Treatment with a Monoclonal Antibody against Methamphetamine and Amphetamine Reduces Maternal and Fetal Rat Brain Concentrations in Late Pregnancy

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ABSTRACT

We hypothesized that treatment of pregnant rat dams with a dual reactive monoclonal antibody (mAb4G9) against (+)-methamphetamine [METH; equilibrium dissociation rate constant (K_D) = 16 nM] and (+)-amphetamine (AMP; K_D = 102 nM) could confer maternal and fetal protection from brain accumulation of both drugs of abuse. To test this hypothesis, pregnant Sprague-Dawley rats (on gestational day 21) received a 1 mg/kg i.v. METH dose, followed 30 minutes later by vehicle or mAb4G9 treatment. The mAb4G9 dose was 0.56 mole-equivalent in binding sites to the METH body burden. Pharmacokinetic analysis showed baseline METH and AMP elimination half-lives were congruent in dams and fetuses, but the METH volume of distribution in dams was nearly double the fetal values. The METH and AMP area under the serum concentration-versus-time curves

from 40 minutes to 5 hours ($AUC_{40\text{ min}}^{5\text{ h}}$) after mAb4G9 treatment increased >7000% and 2000%, respectively, in dams. Fetal METH serum $AUC_{40\text{ min}}^{5\text{ h}}$ did not change, but AMP $AUC_{40\text{ min}}^{5\text{ h}}$ decreased 23%. The increased METH and AMP concentrations in maternal serum resulted from significant increases in mAb4G9 binding. Protein binding changed from ~15% to > 90% for METH and AMP. Fetal serum protein binding appeared to gradually increase, but the absolute fraction bound was trivial compared with the dams. mAb4G9 treatment significantly reduced METH and AMP brain $AUC_{40\text{ min}}^{5\text{ h}}$ values by 66% and 45% in dams and 44% and 46% in fetuses ($P < 0.05$), respectively. These results show anti-METH/AMP mAb4G9 therapy in dams can offer maternal and fetal brain protection from the potentially harmful effects of METH and AMP.

Introduction

Approximately half of the (+)-methamphetamine (METH) users are female (Cohen et al., 2007). Therefore, it is inevitable that some women will use METH during pregnancy. Indeed, 24% of the pregnant women seeking admission to drug treatment programs in 2009 had used METH (Terplan et al., 2009). In contrast, METH accounted for only 8% of the pregnant women seeking admission in 1994.

METH exposure in utero can cause reproductive, developmental, and behavioral toxicity (Golub et al., 2005). In animal and clinical

studies, adverse maternal and fetal outcomes include premature delivery, low birth weight, reduced head circumference, optic defects, neurochemical alterations, and behavioral, motor, and learning deficits (Oro and Dixon, 1987; Acuff-Smith et al., 1996; Cernerud et al., 1996; Slamberová et al., 2006; Chang et al., 2007). METH-related adverse effects in newborns, which include poor feeding, tremors, hypertonia, and abnormal sleep patterns, appear related to withdrawal from METH (Oro and Dixon, 1987). Children (ages 3–16) who are exposed prenatally to METH score lower on attention and memory tests than nonexposed children, which correlates with reductions in subcortical brain volume in areas associated with learning (Chang et al., 2004). Furthermore, neuroimaging studies of adult METH users and children who are exposed to METH in utero show reductions in dopamine (D2) receptors, dopamine transporters, serotonin transporters, and vesicular monoamine transporter-2 in the striatum (Chang et al., 2007).

Protecting the health of both the mother and fetus from harmful METH-induced effects presents a challenging medical problem. The potential for drug interactions and unwanted side effects (Scolnik et al., 1994; Eadie, 2008) adds more challenges. For instance, phenytoin, an anticonvulsant used to treat METH-induced seizures, can elicit teratogenic effects, and children exposed to phenytoin in utero score

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ABBREVIATIONS: λ_n , terminal elimination rate constant; AMP, (+)-amphetamine; AUC, area under the curve; Cl_T , total clearance; GD, gestational day; K_D , equilibrium dissociation rate constant; mAb, monoclonal antibody; METH, (+)-methamphetamine; PCP, phencyclidine; $t_{1/2}$, terminal elimination half-life; V_d , apparent volume of distribution.

significantly lower on intelligence quotient and language tests (Scolnik et al., 1994).

Treatment of adult male Sprague-Dawley rats with an anti-METH monoclonal antibody (mAb) before (pretreatment model) or after (overdose model) METH administration can significantly reduce METH concentrations in the brain and other organs (Byrnes-Blake et al., 2003; Laurenzana et al., 2003; Byrnes-Blake et al., 2005). Anti-METH mAb treatment in male rats also produces significant reductions in METH self-administration, locomotor activity, and hemodynamic effects (Byrnes-Blake et al., 2003; McMillan et al., 2004; Byrnes-Blake et al., 2005; Gentry et al., 2006), suggesting anti-METH mAb could be efficacious for multiple METH-induced effects at multiple sites of action, including neuroprotection of mothers and their fetuses.

Keyler et al. (2003, 2005) report that immunization with a nicotine vaccine or administration of anti-nicotine antibodies can reduce nicotine concentrations in maternal and fetal rat brains. Preclinical studies of active vaccines for METH suggest this therapeutic approach does not appear to generate the high and controllable levels of antibody concentrations needed to sustain neuroprotection (Miller et al., 2013; Rüedi-Bettschen et al., 2013; Shen et al., 2013). Our data show that a murine anti-phencyclidine (PCP) mAb [mAb6B5 equilibrium dissociation rate constant (K_D) = 1.3 nM] can safely protect pregnant rats and fetuses from PCP-induced adverse health effects even after repeated i.v. bolus injections of PCP (1 mg/kg) over several days. Therapeutic and safety endpoints show mAb6B5 treatment produces significant reductions in maternal and fetal PCP brain concentrations. These data also show mAb6B5 treatment does not adversely affect maternal weight gain, pup birth weights, pregnancy outcome, or fetal growth; and, more importantly, mAb6B5 substantially reduces PCP-induced fetal deaths (Hubbard et al., 2011a).

Although the brain penetration of METH appears to be driven by passive processes, the mAb appears to slow, reverse, and prevent METH entry to the brain by an active process mediated through high-affinity mAb binding. We previously suggested that the blood-brain barrier restricts anti-METH mAb (but not METH) to the vasculature, which allows temporary greater drug-mAb occupancy and more removal of METH from the brain with each passage through the brain vasculature (Laurenzana et al., 2009).

For these studies, we hypothesized that treatment of pregnant rat dams with mAb4G9 (IgG2b isotype, κ light chain), an mAb against METH (K_D = 16 nM), and its pharmacologically active metabolite, (+)-amphetamine (AMP) (K_D = 102 nM), could confer maternal and fetal protection from brain accumulation of both drugs. For the studies, we administered METH to pregnant rats on gestation day 21 (GD21), followed 30 minutes later with vehicle or mAb4G9. Maternal and fetal rat sera and brains were then analyzed to evaluate the outcome of mAb4G9 treatment on METH and AMP serum concentrations, protein binding, and brain accumulation of METH and AMP. The results showed significant mAb4G9-induced reductions in brain concentrations of METH and AMP for both the dams and fetuses.

Materials and Methods

Drugs, Chemicals, Reagents, and Monoclonal Antibody

METH HCl and AMP sulfate were obtained from the National Institute of Drug Abuse drug supply program (Bethesda, MD). Chemical structures can be found in a previous publication (Peterson et al., 2007). All doses were calculated as the free base. All other reagents were purchased from Fisher Scientific (Fairlawn, NJ), unless noted.

Purification of the mAb4G9 was achieved by affinity chromatography with a protein G-Sepharose column (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) (Peterson et al., 2007). After purification, mAb4G9 was concentrated on a 500 ml stirred cell (Amicon, Beverly, MA) with a 30,000

mol. wt. cutoff cellulose membrane (Millipore, Bedford, MA). The buffer was exchanged in the same process to 15 mM sodium phosphate containing 150 mM sodium chloride (pH 6.5). This was the administration buffer. To assure that endotoxin concentrations in the final protein solutions were insignificant, a Limulus Amebocyte Lysate kit (QCL-1000; Cambrex, East Rutherford, NJ) was used to assay the final product. The endotoxin levels measured were within acceptable limits. The final antibody product was ultracentrifuged at 100,000g for 90 minutes at 4°C to remove large mol. wt. antibody complexes, which can be highly antigenic. UV absorbance and SDS-PAGE were conducted to determine protein concentrations and to ensure purity of the final preparation. Purified mAb4G9 was stored at -80°C until use, at which point it was quickly warmed to 37°C.

The K_D values of mAb4G9 were originally reported as 34 and 51 nM for METH and AMP, respectively (Peterson et al., 2007). However, an improved affinity assay and a correction of how we calculated AMP concentrations show K_D values of 16 and 102 nM, respectively.

Animals

Adult female Sprague-Dawley rats were impregnated and catheterized with venous cannula at Charles River Laboratories (Wilmington, MA), and arrived on GD3, at approximately 8 weeks of age. To prevent undue temperature stress, the pregnant rats were not shipped between June and September. Each animal had dual indwelling polyurethane jugular venous catheters (0.025" i.d. $\times$ 0.04" o.d.) that were used for drug administration and blood sampling. Catheters were secured in the fascia between the scapulae before shipping and were exposed 1 week after arrival. Catheter patency was maintained by routinely flushing each catheter with 200 μ l saline, followed by 50 μ l glycerol containing 25 U heparin. Pregnant rats were individually housed in a temperature- and light-controlled (12-hour light/dark cycle) animal facility, with free access to normal chow (Harlan, Indianapolis, IN) and water.

Animal use was in accordance with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the US National Institutes of Health and the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences, which is accredited and conforms to international animal welfare standards.

Experimental Procedures

Pharmacokinetic Analysis of Maternal and Fetal Serum and Brain Tissues. METH and AMP brain and serum concentrations on GD21 were determined using a previously published method (White et al., 2011), with some modifications. Chromatographic separation and detection were achieved with a HILIC analytical column (Phenomenex) and an Acquity UPLC system connected to a Premier XE triple quadrupole mass spectrometer system (liquid chromatography coupled to tandem mass spectrometry; Waters, Milford, MA).

The METH and AMP concentrations in the maternal brain samples were corrected for blood in the brain vasculature by using the following two equations. The first equation, $C_b = C \times [1 + H(fu \times \rho - 1)]$, estimates the concentration of drug in the blood (Rowland and Tozer, 1995). C_b is blood drug concentration, C is serum drug concentration, H is hematocrit, fu is fraction of unbound drug, and ρ is affinity measurement of drug in erythrocytes, which was determined from the blood to serum drug ratio in the absence of mAb. From this information, we were able to calculate the correct concentration of drug in the maternal brain by using the following equation (Khor and Mayersohn, 1991): $C_{drug(corr)} = [C_{drug} - C_b \times (V_f)_b] / [1 - (V_f)_b]$, where $C_{drug(corr)}$ and C_b are the corrected brain (ng/g) and blood (ng/ml) drug concentrations, respectively. C_{drug} is the drug concentration uncorrected in the brain, and $(V_f)_b$ is the volume fraction of blood remaining in the brain. Because some of the important variables for calculating these values from pooled fetal brain tissues are not known, METH and AMP concentrations in fetal brains were reported without correcting for drug concentrations in the fetal brain vasculature.

Maternal and Fetal Serum Protein Binding. Serum protein binding of METH and its metabolite AMP were determined in dams and litters on GD21 by equilibrium dialysis in a 96-well high-throughput equilibrium dialysis apparatus (model HTD96b; HT Dialysis LLC, Gales Ferry, CT). Maternal serum samples collected from catheters were only sufficient for a single protein-binding analysis. Maternal and fetal trunk blood collected after death provided sufficient serum for a triplicate analysis.

Maternal and fetal serum samples (30–50 μ l) were placed on one side of a well divided by a size exclusion dialysis membrane (6–8 kDa cutoff). An equal volume of Sorenson's buffer (0.13 M Na_2HPO_4 , 0.13 M KH_2PO_4 , pH 7.35) was added to the other side. Equilibrium was achieved after overnight incubation at 37°C with continuous gentle shaking. At that point, serum and buffer were removed from each well, which contained free METH or AMP on the buffer side and total (bound plus free) METH or AMP on the serum side. METH and AMP concentrations were determined by liquid chromatography coupled to tandem mass spectrometry (see above). Monitoring the volume of sample obtained after equilibrium was achieved, assessed recovery of samples. These data indicated excellent total recovery from all dialysis chambers. The percentage of METH and AMP serum protein bound was determined by the following equation: % bound = $[1 - (\text{free METH or AMP concentration in the buffer side} / \text{total METH or AMP concentration in the serum side})] \times 100\%$.

Experimental Design

Experimentation started 1–2 days prior to expected normal delivery of pups on GD21, when the dams were approximately 10.5 weeks old. Animals were weight-matched prior to the controlled randomization of groups based on treatment and time of sacrifice. On GD21, pregnant rats (318–428 g) received a 15-second i.v. injection of 1 mg/kg METH [calculated as the free base and formulated in phosphate-buffered saline] via the left jugular venous catheter. Blood samples up to the 3-minute time point were collected from each dam through an indwelling venous cannula. Thirty minutes after METH administration, 235 mg/kg anti-METH/AMP mAb4G9 was given to half of the dams (24; $n = 4$ per time point) at 2 ml/min (3 ml total volume). Antibody administration buffer (vehicle) was given to the control group (24; $n = 4$ per time point) under the same dosing regimen. The mAb4G9 dose was calculated as 0.56 mole-equivalent (assuming two binding sites per IgG) to the body burden of METH at 30 minutes. The body burden was determined by the following equation: body burden = dose $\times e^{-\lambda_n t}$ (Rowland and Tozer, 1995), where λ_n (the METH terminal elimination rate constant) was previously determined to be 0.0060/h in timed-pregnant rats on GD21 (White et al., 2011), and t was the 30-minute time point.

The optimal time for vehicle and mAb4G9 treatments was based on previous studies in male and nonpregnant female rats, which show METH-induced locomotor activity (and AMP serum concentrations) is maximal approximately 30 minutes after a 1 mg/kg i.v. METH dose (Milesi-Hallé et al., 2005). The major METH-induced behavioral effects are over in 2–3 hours (Milesi-Hallé et al., 2005), so a 5-hour final sampling time was after the observable METH pharmacological effects in dams.

Blood samples (200 μ l) were collected from the gravid animal's catheter 35 and 45 minutes after METH administration (5 and 15 minutes after mAb or vehicle treatment). Both control and mAb4G9-treated animals were sacrificed by decapitation under isoflurane anesthesia at 40 minutes, 1 hour, 1.5 hours, 2 hours, 2.5 hours, or 5 hours after METH administration ($n = 4$ /time point for each group). An additional blood sample was collected at 3.5 hours post-METH injection from mAb- and vehicle-treated animals that were sacrificed at 5 hours. Adequate depth of anesthesia was determined by carefully monitoring the depth and frequency of respiration and by determining the lack of a corneal reflex and response to a paw pinch. Immediately after decapitation, maternal trunk blood was collected and maternal brains were removed, rinsed with saline, weighed, and quickly frozen in liquid nitrogen.

Afterward, a laparotomy was conducted on the maternal trunk, exposing the uterine horns. After separation from the uterus, live fetuses were placed on a heating pad at 37°C. After all of the fetuses were removed, litter size and weight were determined. The average number of pups was 14 ± 1.6 in the vehicle-treated group and 13 ± 2.6 in the mAb4G9-treated group. Fetal trunk blood was collected and pooled after decapitation. Fetal brains were removed from each pup, pooled, weighed, and frozen in liquid nitrogen. All maternal and fetal blood samples were allowed to clot at room temperature. The coagulated blood samples were centrifuged, and the serum portion was collected. All serum and brain samples were stored at -80°C until analysis.

Data Analysis and Statistical Procedures

Model-dependent pharmacokinetic analyses were conducted using Win-Nonlin software version 6.3 (Pharsight, Mountain View, CA) for the maternal

METH and AMP serum concentration-time data in Table 1 and for the best-fit lines shown for the plots of maternal METH and AMP serum data in Fig. 1, A and B.

For model-dependent analysis, one-, two-, and three-compartmental models were fit to the average maternal serum METH and AMP concentrations from 5 minutes to 5 hours using the appropriate input function with $1/y$ or $1/y^2$ weighting, where y is the predicted concentration. The best-fit line, based on the predicted concentrations, was selected after consideration of visual inspection, analysis of the residuals, and comparisons with model-independent analysis.

The following baseline pharmacokinetic parameters for dams were determined: METH and AMP area under the curve (AUC) from time zero to infinity (AUC_0^∞), METH and AMP terminal elimination half-lives ($t_{1/2}$) and terminal elimination rate constant (λ_n), maximum AMP concentrations, time to reach maximum AMP concentrations (and time to maximum concentration), volume of distribution (Vd), and total clearance (Cl_T). Equation for pharmacokinetic calculations was as follows: $t_{1/2} = 0.693/\lambda_n$, $Cl_T = \text{i.v. dose} / AUC_0^\infty$, and $Vd = Cl_T/\lambda_n$.

Model-independent pharmacokinetic analysis was used for analysis of all other pharmacokinetic data sets from maternal and fetal tissues. METH and AMP tissue pharmacokinetic parameters in controls and mAb4G9-treated rats were calculated from 40 minutes to 5 hours following METH administration. METH and AMP AUC values from 40 minutes to 5 hours ($AUC_{40\text{ min}}^{5\text{ h}}$) for pregnant rats in the presence and absence of mAb were calculated using the equation $AUC = 0.5 \times (C_1 + C_2)/(t_2 - t_1)$, where C is the drug concentration at each time (t) point.

The effect of mAb4G9 treatment on maternal serum METH and AMP concentrations was analyzed by an unpaired Student's t test (SigmaStat Software, Jandel Scientific, San Rafael, CA). A two-way analysis of variance was used to detect significant differences in METH and AMP serum and brain concentrations, unbound METH and AMP serum concentrations, and percentage of METH and AMP bound in vehicle- and mAb-treated dams and litters using SAS Proc Mixed software (SAS Institute, Cary, NC). Since concentrations and S.E. frequently differed between groups, equal variance was not assumed. Therefore, SAS Proc Mixed was used to fit a model that allowed for different variances within the groups. If statistical differences were found, analysis was followed by a Bonferroni correction ($\alpha = 0.05$). A significance level of $P < 0.05$ was used for all studies.

Results

METH and AMP Pharmacokinetic Profile in Pregnant Rats and Litters. The baseline disposition of METH and its pharmacologically active metabolite AMP (without antibody treatment) was determined in maternal and fetal sera and brains (Fig. 1) after the dams received 1 mg/kg i.v. METH on GD21. Table 1 summarizes the METH and AMP baseline pharmacokinetic values in the maternal sera, based on the data in Fig. 1, A and B. An i.v. bolus two-compartment model with $1/y^2$ weighting provided the best-fit line to

TABLE 1

Average METH and AMP metabolite pharmacokinetic parameters for maternal sera after maternal administration of an i.v. 1 mg/kg METH dose, followed 30 minutes later by vehicle treatment

Pharmacokinetic Parameter (units)	METH	AMP
AUC_0^∞ (ng $\times$ h/ml)	367	96
$t_{1/2}$ (hours)	1.7	2.9
T_{max} (hours)	N/A ^a	1.3
C_{max} (ng/ml)	272	17
Vd (L/kg)	6.8	N/A ^a
Cl_T (ml/min/kg)	45	N/A ^a
Molar ratio of AMP/METH AUC_0^∞ ^b		0.29

^a C_{max} , maximum concentration; T_{max} , time to maximum concentration.

^bNot applicable.

^bThe molar ratio of AMP to METH was calculated by dividing AUC_0^∞ values for each drug after converting the AUC_0^∞ units into nmol $\times$ h/ml for each drug.

Fig. 1. Average METH (A and C) and AMP (B and D) concentration-versus-time profiles in maternal and fetal sera (A and B) and brains (C and D) on GD21 in control animals used for calculating baseline pharmacokinetic values (Tables 1 and 2). These animals did not receive antibody. The dams received 1 mg/kg i.v. METH followed 30 minutes later with vehicle (buffer, as indicated by the arrows on the serum graphs). Blood samples up to the 30-minute time point were collected from a venous cannula. Remaining samples were collected after sacrificing the dams. The solid lines represent either a WinNonlin best-fit line to the maternal serum METH (A) and AMP (B) concentration-time data, or a linear regression best-fit line to the terminal log concentration-time data (all other plots). All values are represented by mean $\pm$ S.D.; $n = 4$ per time point. *, Indicates a significant difference ($P < 0.05$) from corresponding maternal serum or brain concentrations.

the maternal METH serum concentration-time data (Table 1; Fig. 1A). A first-order input, one-compartment model with $1/y$ weighting provided the best-fit line to the AMP serum concentration-time data (Table 1; Fig. 1B).

By the first time point of brain tissue collection (40 minutes after METH dosing), the METH distribution phase in serum and brain samples appeared complete in both dams and fetuses, and only the terminal elimination phase remained. Due to slower formation and subsequent distribution (Milesi-Hallé et al., 2005; Cohen et al., 2007), AMP did not achieve the linear terminal elimination phase until about 2 hours after METH administration. Table 2 shows the $t_{1/2}$ values for the maternal and fetal sera and brain tissues calculated from the data shown in Fig. 1.

Although the shape of concentration-versus-time curves for METH or AMP in serum was similar in dams and their fetuses (Fig. 1), the actual concentrations of METH and AMP at each time point were usually significantly higher in the fetuses. METH and AMP brain concentrations remained two- to three-fold higher in dams at all time points. Maternal METH serum V_d and Cl_T were 45% and 28% (respectively) higher than the corresponding fetal values.

Effect of Anti-METH/AMP mAb4G9 on Serum METH and AMP Pharmacokinetics. Treatment with dual reactive mAb4G9 30 minutes after METH administration led to significant increases in both METH and AMP maternal serum concentrations (Fig. 2, left panels) for the remainder of the 5-hour study. Compared with vehicle-treated controls, the METH and AMP $AUC_{40\text{ min}}^{5h}$ values increased by $>7000\%$ and 2000% (respectively) following mAb4G9 treatment. The complete pharmacokinetic profile for METH and AMP could not be accurately determined in the presence of mAb4G9 due to the substantially prolonged $t_{1/2}$ for METH and AMP in the presence of mAb4G9.

Whereas maternal serum concentrations increased significantly after mAb4G9 administration, fetal serum concentrations did not (Fig. 2,

TABLE 2

Baseline METH and AMP $t_{1/2}$ values in maternal and fetal sera and brains based on the concentration-time plots in Fig. 1

The $t_{1/2}$ values were calculated from the best-fit lines to the terminal elimination phase shown in the METH and AMP concentration-time plots in Fig. 1, A–D. All values were calculated by pharmacokinetic model-independent analysis. Data were calculated from the average maternal and fetal brain and sera values from four grouped animals per time point (see open circles in Fig. 1, A and B).

Tissue	Source	$t_{1/2}$ Values (hours)	
		METH (hours)	AMP (hours)
Sera	Maternal	1.7 ^a	2.5 ^a
	Fetal	1.2	2.0
Brain	Maternal	1.1	2.8
	Fetal	1.3	3.2

^aData for these two $t_{1/2}$ values were also calculated by model-dependent analysis in Table 1 as 1.7 hours and 2.9 hours (respectively).

right panels). In fact, METH concentrations were significantly lower at 1 hour, and AMP concentrations were significantly lower at 1, 1.5, and 2 hours in mAb-treated fetal serum compared with vehicle-treated fetal serum. By 5 hours, METH and AMP concentrations were significantly higher than controls. Table 3 summarizes the average METH and AMP AUC values in maternal and fetal rat tissues, and average $AUC_{\text{brain}}/AUC_{\text{serum}}$ ratio from 40 minutes to 5 hours following maternal administration of a 1 mg/kg METH i.v. dose, followed at 30 minutes by vehicle or anti-METH/AMP mAb4G9 treatment. The ratio $AUC_{\text{brain}}/AUC_{\text{serum}}$ was calculated using the $\text{ng} \times \text{h/g}$ (brain) and $\text{ng} \times \text{h/ml}$ (serum) AUC values from 40 minutes to 5 hours. Although significant differences were found with fetal serum concentrations at some time points, METH serum $AUC_{40\text{ min}}^{5h}$ remained virtually the same with and without mAb treatment. Conversely, AMP fetal serum $AUC_{40\text{ min}}^{5h}$ decreased by 23%.

Effect of Anti-METH/AMP mAb4G9 on Serum Protein Binding. The percentage of METH and AMP bound in serum was approximately 15% in all dams at 5 and 15 minutes after METH administration (data not shown). This was before vehicle or mAb4G9 administration. Serum protein binding remained at similar levels throughout the experiment in vehicle-treated dams (Fig. 3, left panels). In contrast, METH and AMP serum protein binding in dams significantly increased to $\geq 92\%$ after mAb administration.

Although mAb4G9 immediately increased METH and AMP serum protein binding in the dams, METH and AMP protein binding in the fetuses remained about 15–25% in control and mAb4G9-treated animals until 2.0 hours (Fig. 3, right panels), when METH protein binding began to increase. AMP protein binding did not significantly increase above control fetal values until the 5-hour time point.

Unlike the dramatic changes induced by mAb4G9 for the percentage of METH and AMP bound in serum, the antibody did not substantially alter free drug concentrations in dams (results not shown). Fetal serum-free drug concentrations (data not shown) were higher than maternal concentrations at all times, following a similar pattern observed for total METH and AMP serum (but not brain) concentrations shown in Fig. 1. Importantly, when compared with control values, mAb4G9 treatment had an apparent greater effect on lowering fetal METH and AMP unbound concentrations than it did in lowering maternal unbound concentrations.

Effect of Anti-METH/AMP mAb4G9 on Brain METH and AMP Pharmacokinetics. Administration of mAb4G9 produced significant reductions in METH and AMP concentrations in maternal and fetal brains (Fig. 4). This was accompanied by substantial reductions in the maternal and fetal brain METH and AMP $AUC_{40\text{ min}}^{5h}$ values (Table 3).

Fig. 2. Average maternal (A and B) and fetal (C and D) serum concentration-versus-time profiles of METH (A and C) and AMP (B and D) in dams administered 1 mg/kg METH followed at 30 minutes by vehicle or 0.56 mole-equivalent of mAb4G9 treatment. The studies were conducted on GD21. The solid lines represent a linear regression best-fit line to the terminal log concentration-versus-time data. A best-fit line to the terminal phase was not performed on animals with mAb4G9 treatment, because previous studies suggest the terminal elimination phase ($t_{1/2} \sim 7$ days) would be substantially longer than the current 5-hour study. The arrows (on the serum graphs) indicate the time of vehicle or mAb4G9 administration. All values are represented by mean $\pm$ S.D. ($n = 4/\text{time point}$). *, Indicates a significant difference from controls ($P < 0.05$).

Discussion

We hypothesized that treatment of rat dams with anti-METH/AMP mAb4G9 could offer protection from accumulation of METH and AMP in both maternal and fetal brains. The rat is an often used model to study neurologic effects of METH and AMP, although the clearance and transport of the IgG across blood organ barriers, such as the blood-brain barrier, by rats and humans are somewhat different (Pentšuk and van der Laan, 2009). Although the 1 mg/kg METH dose was not considered toxic to the rats, the results from these experiments were used to help predict how larger METH doses might significantly attenuate both maternal and fetal METH and AMP brain concentrations during late-stage pregnancy. This will be particularly important for studying more complex, longer-term experiments designed to assess neurologic and behavioral outcomes in rat pups with and without the protective effects of anti-METH mAb.

To individualize and adapt the therapy for pregnant rats, we calculated mAb dose and time of dosing based on previous findings in

rats (Hubbard et al., 2011b). Following mAb4G9 treatment, there was a substantial and statistically significant increase in maternal serum METH and AMP concentrations (Fig. 2). Serum concentrations remained high throughout the study, leading to a $>7000\%$ and 2000% increase in METH and AMP $AUC_{40 \text{ min}}^{5 \text{ h}}$, respectively. This was primarily due to the significant increase in serum protein binding mediated by mAb4G9 binding to METH and AMP (Fig. 3, left panels). This caused rapid redistribution of METH and AMP out of tissues (including the brain) and into the extracellular fluid space where the antibody is confined.

Compared with the profound effect on protein binding in dams (Fig. 3, left and right panels), mAb4G9 initially appeared to have minimal effect on METH and AMP protein binding in the fetal serum. This provided strong evidence that the placental barrier substantially blocked or slowed maternal-fetal passage of mAb4G9, thereby preventing substantial increases in fetal serum protein binding. However, starting 2.5 hours after mAb4G9 treatment, the fetal serum METH protein binding appeared to gradually increase, producing slightly elevated concentrations of METH and AMP, which suggested small amounts of mAb4G9 were present. This could indicate a slow leakage or transport of mAb4G9 into the fetal compartment. This interpretation is also based on similar studies testing the effects of anti-PCP mAb6B5 on PCP disposition in late-stage pregnant rats and their fetuses (Hubbard et al., 2011a) and results from other studies (Arizono et al., 1994; Nekhayeva et al., 2005).

Unlike METH and AMP, which are transported across the placenta in a blood flow-limited manner by the norepinephrine and serotonin transporter (Ramamoorthy et al., 1995), rats have only modest maternofetal IgG transport during late gestation (GD17–GD21; (Roberts et al., 1990). Indeed, most humoral immunity is established in the litters postnatally through ingestion of colostrum and breast milk (Simister et al., 1997). The rate of neonatal Fc receptor-mediated transport of mAb4G9:drug complexes is also likely slow, because several pH-dependent steps are necessary for transport of the mAb4G9:drug complex from the apical to basolateral placental membrane (Lobo et al., 2004). This could account for the later onset of METH and AMP serum protein binding observed in these studies. In a similar manner, Keyler et al. (2005) report nicotine concentration increase in GD20 fetal rat serum following maternal immunization with a nicotine vaccine. They suggest anti-nicotine antibody titers in fetal serum (10% of the maternal serum titers) contribute to the vaccine's effectiveness at reducing fetal brain concentrations.

Maternal mAb4G9 treatment significantly reduced maternal and fetal brain METH and AMP concentrations compared with controls (Fig. 4), confirming our hypothesis that anti-METH/AMP mAb treatment is neuroprotective in pregnant dams and their fetuses.

TABLE 3

Summary of maternal and fetal changes in METH and AMP AUC values (from 40 minutes to 5 hours) for brain and serum samples in control- and mAb4G9-treated dams

		$AUC_{40\ min}^{5\ h}$ (ng $\times$ h/ml or ng $\times$ h/g)				$\frac{AUC_{brain}}{AUC_{serum}}$	
		Drug			Drug		
			Control	mAb4G9-Treated		Control	mAb4G9-Treated
Maternal	Brain	METH	2257	759	METH	10	0.05
		AMP	680	374			
	Serum	METH	215	16,050	AMP	12	0.3
		AMP	57	1076			
Fetal	Brain	METH	735	408	METH	2.5	1.5
		AMP	273	147			
	Serum	METH	293	276	AMP	2.6	1.8
		AMP	105	81

Fig. 3. Average percentage of METH (A and C) and AMP (B and D) protein binding in the maternal serum (A and B) and fetal serum (C and D) over time after maternal administration of a 1 mg/kg i.v. METH dose on GD21. Thirty minutes after the pregnant rats received i.v. METH, mAb4G9 or vehicle was administered. All values are represented by mean $\pm$ S.D. ($n = 4$ /time point). *, Indicates a significant difference compared with control values. †, Indicates a significant difference compared with maternal control dams. ‡, Indicates a significant difference compared with mAb-treated maternal values ($P < 0.05$ in all cases).

Whereas METH and AMP brain concentrations returned to near control levels in mAb4G9-treated dams and fetuses by 5 hours, maternal and fetal brain $AUC_{40\text{ min}}^{5\text{ h}}$ decreased by 66% and 45% for METH and 44% and 46% for AMP ($P < 0.05$), respectively. We think the reason METH and AMP concentrations appeared to rebound back to the very low control levels by 5 hours is due in part to a delayed repartitioning of METH and AMP from membrane-limited tissues, which are much slower to re-equilibrate after antibody treatment than

the rapidly equilibrating brain tissue. Previous studies show similar changes in male rats treated with anti-PCP and anti-METH immunotherapies (Valentine and Owens, 1996; Byrnes-Blake et al., 2003). Specifically, studies in male rats show anti-drug immunotherapy substantially lowers brain concentrations for a longer period of time than the duration of PCP- or METH-induced behavioral activity (Valentine and Owens, 1996; Valentine et al., 1996; Byrnes-Blake et al., 2003). Because the major METH-induced behavioral effects at this METH dose are over in 2–3 hours in female rats (Milesi-Hallé et al., 2005), the re-equilibration of METH brain concentrations back to the low control levels 4.5 hours after antibody treatment would not be expected to produce detectable pharmacological effects. These data also suggest the METH and AMP are cleared from the rats even in the presence of antibody. This clearance of the METH and AMP would presumably help to restore antibody-binding capacity (Stevens et al., 2014).

Previous studies suggest anti-PCP or anti-METH mAb-induced changes in unbound serum drug concentrations are not the most reliable surrogate marker for predicting reductions in adverse central nervous system effects (Valentine and Owens, 1996; Laurenzana et al., 2003, 2009). In the absence of data showing *in vivo* changes like reduction in mAb-induced behavioral or cardiovascular effects, we find reductions in brain concentrations are the best surrogate marker (Hardin et al., 2002; Byrnes-Blake et al., 2003). For instance, Byrnes-Blake et al. (2003) found that male rats treated with 1 mg/kg METH followed 30 minutes later by an equimolar dose of anti-METH mAb6H4 ($K_D = 4$ nM) had approximately 70% lower brain METH $AUC_{38\text{ min}}^{4.5\text{ h}}$ than vehicle-treated rats, which correlated to a 70% reduction in behavioral activity.

One possible explanation for this apparent discrepancy between changes in brain concentrations and inconsistent changes in free serum concentrations is that METH clearance from the brain changes from a nonrestrictive clearance without mAb treatment to a restrictive clearance with mAb. This fundamental change in the type of brain clearance without and with mAb is due to the significant increase in maternal protein binding after mAb treatment. This means that, in the absence of mAb, all (or most) of the METH (and AMP) in the bloodstream enters the brain with each pass through the brain. However, when METH and AMP become highly protein-bound after mAb treatment, only the free fraction has the availability (or potential) to be removed with each pass through the brain vasculature.

We hypothesize that the mAb is not statically occupied with the same drug molecules over time, but rather undergoes a more frequent turnover due to mAb's association and dissociation rates, which drive kinetics of drug binding, release, and rebinding. Unlike the equilibrated free and bound serum concentrations predicted by *in vitro* equilibrium dialysis experiments, free (and bound) METH and AMP are in fact in a constant flux dictated by changes in mAb-, tissue-, and drug-dependent factors. Thus, tissue concentrations most likely vary with the physiologic changes in cardiac output to individual organs, changes in individual organ volumes, and the presence or absence of blood-organ barriers, which are all altered from the dynamic pregnancy-induced physiologic changes and their concomitant impact on pharmacokinetic processes (Mattison et al., 1991). In this unique physiologic environment, mAb4G9 was able to significantly reduce both maternal and fetal METH and AMP brain concentrations (Fig. 4), even though the antibody was mostly confined to the maternal side of the blood-placental barrier.

From previous studies, we know a 5.6 mg/kg i.v. METH dose can cause maternal and fetal death (White et al., 2011). Although not directly toxic, a 1 mg/kg dose produces significant METH-induced behaviors in female rats (Milesi-Hallé et al., 2007). Changes in maternal behaviors resulting from chronic treatment with METH can result in significant changes in the dam's ability to nurse, groom, and

Fig. 4. Average METH (A and C) and AMP (B and D) concentration-versus-time profiles of maternal (A and B) and fetal (C and D) brains after maternal administration of i.v. METH followed 30 minutes later by vehicle or mAb4G9. The solid lines represent the linear regression fit to the terminal log concentration-versus-time data. All values are represented by mean $\pm$ S.D. ($n = 4$ /time point). *, Indicates a significant difference compared with vehicle-treated maternal values. †, Indicates a significant difference compared with mAb-treated maternal values ($P < 0.05$ in all cases).

build nests for their pups (Slamberová et al., 2006). In humans, METH-induced behaviors can cause female METH users to neglect and abuse their children (Connell-Carrick, 2007). Prenatal methamphetamine exposure is also associated with increases in cognitive problems in children that could lead to poor behavioral outcomes and affect academic achievement (Diaz et al., 2014). Therefore, longer-term studies of METH-induced changes in maternal behaviors in rats and their pups, with and without antibody treatment, are needed.

In conclusion, anti-METH/AMP mAb4G9 treatment protected late-stage pregnant rats and their fetuses by substantially reducing maternal and fetal brain concentrations. Reductions of METH and AMP brain concentrations could in turn diminish harmful effects and possibly prevent METH-induced maternal and fetal death. Therefore, passive immunization with anti-METH/AMP mAb4G9 shows promise as an effective and safe treatment of pregnant drug addicts due to its high affinity and specificity, relatively long $t_{1/2}$, and ability to simultaneously reduce METH levels in maternal and fetal brains. These qualities are particularly important when considering the numerous difficulties associated with treating pregnant METH users and, undoubtedly, their unborn child(ren).

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Authorship Contributions

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