-3000 ng/ml. The instrument performance was evaluated by analysis of the calibrator and control samples. Analytes were identified and their concentrations were determined using both the internal deuterated standards and concentration control samples

prepared with normal mouse serum, respectively, as described above. The response factor was determined for each analyte. The response factor was calculated by dividing the area of the analyte peak by the area of the internal standard peak. Calibration curves were then prepared by plotting a linear regression of the analyte/internal standard response factor versus the analyte concentration for all calibrators analyzed. The standard curve was constructed using a set of cocaine/metabolite samples varying over a concentration range of 1-500 ng/ml. The standard curve was used to determine concentrations of analytes in both control and experimental samples.

Chemicals reagents and reference standards: Standard solutions of cocaine, BE and EME (each 1 mg/ml) were prepared in methanol or acetonitrile and served as stock solutions for preparing the reference standard curves. The cocaine-D_3, BE-D_3 and EME-D_3 that were used as the internal standards (0.1 mg/ml each in methanol or acetonitrile) were purchased from Radian International (Austin, TX). The derivatizing reagent MSTFA was purchased from Pierce Chemicals (Rockford, IL). Normal mouse plasma with heparin was obtained from Harlan Bioproducts for Science (Indianapolis, IN). The human hemoglobin standards and control standards were obtained from Pointe Scientific, Inc. (Lincoln Park, MI). All other chemicals and immunoreagents were purchased from Sigma-Aldrich (St. Louis, MO) or Pierce Chemicals (Rockford, IL). All reagents and organic solvents were of analytical grade or HPLC grade.

Data analysis and statistics: Cocaine and 2E2 pharmacokinetic data were analyzed using the program WinNonLin (Pharsight Corporation, Mountain view, CA). The program provides Akaike Information Criterion (AIC) and Schwartz Bayesian Criterion (SBC) measures of

"goodness of fit" of the data to the one or two compartment pharmacokinetic models that were used. Data were first analyzed according to a single compartment pharmacokinetic model. In some experiments a single compartment model gave a poor fit to the cocaine pharmacokinetic data, as assessed by a systematic deviation of the model from the data. In these cases the fit to the data was improved by applying a two compartment pharmacokinetic model that assumed cocaine distributed between a central and a peripheral compartment. In addition to an improvement in the AIC and SBC measures, the improvement of the fit of the model to the data was evaluated by a lack of a systematic deviation from the data points and a concomitant reduction in the sum of squares residuals. Applying pharmacokinetic models that assumed that cocaine distributed between more than two compartments only slightly improved the fit to the observed data and this additional complexity was considered unnecessary. Statistical comparisons of the cocaine and metabolite levels observed in the presence and absence (vehicle) of antibody at the single 5 min time point used non-parametric Mann-Whitney test while the Analysis of Variance (ANOVA) procedure was used to compare the results obtained over different experimental days.

Results

The pharmacokinetics of mAb 2E2. In determining the pharmacokinetics of mAb 2E2 in mice, the first samples of tail vein blood were taken 3 minutes after completion of the i.v. infusion of 2E2 (120 mg/kg) via the jugular vein of mice. The initial mean ± SEM blood concentration of mAb was determined to be 370 ± 17 μg/ml (n = 8 mice). As shown in Fig. 1 there was no evidence for an initial decrease in blood concentrations over the first 24 hours. Indeed, 2E2 concentrations increased slightly over the first four hours and then appeared to plateau for approximately 20 hours. The mean concentration of 2E2 as measured 24 hours after infusion was 422 ± 21 μg/ml. After 24 hours, the concentrations of 2E2 in blood then began to decline and this was adequately described by a single compartment pharmacokinetic model with an elimination $t_{1/2}$ of 8.1 days (Fig. 1). This model gave a calculated volume of distribution at steady state (Vdss) for 2E2 in this group of mice of 0.28 l/kg.

The plasma pharmacokinetics of cocaine. Next, the disposition of cocaine in mouse plasma subsequent to its i.v. injection via the jugular vein was determined. The highest plasma concentrations measured (~110 ng/ml) were observed at the earliest sample time, after which cocaine concentrations declined rapidly (Fig. 4A). A pharmacokinetic model assuming that cocaine distributed between a central and a peripheral compartment improved the fit to the observed data as compared to a single compartment model. This result is similar to that which has previously been reported for i.v. injected cocaine in several species including rats (Booze et al., 1997; Lau and Sun, 2002), non-human primates (Misra et al., 1977; Evans and Foltin, 2004) and for i.p. injected cocaine in mice (Benuck et al., 1987). The simplest pharmacokinetic model

that provided a general description of the data generated parameter estimates for the distribution half-life ($t_{1/2\alpha}$) and terminal elimination half-life ($t_{1/2\beta}$) for cocaine of 1.9 and 26.1 min, respectively. The calculated Vdss was 6.0 l/kg.

Effect of cocaine-specific mAbs on cocaine distribution. In these experiments a single time point, 5 minutes after i.v. cocaine administration, was selected at which to determine the effect of circulating anti-cocaine mAbs on the *in vivo* plasma and brain levels of cocaine. As shown in Table 1, at 5 minutes considerable distribution of cocaine has occurred as the plasma concentration (~ 77 ng/ml) has declined from an initial value of ~110 ng/ml (45 sec, Fig. 4A) and brain levels (~1070 ng/g) are about 10-fold higher than that in plasma. The presence of mAb 2E2 then produced a substantial 29-fold increase in plasma and an almost 5-fold decrease in brain cocaine concentrations (Table 1) in comparison to the vehicle controls. Furthermore, pretreatment with the mouse anti-cocaine mAbs 3P1A6 (K_d = 0.2 nM, Paula et al., 2003) and B4E10 (K_d = 40 nM, unpublished results) also similarly increased cocaine concentrations in plasma, while they were somewhat less effective than 2E2 in decreasing cocaine concentrations in the brain. These results clearly demonstrated the capability of cocaine-specific mAbs for *in vivo* binding of cocaine. In contrast, the pretreatment with non-specific human polyclonal antibodies with no measurable affinity for cocaine produced a small increase in cocaine concentrations in both plasma and brain relative to those in mice pretreated with the vehicle (PBS).

The dose-dependent effect of 2E2 on plasma and brain cocaine concentrations. In view of the magnitude of the effects of stoichiometric doses of the anti-cocaine mAbs on the plasma and brain cocaine concentrations, the dose-dependency for the responses was determined using mAb

2E2. In the absence of 2E2 the mean ± SEM plasma cocaine concentration at 5 min post cocaine injection was 78.5 ± 4.5 ng/ml (Fig. 2A). Infused 2E2 produced a dose-dependent increase in plasma cocaine concentrations (Fig. 2A). The lowest dose of 2E2 (12 mg/kg, a 1:10 mAb:cocaine ratio) produced a significant ($p < 0.01$, one-way ANOVA with post-hoc test) 5.1-fold increase in plasma cocaine concentrations while the highest dose (360 mg/kg, a 3:1 ratio) produced a dramatic 46.1-fold increase in cocaine concentrations. The calculated dose of 2E2 that produced 50 % of the highest effect for the range of 2E2 doses used (ED_{50}) was approximately 80 mg/kg, a somewhat less than stoichiometric amount of 2E2.

In the absence of 2E2 the mean ± SEM brain cocaine concentration at 5 min post injection, corrected for cocaine present in cerebral blood, was 796.8 ± 50 ng/ml (Fig. 2B). This represented a brain:plasma cocaine concentration ratio of 10:1. 2E2 then produced a dose-dependent decrease in brain cocaine concentrations (Fig. 2B). At the dose of 24 mg/kg, 2E2 produced a significant 35% decrease in cocaine concentrations. At the 2E2 dose of 360 mg/kg, after correction for cocaine present in the residual blood, the brain cocaine concentration was negligible. The ED_{50} for the range of 2E2 doses used was approximately 60 mg/kg.

The effect of 2E2 on cocaine metabolite concentrations in plasma and brain. An additional point of interest was the determination of the effects of circulating 2E2 on the *in vivo* metabolism of cocaine. As shown in Fig. 3, at 5 min after the injection of cocaine in the absence of 2E2, mean concentrations of the predominant cocaine metabolite in mice, EME, which results largely from plasma butyrylcholinesterase activity, were 25 ng/ml and 267 ng/g in plasma and brain, respectively. This represented a brain:plasma ratio for EME of 10.7:1, a ratio similar to that of

cocaine in these mice (Fig. 2), thus the cocaine:EME ratio was ~3:1 in both plasma and brain. Produced by non-specific liver carboxylesterase activity, BE levels were lower with mean concentrations of 7 ng/ml and 16 ng/g in plasma and brain, respectively, representing a brain:plasma ratio of 2.3:1. A modest increase (~3-fold) in plasma BE concentrations was observed with increasing doses of 2E2 but the effect approached a plateau at 2E2 doses above 40 mg/kg. There was a concomitant decrease in brain BE concentrations which was observed at doses above 40 mg/kg. These results were consistent with mAb 2E2 having no effect on BE production but a sufficiently high affinity for BE to sequester some in the plasma but its levels are limited. In contrast, plasma EME concentrations appeared unaffected at the lower doses of 2E2, but an approximate 2-fold reduction was observed at the highest dose of 2E2. There was no systematic effect of 2E2 dose on brain EME concentrations (Fig. 3). It is of note, that despite 2E2's effective *in vivo* binding of cocaine, its alterations in cocaine's initial metabolism appeared modest.

Effect of 2E2 on the pharmacokinetics of cocaine in plasma and brain. Next, we determined the effects of a stoichiometric dose of 2E2 on the pharmacokinetics of a single injection of cocaine. As shown in Fig. 4A, in the presence of 2E2, the peak plasma concentrations (~1,100 ng/ml) of cocaine were observed at the earliest time point (45 sec) sampled after its injection. This is similar to what was observed in the absence of 2E2. However, the peak plasma concentration was 11.3-fold higher in the presence than in the absence of 2E2. Furthermore, in contrast to the biexponential decrease in the concentrations of cocaine observed in the absence of 2E2, the decrease in the cocaine concentration was well described by a pharmacokinetic model that assumes a single compartment and no initial distribution phase. Thus, the calculated $t_{1/2}$ for

the disappearance of cocaine from plasma in the presence of 2E2 was 17.1 min and this contrasts with the distribution and elimination phases, with parameter estimates for $t_{1/2\alpha}$ and $t_{1/2\beta}$ of 1.9 and 26 min, respectively, obtained in the control study. 2E2 also produced a sustained increase in the plasma cocaine concentration that resulted in 26-fold increase in the area under the concentration-time curve (AUC) in plasma. Consistent with this result, the calculated Vdss of cocaine in the presence of 2E2 was 0.2 l/kg as compared to 6.0 l/kg in the absence of 2E2.

As shown in Fig. 4B, the cocaine concentration-time profile in brain differed substantially from that observed in the plasma (Fig. 3A). The concentration of cocaine in the brain (corrected for cocaine present in residual blood) at 45 sec (~650 ng/g) after the injection was approximately 6-fold higher than that measured in plasma. The brain cocaine concentrations subsequently increased further and the highest measured concentration was observed at 3 min (~1,500 ng/g), after which concentrations then rapidly declined. A pharmacokinetic model that assumed a first-order input to the brain and a first-order output was used to describe the increase and subsequent decrease in brain cocaine concentrations. The estimated $t_{1/2}$ for entry into the brain was approximately 2.0 min. Furthermore, a pharmacokinetic model assuming two compartments described the disappearance of cocaine from the brain. The parameter estimates for $t_{1/2\alpha}$ and $t_{1/2\beta}$ were 2.0 min and 14.5 min, respectively (Fig. 4B), values similar to those obtained for the plasma. However, given the relatively few data points and the relatively complex time/concentration profile, these parameter estimates should be considered tentative.

In the presence of 2E2, the peak cocaine concentration (~490 ng/g) was observed at the earliest sample time and it subsequently declined rapidly over time (Fig. 4B). There was no indication of

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the normal delayed influx and peak of the cocaine concentrations in the brain and a single compartment model approximated the decline in cocaine concentrations. The estimated $t_{1/2}$ was 3.8 min, a value considerably faster than the $t_{1/2\beta}$ value obtained in the absence of 2E2. Importantly, 2E2 produced an approximately 4.5-fold (78 %) decrease in the cocaine AUC in the brain.

Discussion

The low volume of distribution of 2E2 observed in mice is similar to that previously reported for several murine and rat monoclonal IgG_1 antibodies and human polyclonal IgG_1 antibodies in rats (Bazin-Redureau et al., 1997) and is consistent with 2E2's distribution being predominantly restricted to the blood volume. Additionally, the elimination $t_{1/2}$ value for 2E2 was relatively long and similar to that reported for other murine, rat and human antibodies in rats (Bazin-Redureau et al., 1997). This indicates that 2E2's effects on cocaine pharmacokinetics could persist for several days after a single injection. Furthermore, the terminal elimination $t_{1/2}$ of cocaine is more than 400-fold faster than that of 2E2 and, therefore, it can be assumed that the plasma concentration of 2E2 was constant during the study of cocaine pharmacokinetics. Interestingly, although the V_d and $t_{1/2}$ for 2E2 were similar to those previously described for antibodies in rodents, there was no evidence for an initial distribution of 2E2 from the blood to the interstitial spaces. Determining whether this reflects a particular property of 2E2 will require additional studies.

As to 2E2's *in vivo* binding of cocaine, its effect on the plasma concentration of cocaine was saturable, which is consistent with the limited number of cocaine molecules present. Furthermore, doses of 2E2 that were only 10% to 30% of the dose of cocaine still provided a measurable increase in plasma cocaine concentrations and a decrease in exposure of the brain to cocaine. This is consistent with reports that a 0.3 molar ratio of anti-phencyclidine (PCP) Fabs decreased the behavioral effects of PCP in rats (Valentine et al., 1996). Furthermore, a 4 mg dose of an anti-cocaine mAb, representing a molar ratio of approximately 0.005, has been reported to antagonize the behavioral effects of repeated 1 mg/kg doses of cocaine HCl (Fox et

al., 1996; Fox, 1997). While, 30 and 40 mg/kg doses of another murine anti-cocaine mAb decreased the self-administration of cocaine at molar ratios of approximately 0.2 for each cocaine injection (Carrera et al., 2000). The finding that 2E2 produced a substantial reduction in the brain's exposure to cocaine at equimolar ratios and has measurable effectiveness at lower molar ratios indicates that 2E2 will reduce brain concentrations even after a mAb dose has been partially eliminated. Thus the efficacy of a given dose of 2E2 will be prolonged.

The demonstration that an equimolar dose of a nonspecific antibody did not significantly alter either plasma or brain concentrations of cocaine ruled out the possibility of nonspecific effects of infused IgG proteins as an explanation for the mAb effects on cocaine pharmacokinetics. Therefore, the efficacy of anti-cocaine mAbs requires specificity of the binding interaction between the drug/antibody molecules. However, the three anti-cocaine mAbs with affinities ranging from very high (K_d = 0.2 nM) to modest (K_d = 40 nM, as measured *in vitro* at equilibrium) were approximately equipotent under the limited *in vivo* experimental conditions tested. This suggests that the ability of an antibody to influence the pharmacokinetics of cocaine may not be highly affinity sensitive. Therefore, antibodies with a fairly broad range of affinities may have clinical efficacy. Whether antibodies with low affinity, that is having K_ds in the μM range, can also influence cocaine distribution to the brain has yet to be determined, though such antibodies have been reported to ameliorate some behavioral effects of cocaine in rodents (Mets et al., 1998).

In the presence of 2E2, the initially observed approximately 10-fold increase in the concentration of cocaine in plasma, the lack of an initial distribution phase from the plasma and the reduction

of the Vdss of cocaine to essentially that of 2E2 are all consistent with 2E2 restricting cocaine's distribution predominantly to the blood volume. Therefore, the 2E2-induced decrease in brain cocaine concentrations is due to an inhibition of cocaine distribution from the blood to the brain. Furthermore, the reduction in the peak levels and the distribution of cocaine to the brain occurred at all time points, indicating that 2E2 did not simply delay cocaine's distribution to the brain. This report is the first to demonstrate that an anti-cocaine mAb can prevent the entry of cocaine into the brain and it is consistent with previous reports that active immunization-induced anti-cocaine antibodies decreased cocaine levels in brain after i.v., intranasal or i.p. cocaine administration (Fox et al., 1996, 1997; Carrera et al., 2000). The ability of 2E2 to decrease brain concentrations of cocaine is also consistent with the mAb-induced reductions observed for other psychoactive drugs such as phencyclidine (Valentine et al., 1996; Proksch et al., 2000), methamphetamine (Laurenzana et al., 2003) and nicotine (Keyler et al., 2005) in rats.

The markedly altered distribution of cocaine was presumably the result of cocaine binding to 2E2. This mAb binding of cocaine might be expected to also restrict cocaine's access to the enzymes that metabolize it, thereby decreasing its clearance. However, there was no evidence of an increase in the elimination $t_{1/2}$ of cocaine in plasma. This is consistent with the reported lack of effect of active immunization on either the elimination of cocaine from plasma (Fox et al., 1996; Fox, 1997) or on the rate of metabolism of nicotine (de Villiers et al., 2004). However, a murine anti-nicotine mAb (Keyler et al., 2005) and active immunization against nicotine (Keyler et al., 1999) have also been reported to significantly increase the elimination $t_{1/2}$ of nicotine in rats. The reasons for these discrepant results are not clear at present but do not appear to be related to different affinities of the mAbs or the polyclonal antibodies for their target molecules.

If anti-drug antibodies can inhibit the metabolism and slow the rate of drug elimination this would increase the *in vivo* concentrations resulting from repeated drug doses and may not be desirable for an immunotherapeutic agent. Importantly, the lack of effect of 2E2 on cocaine elimination from plasma should minimize the potential for 2E2 to become saturated following repeated doses of cocaine.

In contrast to the rat, where BE is the major metabolite of cocaine (Booze et al., 1997; Warner and Norman, 2000), in the present studies it was found that in mice at 5 min following the administration of cocaine, EME concentrations were approximately 2-3-fold higher than BE in both plasma and brain. Importantly, while these data only provide information at a single time point of cocaine's disposition, they indicate that the metabolism of cocaine to both BE and EME is generally unaffected by 2E2's binding of cocaine. The modest increase in plasma BE concentrations and decrease in brain BE concentrations accompanying the increasing 2E2 doses may indicate that 2E2 has a sufficient affinity for BE to alter its distribution as well as that of cocaine. The apparent plateau in this effect at the higher doses of 2E2 may reflect the saturation of the limited amounts of BE formed. Additionally, the lack of any significant effect of 2E2, at doses up to 120 mg/kg, on the plasma and brain concentrations of EME is consistent with its having no effect on cocaine's metabolism and a low affinity for EME (Paula et al., 2004). The apparent decrease in plasma EME concentration observed at a dose of 2E2 (360 mg/kg) that is greater than equimolar may indicate that cocaine's metabolism to EME was decreased. If so, then the stoichiometry between the antibodies and their target molecules may influence the antibody's effects on the rate of elimination/metabolism of drugs. Based on their physicochemical properties, EME and BE should be charged and relatively lipophobic.

Consequently, EME and BE formed in the periphery would not be expected to readily cross the blood-brain barrier and the brain:plasma ratio of EME and BE should be low. However, since brain:plasma concentration ratios for cocaine and EME were similar this may indicate that cocaine is metabolized to EME in the brain. Unexpectedly, at the highest dose of 2E2 given, cocaine could not be detected in the brain yet the brain concentration of EME was not affected. Clearly, the metabolism of cocaine and the disposition of its metabolites require additional study.

In summary, the high affinity anti-cocaine mAb 2E2 limited the distribution of cocaine to the plasma thus decreasing the levels of cocaine reaching the brain without any detectable effect on the rate of elimination of cocaine. These data further support the general concept of the usefulness of immunotherapy for the treatment of drug abuse and they are consistent with mAb 2E2 being effective as a passive immunotherapy for the prevention of relapse in cocaine abuse.

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Legends For Figures

Figure 1. The pharmacokinetics of the anti-cocaine mAb 2E2. Mice received an i.v. infusion of 120 mg/kg of 2E2. Samples of blood (10 µl) were obtained from tail veins at the indicated times after the completion of the mAb infusion. Concentrations of 2E2 in blood were determined using an ELISA. Data points represent the mean ± SEM from 8 mice. The Vdss was approximately 0.28 l/kg. A single compartment model with a $t_{1/2}$ of 8.1 days adequately described the elimination phase, represented by the best-fit regression line through the data points.

Figure 2. The dose-dependent effect of 2E2 on plasma (A) and brain (B) concentrations of cocaine. Mice were injected with vehicle or 2E2 at doses of 12, 24, 40, 120 or 360 mg/kg. One hour after the infusion of vehicle or 2E2 was completed, an i.v. bolus of cocaine HCl was administered and after five min the samples were collected. Cocaine concentrations were measured using GC/MS. Symbols represent the mean ± SEM from three mice. The line through the data points represents the best fit according to a hyperbolic function. The ED_{50}s of 2E2 for decreasing the cocaine concentration in the brain and increasing the plasma cocaine concentration were approximately 50 and 60 mg/kg, respectively.

Figure 3. The effect of 2E2 on plasma and brain concentrations of cocaine metabolites. The concentrations of BE and EME were measured in the same tissue samples used in Figure 2.

Plasma and brain concentrations of BE are represented by closed and open squares, respectively. Plasma and brain concentrations of EME are represented by closed and open triangles, respectively.

Figure 4. The effect of 2E2 on the pharmacokinetics of cocaine in plasma (A) and brain (B). Mice received an i.v. infusion of 120 mg/kg of 2E2. One hour later the mice received an i.v. injection of cocaine HCl (0.56 mg/kg). The animals were sacrificed at the indicated times and samples were collected. Cocaine concentrations were determined by GC/MS. The data points represent the mean ± SEM from three mice. In the absence of 2E2 (open circles), the cocaine concentration-time profile in plasma (A) was described by a two-compartment pharmacokinetic model with a $t_{1/2\alpha}$ of 1.9 min and a $t_{1/2\beta}$ of 26.1 min. In the presence of 2E2 (closed circles), a single compartment pharmacokinetic model indicated a $t_{1/2}$ of 17.1 min. In the brain (B) in the absence of 2E2 (open circles) a two-compartment pharmacokinetic model with first order input into the first compartment described the cocaine concentration-time profile. The calculated input $t_{1/2}$ was 2.0 min and the $t_{1/2\alpha}$ and $t_{1/2\beta}$ values were 2.0 min and 14.5 min, respectively. In the presence of 2E2 (closed circles), a single compartment pharmacokinetic model with first order elimination of cocaine indicated an elimination $t_{1/2}$ value of 3.8 min.

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Table 1. The effect of anti-cocaine monoclonal antibodies on cocaine concentrations in plasma and brain

	Plasma cocaine concentration (ng/ml)	Change from vehicle	Brain cocaine concentration (ng/g)	Change from vehicle
Vehicle	76.6 ± 3.3 (n = 23)		1070.5 ± 32.1 (n = 22)	
Human IgG	121.2 ± 5.6* (n = 6)	+ 1.6-fold	1568 ± 130.5* (n = 5)	+ 1.5-fold
2E2	2197.7 ± 75.8** (n = 6)	+ 28.7-fold	223.7 ± 25.5** (n = 6)	- 4.8-fold
3P1A6	2215.5 ± 157.2** (n = 6)	+ 28.9-fold	469.2 ± 68.9** (n = 6)	- 2.3-fold
B4E10	1591.5 ± 57.8** (n = 6)	+ 20.8-fold	560.5 ± 62.4** (n = 6)	- 1.9-fold

Mice were injected with vehicle (PBS) or antibody (120 mg/kg i.v.) and 1 hour later were injected with cocaine HCl (0.56 mg/kg i.v.). At 5 min after the injection of cocaine the mice were sacrificed and plasma and brain were collected. Non-specific human IgG served as a control. Values represent the mean ± SEM as obtained from the number of mice shown in parentheses. There were no significant differences of the values for the vehicle treated mice between experiments ($p > 0.1$, one-way ANOVA) and the values were pooled. Statistically different from the corresponding control value, *$p < 0.01$, **$p < 0.001$ Mann-Whitney test. Statistical comparisons between vehicle and antibody-treated mice were performed within experiments. The + and – designate an increase or a decrease, respectively, from the corresponding values from the vehicle treated mice.

Figure 1. Norman et al.

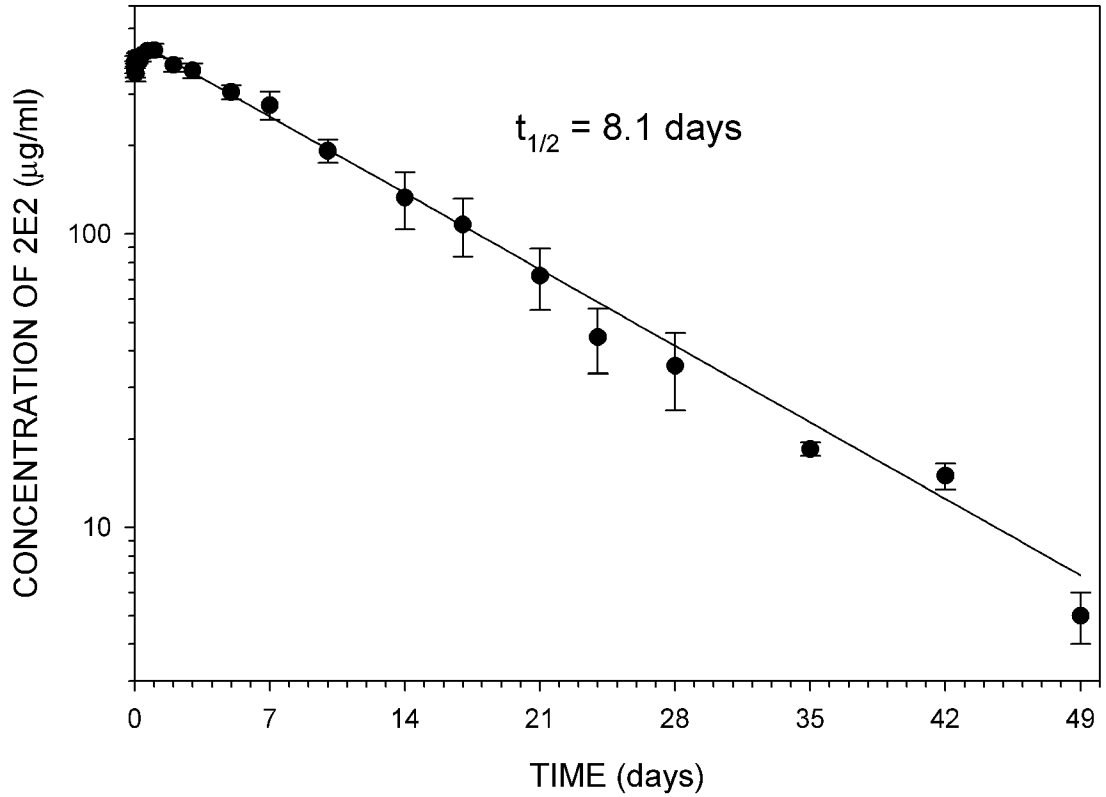
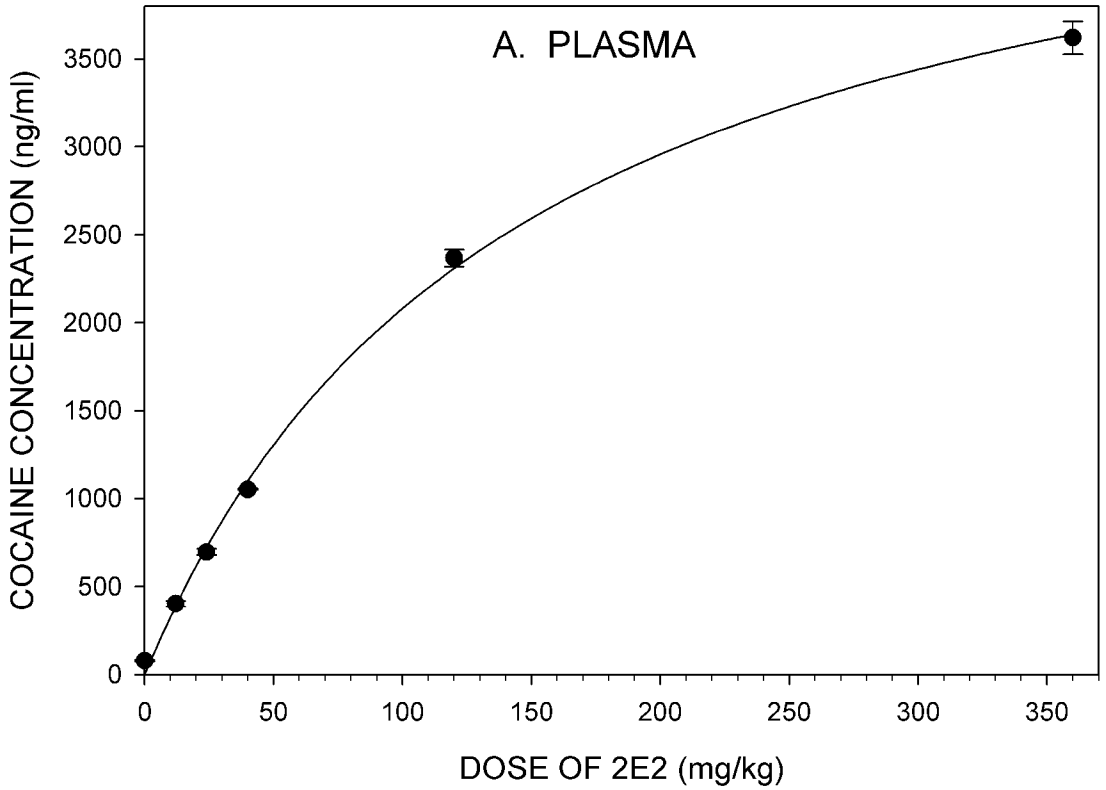


Figure 2. Norman et al.



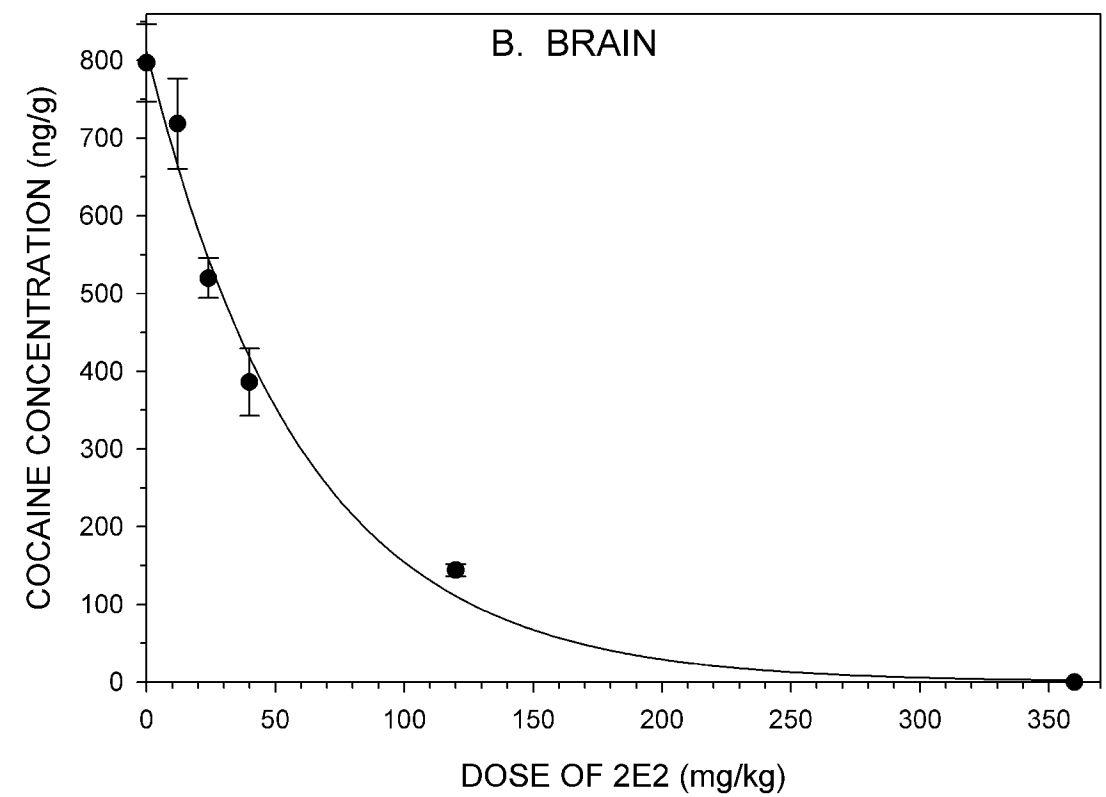


Figure 3, Norman et al.

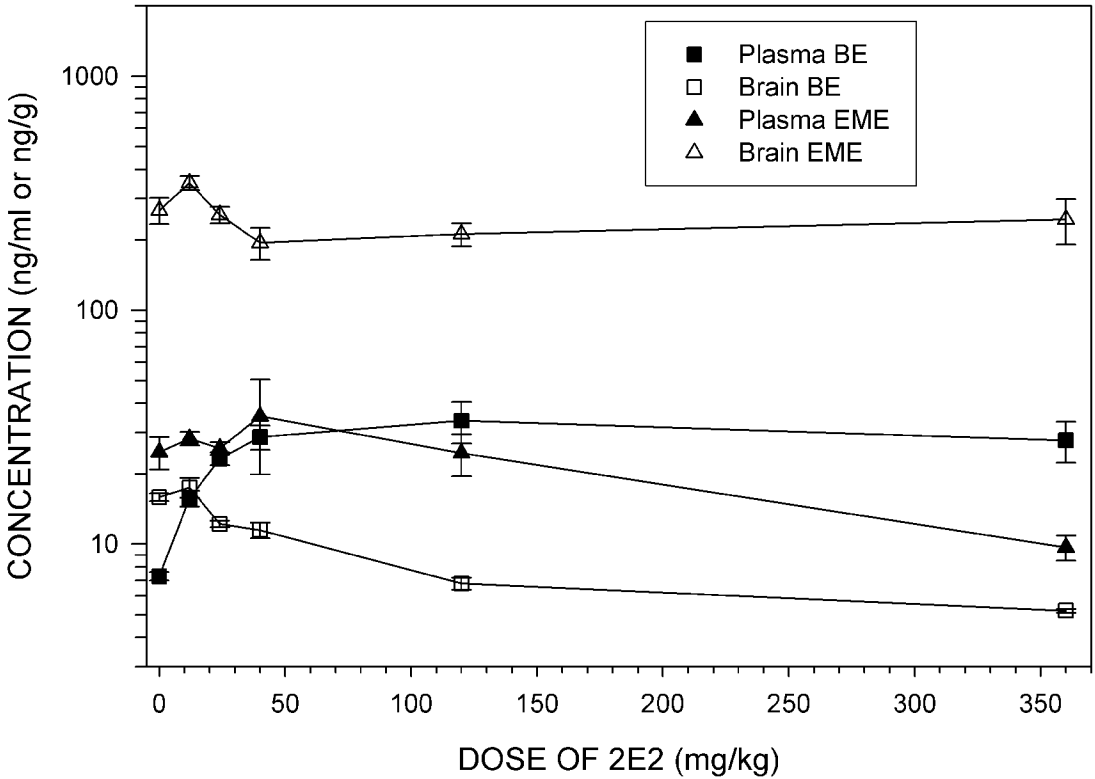


Figure 4. Norman et al.