Impact of Nicotine Metabolism on Nicotine's Pharmacological Effects and Behavioral Responses: Insights from a *Cyp2a(4/5)bgs*-Null Mouse^S

Lei Li, Kunzhi Jia, Xin Zhou, Sarah E. McCallum, Lindsay B. Hough, and Xinxin Ding

Wadsworth Center, New York State Department of Health, and School of Public Health, State University of New York at Albany (L.L., K.J., X.Z., X.D.) and Center for Neuropharmacology and Neuroscience, Albany Medical College (S.E.M., L.B.H.), Albany, New York

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ABSTRACT

Nicotine metabolism is believed to affect not only nicotine's pharmacological effects but also nicotine addiction. As a key step toward testing this hypothesis, we have studied nicotine metabolism and nicotine's pharmacological and behavioral effects in a novel knockout mouse model [named *Cyp2a(4/5)bgs*-null] lacking a number of cytochrome P450 genes known to be or possibly involved in nicotine metabolism, including two *Cyp2a* and all *Cyp2b* genes. We found that, compared with wild-type mice, the *Cyp2a(4/5)bgs*-null mice showed >90% decreases in hepatic microsomal nicotine oxidase activity in vitro, and in rates of systemic nicotine clearance in vivo. Further comparisons of nicotine metabolism between *Cyp2a(4/5)bgs*-null and *Cyp2a5*-null mice revealed significant roles of both CYP2A5 and CYP2B enzymes in nicotine clearance. Compared

with the behavioral responses in wild-type mice, the decreases in nicotine metabolism in the *Cyp2a(4/5)bgs*-null mice led to prolonged nicotine-induced acute pharmacological effects, in that null mice showed enhanced nicotine hypothermia and antinociception. Furthermore, we found that the *Cyp2a(4/5)bgs*-null mice developed a preference for nicotine in a conditioned place preference test, a commonly used test of nicotine's rewarding effects, at a nicotine dose that was 4-fold lower than what was required by wild-type mice. Thus, CYP2A/2B-catalyzed nicotine clearance affects nicotine's behavioral response as well as its acute pharmacological effects in mice. This result provides direct experimental support of the findings of pharmacogenetic studies that suggest linkage between rates of nicotine metabolism and smoking behavior in humans.

Introduction

Nicotine is the main addictive component of cigarette smoke. During cigarette smoking, nicotine is readily absorbed and reaches the brain quickly. Nicotine binds with high affinity to nicotinic acetylcholine receptors in the ventral tegmental area, stimulating dopamine release in the nucleus accumbens, thereby effecting reward (Corrigall et al., 1992).

Nicotine is metabolized rapidly in the liver, mainly via cytochrome P450 (P450)-mediated C-oxidation, yielding cotinine. The major P450 enzyme involved in nicotine metabolism in the human liver is CYP2A6 (Hukkanen et al., 2005), followed by CYP2B6 (Yamazaki et al., 1999). CYP2A6 genetic polymorphisms have been found to influence the metabolism of nicotine (Ray et al., 2009). In mice, the role of CYP2A5 in nicotine clearance has been demonstrated (Siu and Tyndale, 2007; Zhou et al., 2010). One or more of the mouse CYP2B enzymes may also be active toward nicotine

(Siu and Tyndale, 2007), although direct evidence of a role for mouse CYP2B in nicotine metabolism in vivo has yet to be obtained.

Previous works have led to the hypothesis that nicotine metabolism influences the risks of smoking-related lung carcinogenesis, as well as nicotine's pharmacological actions (Damaj et al., 2007; Wassenaar et al., 2011). Presumably, a more rapid clearance of nicotine would make addicted smokers smoke more cigarettes per day, with consequently higher exposure to cigarette-derived carcinogens, such as the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (Hecht, 1998). Variations in rates of nicotine clearance, e.g., as a result of variations in CYP2A/2B expression, may also affect the development and/or maintenance of nicotine dependence (Ray et al., 2009). The latter hypothesis has been tested in both animal models and human smokers. In a study of F2 progenies of intercrossed high- and low-nicotine-consuming mice, the amount of nicotine oral self-administration correlated positively with hepatic microsomal CYP2A5 protein levels (Siu et al., 2006). A study on current human smokers found that smokers with "normal" CYP2A6 activity smoked more cigarettes per day than the smokers with reduced CYP2A6 activity (Wassenaar

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ABBREVIATIONS: ANOVA, analysis of variance; AUC, area under the concentration; B6, C57BL/6; CL, clearance; cotinine- d_3 , ($\pm$)-cotinine-methyl- d_3 ; CPP, conditioned place preference; F, bioavailability; HPLC, high-performance liquid chromatography; % MPE, percentage maximum possible effect; P450, cytochrome P450; T_{max} , time at maximal concentration; $t_{1/2}$, elimination half-life; WT, wild type.

et al., 2011). It is noteworthy that, although these findings support the importance of nicotine metabolism in the development and/or maintenance of nicotine addiction, more direct proof for the role of nicotine metabolism is needed.

There have also been a few prospective pharmacogenetics studies on the association between CYP2A6 variations and development of nicotine dependence in adolescent novice smokers (O'Loughlin et al., 2004; Karp et al., 2006; Audrain-McGovern et al., 2007). However, the results from these studies are inconsistent in that, whereas Audrain-McGovern et al. (2007) suggested that normal metabolizers progressed more quickly to nicotine dependence than slow metabolizers, Karp et al. (2006) and O'Loughlin et al. (2004) concluded that slow metabolizers showed substantially increased risk of becoming dependent. Differences in age, degree of smoking, or methods of assessment of nicotine dependence might have contributed to the inconsistency; however, it is clear that additional studies in human smokers and in animal models are needed.

Here, we used a *Cyp2a(4/5)bgs*-null mouse model (Wei et al., 2013) to study the impact of nicotine metabolism on nicotine's pharmacological and behavioral effects. In this mouse model, a number of P450 genes known or potentially involved in nicotine metabolism are deleted, including two *Cyp2a* (*2a4* and *2a5*) and all five *Cyp2b* (*2b9*, *2b10*, *2b13*, *2b19*, and *2b23*). We first validated the role of mouse CYP2A/2B enzymes in nicotine metabolism and clearance by comparing wild-type (WT) and *Cyp2a(4/5)bgs*-null mice. We also delineated the relative roles of CYP2A5 and the CYP2B enzymes in nicotine clearance by comparing *Cyp2a(4/5)bgs*-null and *Cyp2a5*-null mice. We then compared WT and *Cyp2a(4/5)bgs*-null mice for responsiveness in nicotine-induced hypothermia and nicotine-induced antinociception to prove that P450-mediated nicotine metabolism impacts nicotine pharmacodynamics as well as pharmacokinetics. Finally, we compared WT and *Cyp2a(4/5)bgs*-null mice for performance in a conditioned place preference (CPP) test, a commonly used test of nicotine's rewarding effects. Our results provide direct proof of the impact of nicotine clearance on nicotine's effects.

Materials and Methods

Reagents and Animals

(-)-Cotinine, ($\pm$)-cotinine-methyl- d_3 (cotinine- d_3), (-)-nicotine hydrogen tartrate, (-)-nicotine, ammonium acetate, and reduced β -nicotinamide adenine dinucleotide phosphate were purchased from Sigma-Aldrich (St. Louis, MO). All solvents (acetonitrile, methanol, and water) were of high-performance liquid chromatography (HPLC) grade (Thermo Fisher Scientific, Houston, TX). All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the Wadsworth Center. WT C57BL/6J (B6), *Cyp2a5*-null (on B6 genetic background; Zhou et al., 2010), and *Cyp2a(4/5)bgs*-null (on B6 genetic background; Wei et al., 2013) mice were obtained from breeding stocks maintained at the Wadsworth Center.

Pharmacokinetics Studies

For determination of nicotine pharmacokinetics, 2-month-old WT, *Cyp2a5*-null, and *Cyp2a(4/5)bgs*-null male mice were given a single i.p. injection of nicotine tartrate (at 1.0 mg/kg, free base) in saline. Blood samples (~20 μ l each) were collected from the tails of individual mice, at various times (5 minutes to 4 hours) after the injection, for preparation of plasma. For studies on tissue nicotine and cotinine levels, tissue samples (brain and liver), as well as blood samples (from

the heart), were obtained after animals were killed by CO₂ asphyxiation. The method for processing plasma and tissue samples was described elsewhere (Zhou et al., 2010).

Quantitative analysis of nicotine and cotinine in plasma and tissue was performed using a liquid chromatography–tandem mass spectrometry system consisting of an Agilent 1200 Series HPLC, fitted with a Gemini C18 column (2.0 $\times$ 50 mm, 5 μ m; Phenomenex, Torrance, CA), and an ABI 4000 Q-Trap mass spectrometer (Applied Biosystems, Foster City, CA). Linear gradients for HPLC and the mass spectrometry conditions were set up essentially as previously described (Zhou et al., 2010). The parent/product ion pairs of *m/z* 163/130 (for nicotine), *m/z* 177/80 (for cotinine), and *m/z* 180/80 (for cotinine- d_3) were monitored in the multiple reaction monitoring scan mode. To construct calibration curves, authentic nicotine and cotinine (0.05–5 ng for plasma and 0.5–500 ng for tissue) as well as 0.1 ng of cotinine- d_3 were added to 10 μ l of blank mouse plasma or tissue homogenate before extraction. The recovery of added standards was >85% for plasma samples and >75% for tissue samples at all concentrations tested.

In Vitro Metabolism of Nicotine

The in vitro microsomal catalytic activity toward nicotine was determined in liver microsomes from 2-month-old WT, *Cyp2a5*-null, and *Cyp2a(4/5)bgs*-null male mice. The postmicrosomal supernatant fraction was used as cytosol. Nicotine C-5'-oxidation assay was performed essentially as described previously (Zhou et al., 2010). Reaction mixtures contained 1.0 or 10 μ M nicotine, 0.5 mg/ml liver microsomal protein, and 1.0 mg/ml cytosolic protein, and the reactions were carried out at 37°C in a final volume of 0.5 ml for 15 minutes. Cotinine formation was quantified by liquid chromatography–tandem mass spectrometry analysis, as described for the pharmacokinetics of plasma cotinine.

Nicotine-Induced Pharmacological Effects

Two-month-old WT and *Cyp2a(4/5)bgs*-null male mice received an intraperitoneal injection of saline or nicotine at 5.0 mg/kg and were tested for hypothermia response (body temperature) and antinociceptive response (two assays, tail-immersion and hot-plate tests). Groups of 8 animals were used for each treatment.

Body Temperature. Rectal temperature was measured by a thermistor probe (inserted to 24 mm) and a digital thermometer (Harvard Apparatus, Holliston, MA). Readings were taken before and 5, 15, 30, 45, 60, 120, and 180 minutes after the intraperitoneal injection of either saline or nicotine. The difference in rectal temperature before and after treatment was calculated for each mouse. The ambient temperature in the laboratory varied from 22°C to 24°C.

Tail-Immersion Test. Antinociception was assayed by the tail-immersion method (Sewell and Spencer, 1976). In brief, mice were loosely restrained in a conical polypropylene tube. The tip (2–3 cm) of the tail was immersed in a 50°C water bath, and the latency of flick or removal of the tail from the water was recorded. At this temperature, the baseline latencies were approximately 1–3 seconds. On test day, each animal underwent two tail-immersion tests performed at a 1-minute interval, for determination of the baseline latencies. The animals were then injected (intraperitoneally) with saline or nicotine, and tested repeatedly at 5, 15, 30, 45, 60, 120, and 180 minutes after treatment. To minimize tissue damage, a maximal latency of 8 seconds was imposed. Antinociceptive response was converted to percentage maximum possible effect (% MPE), where % MPE = [(test – baseline) / (8 – baseline)] $\times$ 100. The results are expressed as means $\pm$ S.E.M.

Hot-Plate Test. The mice were placed on a 52°C metal surface, and the latency for a hind paw lift or lick was recorded (Hough et al., 2006). On the test day, each animal underwent a single baseline hot-plate test (the baseline latencies were ~8–14 second), and then test latencies were determined at 5, 15, 30, 45, 60, 120, and 180 minutes

after nicotine or saline treatment. Mice not responding within 40 seconds were removed from the hot plate. Antinociceptive response was calculated as % MPE, where % MPE = [(test – baseline)/(40 – baseline)] × 100. The results are expressed as means ± S.E.M.

Nicotine-Induced Conditioned Place Preference Test

The CPP test was designed using an unbiased procedure, as previously reported (Grabus et al., 2006). The testing boxes (Med Associates, Inc., St. Albans, VT) consisted of three distinct conditioning compartments. The center compartment (7.5 × 16.0 cm) separated the two choice sides (16.0 × 16.0 cm), with manual doors that allowed access to either side of the compartment, which could be closed off for pairing days. One choice compartment was white with a stainless steel grid rod floor, and the other choice compartment was striped with a stainless steel mesh floor. The mouse transition from one compartment to the other was monitored by light beam, and the time spent in each compartment was recorded using Med-PC software (Med Associates, Inc.).

Male, 2-month-old WT mice and *Cyp2a(4/5)bgs*-null mice (8–12/group) were used for the CPP test. During the 3 days before the start of the test, mice were handled once per day for ~2 minutes each. On day 1 of the test, mice were placed in the CPP boxes, one at a time, and allowed to roam freely from side to side for 15 minutes; time spent in each side chamber was recorded. For all groups, mice spending less than 65% of the time in either chamber (i.e., unbiased) during preconditioning were selected for the CPP test. The following morning (day 2; ~9:00 AM), mice were injected intraperitoneally with either nicotine (the “drug” condition) or saline (the “nondrug” condition), and immediately placed in one of the two compartments for 20 minutes. Five hours later, the mice were injected with the alternate substance relative to its first injection (saline or nicotine), and immediately confined to the opposite chamber for 20 minutes. Mice stayed in their home cages between the two injections, or when not being tested in the CPP apparatus. This procedure was repeated on days 3 and 4, so that each animal received a total of three drug (nicotine) and three saline conditioning trials. Randomization was achieved at two levels: for each nicotine dose or mouse strain, the chamber that was paired with nicotine was randomly selected; for each mouse, the order in which the two differing conditions were presented within a day was also randomly selected. Mice injected with only saline in both side chambers were used as the control group. On the test day (day 5), no injections were given. Animals were placed in the center compartment and allowed access to all chambers for 15 minutes. Time spent on each side was recorded, and data were calculated as a preference score [= time (seconds) in drug-paired compartment during test minus time (seconds) in drug-paired compartment during preconditioning].

Other Methods

Protein concentration was determined by the bicinchoninic acid method (Pierce Chemical, Rockford, IL) with bovine serum albumin as the standard. Statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software, Inc., San Diego, CA). Statistical significance of differences between two groups was examined with Student's *t* test. Pharmacokinetic parameters were calculated using the noncompartmental method in the PKSolver software (Zhang et al., 2010). For total body clearance, given that bioavailability (*F*) is not known, the hybrid constant clearance (CL)/*F* was used (Statler et al., 2007), instead of CL. Statistical significance in the CPP test was determined using 2-way analysis of variance (ANOVA; with Bonferroni post-hoc test) with genotype and treatment as variables. One-way ANOVA (followed by Tukey's test) was used to assess the significance of differences between nicotine and saline groups. Paired *t* test was performed to compare behavior of the same groups of animals on different days, and unpaired *t* test was performed for all other comparisons between two groups.

Results

Nicotine Metabolism and Clearance in WT and *Cyp2a(4/5)bgs*-Null Mice

Rates of hepatic microsomal cotinine formation, by P450-mediated nicotine C-5' oxidation, were drastically decreased in *Cyp2a(4/5)bgs*-null mice compared with WT mice at both 1 and 10 μM nicotine (Fig. 1). Corresponding decreases in rates of nicotine clearance and amounts of cotinine formation were found in vivo, in mice injected with nicotine (intraperitoneally) at 1.0 mg/kg (Fig. 2; Table 1) or 5.0 mg/kg (Supplemental Fig. 1; Supplemental Table 1). The pharmacokinetic parameters for plasma nicotine in WT mice were comparable to what were previously reported (Zhou et al., 2010). Compared with WT mice, the *Cyp2a(4/5)bgs*-null mice had a ~93% decrease in CL/*F* values, accompanied by increases in area under the concentration (AUC; 13-fold), *C*_{max} (7-fold), and elimination half-life (*t*_{1/2}; 5-fold) values (Table 1). Consistent with the decrease in nicotine metabolism, plasma levels of cotinine, the main circulating metabolite of nicotine, were much decreased in the *Cyp2a(4/5)bgs*-null mice, as indicated by decreases in AUC (4-fold) and *C*_{max} (7.5-fold) values compared with WT.

Levels of nicotine and cotinine were also determined for the liver, the main metabolic organ, and the brain, the target organ, and compared between WT and *Cyp2a(4/5)bgs*-null mice (Fig. 2, B and C). The concentration-time curves for nicotine and cotinine are similar in the liver, brain, and plasma of either mouse strain. The AUC and *C*_{max} values for brain or liver nicotine were both increased in *Cyp2a(4/5)bgs*-null mice over WT mice (by ~3- and ~1.5-fold, respectively), whereas the AUC and *C*_{max} values for brain or liver cotinine were both decreased (by ~2-fold and ~6.5-fold, respectively) (Table 1).

We also determined the relative contributions of *Cyp2a5* and the *Cyp2b* genes to nicotine clearance by directly comparing nicotine and cotinine levels between male *Cyp2a(4/5)bgs*-null mice and male *Cyp2a5*-null mice (Fig. 3; Table 2). Given that *Cyp2s1* and *Cyp2g1* are not expressed in hepatocytes, and *Cyp2a4* is female-predominant, the

Fig. 1. Rates of in vitro metabolism of nicotine by liver microsomes of WT and *Cyp2a(4/5)bgs*-null mice. Rates of cotinine formation were determined as described in *Materials and Methods*. Reaction mixtures contained 50 mM potassium phosphate buffer (pH 7.4), 1.0 or 10 μM nicotine, 0.5 mg/ml liver microsomal protein, and 1.0 mg/ml liver cytosol protein (as a source of aldehyde oxidase) from 2-month-old male mice, and 1.0 mM reduced β-nicotinamide adenine dinucleotide phosphate, and the reactions were carried out for 15 minutes. The values presented are means ± S.D. (*n* = 3). ***P* < 0.01, compared with WT; Student's *t* test (unpaired).

Fig. 2. Plasma and tissue levels of nicotine and cotinine in mice treated with nicotine. WT and *Cyp2a(4/5)bgs*-null mice (2 months old, male) were injected with nicotine at 1.0 mg/kg i.p., and levels of nicotine and cotinine were determined in the plasma (A), liver (B), and brain (C) at various times after the injection. Data represent means $\pm$ S.D. ($n = 4$ for plasma; $n = 3-4$ for tissue). * $P < 0.05$ and ** $P < 0.01$ compared with WT mice; Student's t test (unpaired). Pharmacokinetic parameters are shown in Table 1.

difference between male *Cyp2a(4/5)bgs*-null mice and male *Cyp2a5*-null mice is the presence of the five *Cyp2b* genes (*2b9*, *2b10*, *2b13*, *2b19*, and *2b23*). In vitro studies of hepatic microsomal nicotine metabolism showed that rates of cotinine formation were not different between *Cyp2a(4/5)bgs*-null and *Cyp2a5*-null mice at a nicotine concentration of 1 μ M (7.1 ± 0.8 vs. 7.2 ± 1.3 ; mean $\pm$ S.D., $n = 3$) but were $\sim 60\%$ lower in the *Cyp2a(4/5)bgs*-null mice than in *Cyp2a5*-null mice at a nicotine concentration of 10 μ M (28.7 ± 0.7 vs. 66.2 ± 2.3 ; mean $\pm$ S.D., $n = 3$, $P < 0.01$), suggesting that the mouse CYP2B enzymes have higher K_m values than CYP2A5 toward nicotine metabolism. In vivo studies of plasma nicotine and cotinine showed that, in the *Cyp2a(4/5)bgs*-null mice, there were further decreases (versus *Cyp2a5*-null mice) in nicotine clearance ($\sim 55\%$ in CL/F values) and increases in AUC and C_{max} ($>65\%$), as well as $t_{1/2}$ values (1.4-fold), and corresponding decreases in abundance of cotinine (by $>40\%$ in AUC and C_{max}) (Table 2). Interestingly, the $t_{1/2}$ values were comparable between the two null strains for cotinine. Thus, the mouse CYP2B enzymes also play a significant role in converting

nicotine to cotinine in vivo, but they may not contribute to further clearance of cotinine.

Nicotine's Acute Pharmacological Effects on WT and *Cyp2a(4/5)bgs*-Null Mice. Nicotine-induced acute pharmacological effects, including antinociception and hypothermia, were observed after intraperitoneal injection of nicotine at 5.0 mg/kg in *Cyp2a(4/5)bgs*-null mice and WT mice. Mice were tested at various times (up to 180 minutes) after nicotine dosing for rectal temperature (Fig. 4A) and for thermal nociceptive responses in tail-immersion (Fig. 4B) and hot-plate (Fig. 4C) trials.

As illustrated in Fig. 4A, although nicotine-induced hypothermia lasted ~ 180 minutes in both WT and *Cyp2a(4/5)bgs*-null mice, the *Cyp2a(4/5)bgs*-null mice had a greater body temperature decrease than WT mice did at all time points examined. The maximal decrease was observed at 30 minutes for WT and 45 minutes for *Cyp2a(4/5)bgs*-null mice after nicotine dosing.

Similar time-course patterns were observed in the tail-immersion and hot-plate tests. The onset of action for

TABLE 1

Pharmacokinetic parameters for nicotine and cotinine in mice treated with nicotine at 1.0 mg/kg

Values from Fig. 2 were used for calculating pharmacokinetic parameters. Data for plasma represent means $\pm$ S.D. ($n = 4$ for each group of mice). For liver and brain, pharmacokinetic parameters were calculated using mean concentration values for each time point ($n = 3-4$); different mice were used for each time point.

Tissue	Analyte	Mouse Strain	AUC _{0-240 min}	$t_{1/2}$	T_{max}	C_{max}	CL/F
			ng·h/ml	min		ng/ml	ml/min
Plasma	Nicotine	WT	16.7 $\pm$ 4.6	16.6 $\pm$ 0.4	5.0 $\pm$ 0.0	47.3 $\pm$ 14.9	21.6 $\pm$ 5.6
		<i>Cyp2a(4/5)bgs</i> -null	220 $\pm$ 52*	83.6 $\pm$ 13.2*	5.0 $\pm$ 0.0	335 $\pm$ 54*	1.5 $\pm$ 0.4*
	Cotinine	WT	487 $\pm$ 53	30.8 $\pm$ 2.0	23.8 $\pm$ 12.5	297 $\pm$ 30	N/A
		<i>Cyp2a(4/5)bgs</i> -null	119 $\pm$ 3*	120 $\pm$ 12*	60.0 $\pm$ 0.0*	39.9 $\pm$ 2.9*	N/A
Liver	Nicotine	WT	172	32.5	5.0	680	N/A
		<i>Cyp2a(4/5)bgs</i> -null	428	41.2	5.0	889	N/A
	Cotinine	WT	333	29.7	5.0	450	N/A
		<i>Cyp2a(4/5)bgs</i> -null	142	125	30.0	65.3	N/A
Brain	Nicotine	WT	101	16.1	5.0	298	N/A
		<i>Cyp2a(4/5)bgs</i> -null	333	33.7	5.0	566	N/A
	Cotinine	WT	205	30.9	15.0	209	N/A
		<i>Cyp2a(4/5)bgs</i> -null	98.6	211	30.0	33.6	N/A

N/A, not applicable.

* $P < 0.05$ compared with corresponding WT values; Student's t test (unpaired).

nicotine-induced antinociceptive responses was rapid in both strains of mice, with maximal responses occurring between 0 and 5 minutes (Fig. 4, B and C). However, the antinociceptive effect disappeared ~ 60 minutes after nicotine dosing in WT mice, but it lasted ~ 180 minutes in the *Cyp2a(4/5)bgs*-null mice. Furthermore, the magnitude of the effect was significantly greater in *Cyp2a(4/5)bgs*-null mice than in WT mice, at multiple time points and in both tests. In control experiments (data not shown), saline (vehicle) injection did not produce any hypothermia or antinociceptive responses in either strain of mice.

The increases in both duration and strength of nicotine's acute pharmacological effects in the *Cyp2a(4/5)bgs*-null mice, relative to WT mice, are consistent with the observed decrease in nicotine clearance (Fig. 2; Table 1; Supplemental Fig. 1; Supplemental Table 1). These results confirm greater nicotine bioavailability in the target tissue, the brain, of the *Cyp2a(4/5)bgs*-null mice.

Nicotine's Rewarding Effects on WT and *Cyp2a(4/5)bgs*-Null Mice. Nicotine administration can produce rewarding effects in mice in a dose-dependent manner (Acquas et al., 1989). Here, we used an unbiased CPP paradigm (Grabus

Fig. 3. A comparison of plasma levels of nicotine and cotinine between *Cyp2a5*-null and *Cyp2a(4/5)bgs*-null mice. *Cyp2a5*-null and *Cyp2a(4/5)bgs*-null mice (2 months old, male) were injected with nicotine at 1.0 (A) or 5.0 (B) mg/kg (i.p.), and levels of nicotine and cotinine were determined in the plasma at various times after the injection. Data represent means $\pm$ S.D. ($n = 4$). * $P < 0.05$ and ** $P < 0.01$ compared with *Cyp2a5*-null mice; Student's t test, unpaired. Pharmacokinetic parameters are shown in Table 2.

TABLE 2

A comparison of pharmacokinetic parameters for nicotine and cotinine between *Cyp2a5*-null and *Cyp2a(4/5)bgs*-null mice. Values from Fig. 3 were used for calculating pharmacokinetic parameters. Data represent means $\pm$ S.D. ($n = 4$).

Nicotine Dose	Analyte	Mouse Strain	AUC _{0-240 min}	$t_{1/2}$	T_{max}	C_{max}	CL/F
			ng·h/ml	min		ng/ml	ml/min
1 mg/kg	Nicotine	<i>Cyp2a5</i> -null	116 $\pm$ 2.1	61.1 $\pm$ 1.5	5.0 $\pm$ 0.0	201 $\pm$ 35	3.38 $\pm$ 0.2
		<i>Cyp2a(4/5)bgs</i> -null	220 $\pm$ 52*	83.6 $\pm$ 13.2*	5.0 $\pm$ 0.0	335 $\pm$ 54*	1.5 $\pm$ 0.4*
	Cotinine	<i>Cyp2a5</i> -null	229 $\pm$ 27	148 $\pm$ 36	50.0 $\pm$ 17.3	72.6 $\pm$ 10.8	N/A
		<i>Cyp2a(4/5)bgs</i> -null	119 $\pm$ 3*	120 $\pm$ 12	60.0 $\pm$ 0.0	39.9 $\pm$ 2.9*	N/A
5 mg/kg	Nicotine	<i>Cyp2a5</i> -null	297 $\pm$ 31	102 $\pm$ 36	5.0 $\pm$ 0.0	356 $\pm$ 66	6.78 $\pm$ 0.7
		<i>Cyp2a(4/5)bgs</i> -null	499 $\pm$ 97*	158 $\pm$ 28*	5.0 $\pm$ 0.0	592 $\pm$ 149*	3.16 $\pm$ 0.6*
	Cotinine	<i>Cyp2a5</i> -null	1870 $\pm$ 160	224 $\pm$ 48	75.0 $\pm$ 30.0	371 $\pm$ 25	N/A
		<i>Cyp2a(4/5)bgs</i> -null	1130 $\pm$ 30*	204 $\pm$ 14	105 $\pm$ 30	225 $\pm$ 12*	N/A

N/A, not applicable.

* $P < 0.05$ compared with corresponding *Cyp2a5*-null values.

et al., 2006) to compare WT and *Cyp2a(4/5)bgs*-null mice for the doses of nicotine required to establish nicotine-induced reward (Fig. 5). On the basis of the previously described

pharmacological results, we hypothesized that a lower nicotine dose would be required to produce reward in the *Cyp2a(4/5)bgs*-null mice than in WT mice.

Mice selected for the CPP test did not show a significant inherent preference (bias) to either compartment, as indicated by the typical example shown in Fig. 5A, where mice of either genotype spent approximately equal time in the two chambers in the preconditioning phase. In agreement with previous reports (Matta et al., 2007), WT mice displayed an inverted U-shaped dose-response curve, with maximal, significant ($P < 0.01$ compared with saline group) place preference elicited at a nicotine dose of 0.5 mg/kg (Fig. 5, B and C). As shown in Fig. 5B, WT mice spent significantly more time in the nicotine-paired chamber after (day 5), compared with before (day 1), nicotine conditioning at 0.5 mg/kg ($P < 0.05$, either paired t test or 2-way ANOVA, for day and chamber). There was no significant preference observed at other doses tested in WT mice (Fig. 5C).

As predicted, the dose response in the *Cyp2a(4/5)bgs*-null mice shifted noticeably to the left, compared with that in WT mice, and a significant behavioral reinforcement response (place preference) was detected in *Cyp2a(4/5)bgs*-null mice, but not in WT mice, at a nicotine dose of 0.1 mg/kg (Fig. 5C). Thus, the nicotine dose required to produce CPP in the *Cyp2a(4/5)bgs*-null mice was 4-fold lower than that in WT mice. Further analysis of the data in Fig. 5C using 2-way ANOVA (for treatment and genotype) indicated a significant effect of nicotine treatment ($F = 5.38$, $P < 0.01$), but not genotype ($F = 1.40$, not significant), and a significant interaction between the two ($F = 11.01$, $P < 0.01$). In addition, paired t tests (data not shown) performed for each treatment group, comparing the number of transitions through the compartments between preconditioning (day 1) and postconditioning (day 5), showed that there was no drug treatment-related difference, indicating that the treatments did not change animal mobility.

Discussion

Several pharmacogenetics studies in humans have suggested that smoking behaviors are affected by alterations in nicotine metabolism (see review in Amos et al., 2010). This idea is also supported by data from animal studies. The behavioral and pharmacological effects of nicotine in mice have been well documented (Faraday et al., 2005; Grabus et al., 2005). Inhibition of nicotine metabolism by methoxsalen, a somewhat selective inhibitor of CYP2A, led to a

Fig. 4. Time course of nicotine-induced hypothermia and antinociception in WT and *Cyp2a(4/5)bgs*-null mice. WT and *Cyp2a(4/5)bgs*-null mice (2 months old, male) were injected with nicotine at 5.0 mg/kg and then evaluated for body temperature (A) and thermal nociceptive responses in a tail-immersion test (B) and hot-plate test (C). Mice were tested at various time points after injection. Data represent means $\pm$ S.E. of 8 mice per group. %MPE, percentage of maximal possible effect. * $P < 0.05$ compared with WT values; Student's t test (unpaired).

Fig. 5. Performance of WT and *Cyp2a(4/5)bgs*-null mice in CPP test. The CPP test was performed as described in *Materials and Methods*. For each group, time spent in white or color-striped chambers was recorded on day 1 (preconditioning) and day 5 (after nicotine conditioning). (A) Representative data on total time spent in white chamber or color-striped chamber on day 1 by WT and *Cyp2a(4/5)bgs*-null mice that were selected for the test (means $\pm$ S.E., $n = 10$; $P > 0.05$, comparing between strains for each chamber; Student's t test, paired). (B) An example (from WT mice) of the effects of nicotine conditioning on place preference. Mice were treated with nicotine at 0.5 mg/kg and saline for 3 consecutive days, following preconditioning on day 1. The injection of nicotine was paired with placement in the white chamber, and saline with the color-striped chamber, in this example. Time spent in each chamber when tested on day 1 and day 5 is shown (means $\pm$ S.E., $n = 9$). **/* $P < 0.01$ compared with day 1; Student's t test, paired; or $P < 0.05$, 2-way ANOVA, for day and chamber. # $P < 0.05$ compared with white chamber for day 5, 2-way ANOVA, for day and chamber. (C) Effectiveness of different nicotine doses in producing CPP in WT and *Cyp2a(4/5)bgs*-null mice. A specific nicotine dose was paired with one randomly selected chamber, and saline with the other. Saline was paired with both chamber choices for the control group (0 mg/kg). Data represent mean preference scores $\pm$ S.E. ($n = 8$ –12 per group). ** $P < 0.01$ compared with corresponding saline group (1-way ANOVA, followed by Tukey's test).

prolongation of nicotine's acute pharmacological effects as well as a decrease in nicotine clearance (Damaj et al., 2007). Our present study using the *Cyp2a(4/5)bgs*-null mouse, an engineered mouse model with slow nicotine metabolism, provides further, more definitive evidence for the connection between nicotine metabolism and nicotine's actions on the central nervous system. The *Cyp2a(4/5)bgs*-null mice, with a considerably (>90%) decreased rate of nicotine clearance, showed remarkable increases in the magnitude and persistence of responses to nicotine's acute pharmacological effects, compared with WT mice. The slow nicotine metabolism in the *Cyp2a(4/5)bgs*-null mouse was also accompanied by an increase in sensitivity to nicotine-induced rewarding effects in the CPP test, a result supporting the hypothesis that rates of nicotine clearance can influence the rewarding effects of nicotine.

There are two commonly used models for assessing the rewarding properties of nicotine: the CPP test, in which animals are subjected to drug administration to elicit reward effects, and the self-administration test, which requires surgical implantation of a catheter, for intravenous drug administration, and extensive operant training (Matta et al., 2007). The CPP test offers several advantages over the self-administration model, including a simple methodology and well established protocols (Cunningham et al., 2006; Grabus

et al., 2006); however, the self-administration test, in which animals learn a task in which the responses produce reward effects from drug administration, appears to be more similar to drug use in humans. Both tests are capable of assessing reward effects in an unbiased fashion. In that regard, the current CPP paradigm used an unbiased design, in which only those subjects that did not show a significant preference to either compartment were selected for testing. Furthermore, the preference score was calculated as "time (seconds) in drug-paired compartment during test minus time (seconds) in drug-paired compartment during preconditioning," instead of as "time (seconds) in drug-paired compartment during test minus time (seconds) in saline-paired compartment during test," to eliminate any residual preconditioning bias.

Our findings regarding the impact of the *Cyp2a/2b* genotype on nicotine reward in naive *Cyp2a(4/5)bgs*-null and WT mice, all of the same genetic background, age, sex, and environmental exposures, support the results of previous human studies (O'Loughlin et al., 2004; Karp et al., 2006), which suggests that novice subjects with slow nicotine metabolism are at a higher risk of becoming dependent on nicotine. However, it remains to be determined whether our results support the findings of a more recent study (Audrain-McGovern et al., 2007), which suggested that normal nicotine

metabolizers progressed more quickly to nicotine dependence than slow nicotine metabolizers, given that our study was not designed to test how quickly nicotine dependence was established in *Cyp2a(4/5)bgs*-null and WT mice. Additionally, our study does not address the relationship between rates of nicotine metabolism and amounts of cigarette smoking (i.e., nicotine intake) in subjects that are already dependent on nicotine. The latter relationship, which could be studied using the *Cyp2a(4/5)bgs*-null mouse and the nicotine self-administration model, is already strongly supported by data from human studies (Amos et al., 2010).

This is also the first study to demonstrate the role of CYP2B in nicotine metabolism in vivo, as indicated by the ~55% decrease in rates of nicotine clearance in male *Cyp2a(4/5)bgs*-null mice, relative to male *Cyp2a5*-null mice. Our previous findings indicated that CYP2A5 plays an important role as a low- K_m enzyme in nicotine oxidation in mouse liver (Zhou et al., 2010). The present study suggested that the mouse CYP2B enzymes have higher K_m values than CYP2A5 toward nicotine metabolism, as differences between the two mouse strains in hepatic microsomal activity toward nicotine were only apparent at the higher nicotine concentration studied (10 μ M). Our in vivo pharmacokinetics data also suggest that the mouse CYP2B enzymes do not play a significant role in cotinine metabolism, given that the $t_{1/2}$ values for cotinine were comparable between *Cyp2a(4/5)bgs*-null and *Cyp2a5*-null mice, following nicotine treatment. This result is in contrast with the large increase in $t_{1/2}$ values in *Cyp2a5*-null mice, compared with WT mice, and the demonstrated role of CYP2A5 in cotinine clearance (Zhou et al., 2010).

Previous studies have reported that CYP2B is expressed and inducible in human and rodent brain and that smokers and alcoholics have elevated brain CYP2B levels (Miksys and Tyndale, 2002; Miksys et al., 2003). In contrast, neither human nor mouse CYP2A enzymes have been found to have remarkable expression in the brain (Ferguson and Tyndale, 2011). Brain CYP2B has been shown to participate in propofol metabolism and modification of propofol's anesthetic response (Khokhar and Tyndale, 2011). Thus, it is worthwhile to further establish whether brain CYP2B plays a role in nicotine metabolism within the central nervous system, thereby modifying nicotine's acute pharmacological effects and behavior responses. In that regard, our current study, utilizing whole-body knockout mouse models, was not designed to address tissue-specific contributions to modulation of nicotine's effects, although the differences seen among the mouse models are most likely indicative of hepatic contributions. Additional studies addressing the specific role of brain CYP2B in modulating nicotine's effects are warranted, and can use the *Cyp2a(4/5)bgs*-null mouse model in conjunction with localized drug treatment paradigms, such as intracerebroventricular injection, or a brain-specific *Cyp2a(4/5)bgs*-null mouse model. Notably, these mouse models can be used to study the role of brain CYP2B in the metabolism of a variety of centrally acting drugs or neurochemicals, in addition to nicotine, such as bupropion, ecstasy, and serotonin (Ekens et al., 2008).

In summary, we proved that CYP2B enzymes, as well as CYP2A5, play significant roles in nicotine metabolism in vitro and in nicotine clearance in vivo. We further showed that the decrease in nicotine clearance, resulting from genetic ablation of the major nicotine-metabolizing P450 enzymes, in the

Cyp2a(4/5)bgs-null mouse led to increased sensitivity to nicotine's pharmacological effects and nicotine-induced acute reward actions in the CPP test. Our findings support the notion that decreases in nicotine clearance (e.g., as a result of CYP genetic polymorphism) will not only affect nicotine's pharmacological actions, but will also likely modify smoking behavior in humans. Nonetheless, additional studies using the *Cyp2a(4/5)bgs*-null mouse model, including nicotine self-administration tests and analysis of nicotine metabolism in the brain, are required to provide a definitive understanding of how pharmacokinetic parameters may alter nicotine's rewarding effects.

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Authorship Contributions

Participated in research design: Li, Zhou, McCallum, Hough, Ding.

Conducted experiments: Li, Jia.

Performed data analysis: Li, Ding.

Wrote or contributed to the writing of the manuscript: Li, McCallum, Hough, Ding.

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Address correspondence to: Dr. Xinxin Ding, Wadsworth Center, New York State Department of Health, Empire State Plaza, Box 509, Albany, NY 12201-0509. E-mail: xding@wadsworth.org