Effects of Hydroxymetabolites of Bupropion on Nicotine Dependence Behavior in Mice

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ABSTRACT

Bupropion is an atypical antidepressant that also has utility as a smoking cessation aid. Hydroxybupropions are major metabolites of bupropion and are believed to contribute to antidepressant and perhaps smoking cessation activities. Because bupropion metabolism is more similar in humans and mice than in humans and rats, the present study investigated effects of hydroxybupropion enantiomers in mouse behavioral models measuring various aspects of nicotine dependence. Bupropion and (2S,3S)-hydroxybupropion, but not (2R,3R)-hydroxybupropion, significantly decreased the development of nicotine reward as measured in the conditioned place preference and

withdrawal paradigm in mice. Bupropion and both of its metabolites reversed affective and somatic withdrawal signs in nicotine-dependent mice, but the (2S,3S)-hydroxymetabolite had higher potency. Bupropion and (2S,3S)-, but not (2R,3R)-hydroxybupropion, produced partial substitution for nicotine in drug discrimination tests. Our findings support the hypothesis that the effects of bupropion on measures of nicotine dependence reflect actions of bupropion itself, its hydroxymetabolites, or a combination and suggest that the (2S,3S)-hydroxy isomer is the most active principle, making it a potentially better drug candidate for smoking cessation than bupropion.

Introduction

Tobacco use is the leading cause of premature death in the United States. The majority of smokers report a desire to quit smoking, but poor smoking cessation results indicate a need to explore innovative approaches to treating nicotine addiction. Pharmacotherapies for treating tobacco addiction are limited, and they include nicotine-replacement therapies, bupropion and varenicline. The efficacy of bupropion in the treatment of nicotine dependence was thought to involve modulation of DA and NE systems. Indeed, bupropion is a relatively weak DA reuptake inhibitor and inhibits the firing of locus coeruleus NE neurons at high concentrations in rodents (Cooper et al., 1994). The inhibition of transporter function by bupropion is associated with increases in extracellular DA and NE concentrations, which may substitute for nicotine-evoked neurotransmitter release during smoking,

mimicking nicotine reinforcement and alleviating withdrawal symptoms stemming from the absence of nicotine.

However, recent findings from our laboratories showed that bupropion also acts as a relatively potent, noncompetitive antagonist at various natural and expressed nAChR subtypes (Fryer and Lukas, 1999; Slemmer et al., 2000; Damaj et al., 2004). Other work demonstrated antagonism of nicotine-evoked DA and NE release in rat striatal slices (Miller et al., 2002). Moreover, bupropion blocks several of the behavioral effects of nicotine at doses similar to or lower than those having activity in antidepressant behavioral tests in mice (Martin et al., 1990; Slemmer et al., 2000; Damaj et al., 2004).

Various preclinical studies investigating the effects of bupropion in animal models of nicotine dependence indicated that bupropion altered nicotine-associated changes in reward, nicotine self-administration, nicotine drug discrimination, nicotine withdrawal-associated conditioned place aversion, and somatic and affective signs of withdrawal (for review, see Paterson, 2009). However, most of these studies were conducted in rats. Although these results have been valuable in understanding the mechanisms of action of bu-

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ABBREVIATIONS: DA, dopamine/dopaminergic; NE, norepinephrine/noradrenergic; nAChR, nicotinic acetylcholine receptor; CPP, conditioned place preference; FR, fixed ratio; DAT, dopamine transporter; NET, norepinephrine transporter.

propion in nicotine dependence, metabolism studies indicated that bupropion metabolism is closer in humans and mice than in humans and rats. Specifically, there is much lower formation and more rapid elimination of hydroxybupropion in rats versus humans (Suckow et al., 1986; Welch et al., 1987). Furthermore, bupropion is extensively metabolized to (2*R*,3*R*)- and (2*S*,3*S*)-hydroxybupropion, (*R*,*R*)- and (*S*,*S*)-threohydrobupropion, and (*R*,*S*)- and (*S*,*R*)-erythrohydrobupropion in humans and mice (Cooper et al., 1984). The concentrations of hydroxybupropion isomers present in human cerebrospinal fluid are six times greater than those of the parent bupropion (Cooper et al., 1984). Indeed, plasma levels of hydroxybupropion greatly exceed those of the parent drug, reaching 10 to 100 times the concentration of bupropion (Findlay et al., 1981; Welch et al., 1987; Golden et al., 1988; Hysu et al., 1997). This is an important point because hydroxymetabolites of bupropion were shown to possess interesting neurobiological effects.

We reported previously that racemic bupropion and its (2*S*,3*S*)-hydroxymetabolite, but not (2*R*,3*R*)-hydroxybupropion (which is inactive; $IC_{50} > 10 \mu M$), inhibit [3H]DA uptake with similar potency and that (2*S*,3*S*)-hydroxybupropion is 6-fold more active than bupropion, whereas the (2*R*,3*R*)-hydroxy isomer is inactive as an inhibitor of [3H]NE uptake (studies in vitro of heterologously expressed, human transporters; Damaj et al., 2004). We also showed that whereas bupropion has higher functional inhibitory potency at human $\alpha_3\beta_4$ nAChRs than at other nAChR subtypes, the (2*S*,3*S*)-isomer is more potent than the (2*R*,3*R*)-isomer or racemic bupropion as an antagonist of $\alpha_4\beta_2$ nAChRs (Damaj et al., 2004). In addition, (2*S*,3*S*)-hydroxybupropion is 5- to 15-fold more potent than bupropion and even more potent than the sometimes inactive (2*R*,3*R*)-hydroxybupropion in blocking acute effects of nicotine on body temperature and locomotion and acute, nicotine-mediated analgesia in mice (Damaj et al., 2004).

Given the extensive metabolism of bupropion to hydroxybupropion in humans, which has a long half-life and substantial neurobiological activity, we hypothesized that (2*S*,3*S*)-hydroxybupropion isomer plays an important role in effects of bupropion on nicotine dependence. In the current study, we compared the effects of bupropion and enantiomers of hydroxybupropion [(2*R*,3*R*)- and (2*S*,3*S*)-hydroxybupropion isomers] on several behavioral aspects of nicotine dependence in mice. Specifically, we measured the effects of these drugs on nicotine reward using the CPP paradigm, nicotine drug discrimination, and nicotine withdrawal (both somatic and affective signs).

Materials and Methods

Animals

Male ICR (Harlan, Indianapolis, IN) and C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were housed in a 21°C humidity-controlled Association for Assessment and Accreditation of Laboratory Animal Care-approved animal care facility with food and water available ad libitum. The rooms were on a 12-h light/dark cycle (lights on at 7:00 AM). Mice were 8 to 10 weeks of age and weighed approximately 20 to 25 g at the start of all the experiments. For the drug discrimination studies, mice were maintained at 85 to 90% of free feeding body weights by restricting daily rations of standard rodent chow. All experiments were performed during the light cycle (between 7:00 AM and 7:00 PM) and were approved by the Institu-

tional Animal Care and Use Committee of Virginia Commonwealth University and in accordance with the National Institutes of Health Guide for Animal Care and Use.

Drugs

(-)-Nicotine hydrogen tartrate salt [(-)-1-methyl-2-(3-pyridyl)pyrrolidine (+)-bitartrate salt] and mecamylamine HC were purchased from Sigma-Aldrich (St. Louis, MO). Bupropion HCl was purchased from Sigma/RBI (Natick, MA). (+)-(2*S*,3*S*)- and (-)-(2*R*,3*R*)-, (+)-(2*S*,3*S*)-, and (-)-(2*R*,3*R*)-hydroxybupropion [2-(3-chlorophenyl)-3,5,5-trimethylmorpholin-2-ol] tartrates were synthesized using previously reported methods (Fang et al., 2000). All drugs were dissolved in physiological saline (0.9% sodium chloride) and given in a total volume of 1 ml/100 g body weight for subcutaneous injections. All doses are expressed as the free base of the drug.

Nicotine CPP Assessment

An unbiased CPP paradigm was used in this study as described in Kota et al. (2007). In brief, place-conditioning chambers consisted of two distinct compartments separated by a smaller intermediate compartment with openings that allowed access to either side of the chamber. On day 1, animals were confined to the intermediate compartment for a 5-min habituation period and then allowed to move freely between compartments for 15 min. Time spent in each compartment was recorded. These data were used to separate the animals into groups of approximately equal bias. Days 2 to 4 were the conditioning days during which the saline group received saline in both compartments and drug groups received subcutaneous vehicle, bupropion, (2*S*,3*S*)-hydroxybupropion, or (2*R*,3*R*)-hydroxybupropion 15 min before nicotine (0.5 mg/kg s.c.) in one compartment and saline in the opposite compartment for 20 min. Drug-paired compartments were randomized among all groups. Day 5 was the drug-free test day, and the procedure was the same as day 1. Activity counts and time spent on each side were recorded via photosensors using MED Associates (St. Albans, VT) interface and software. Separate groups of mice were conditioned with saline, bupropion, (2*S*,3*S*)-hydroxybupropion, or (2*R*,3*R*)-hydroxybupropion alone to investigate whether they induce CPP by using the same procedure described above. Data are expressed as time spent on drug-paired side minus time spent on saline-paired side. A positive number indicated a preference for the drug-paired side, whereas a negative number indicated an aversion to the drug-paired side. A number at or near zero indicated no preference for either side.

Chronic Nicotine Administration Protocol

Mice were anesthetized with sodium pentobarbital (45 mg/kg i.p.) and implanted with Alzet osmotic minipumps [model 2004 (14 days); Durect Corporation, Cupertino, CA] filled with (-)-nicotine or saline solution as described in Jackson et al. (2008). The concentration of nicotine was adjusted according to animal weight and minipump flow rate. For withdrawal studies, mice received 36 mg/kg/day for 14 days.

Nicotine Withdrawal Assessment

Withdrawal studies were conducted as described previously in Jackson et al. (2008). In brief, minipumps were removed on the evening of day 14, and testing was initiated on day 15, approximately 18 to 24 h after minipump removal. Mice were then injected with subcutaneous vehicle, bupropion, (2*S*,3*S*)-hydroxybupropion, or (2*R*,3*R*)-hydroxybupropion and 30 min after bupropion or its metabolites treatment, animals were tested. The mice were first evaluated for 5 min in the plus maze test for anxiety-related behavior, followed by a 20-min observation of somatic signs. Hyperalgesia was evaluated immediately after the somatic sign observation period. The specific testing sequence was chosen based on our prior studies showing that this order of testing reduced within-group variability and produced the most consistent results.

Somatic Signs. Animals were placed in individual, Plexiglas containers [28.5 cm (length) $\times$ 18 cm (width) $\times$ 13 cm (height)] and observed for 20 min for occurrences of headshakes, body tremors, involuntary paw tremors, retropulsion (involuntary backing), writhing, and jumping. For each animal, the total score for this assay was the sum of the individual measures.

Elevated Plus Maze. The elevated plus maze consisted of two closed arms and two open arms on a base raised 60 cm above the floor. The overhead lights were dimmed or removed before placing the mouse in the center of the maze. Each animal was allowed to roam freely between the open and the closed arms for 5 min. The duration of time that the mouse spent in the open arms was obtained through the use of photocell beams connected to a timing device. A change in the amount of time spent in the open arms is considered to be an affective sign of withdrawal.

Plantar Stimulation. Subjects were placed in clear Plexiglas compartments [13 cm (length) $\times$ 6.5 cm (width) $\times$ 25.5 cm (height)]. A radiant heat source was applied to the rear right paw, and withdrawal latency was recorded (three to four measurements per animal). The intensity of the heat source was adjusted to yield withdrawal latencies between 9 and 12 s in experimentally naive ICR mice (data not shown). A cut-off time of 20 s was used to minimize tissue damage. A change in response latency is indicative of a physical sign of withdrawal.

Nicotine Drug Discrimination in the Mouse

Apparatus. Eight standard mice operant conditioning chambers that were sound- and light-attenuated (MED Associates) were used for behavioral training and testing. Each operant conditioning chamber (18 $\times$ 18 $\times$ 18 cm) was equipped with a house light, two levers (left and right), and a recessed dipper receptacle centered between the levers. A dipper arm delivered sweetened milk in a 0.05-ml cup, which was available for 5 s. Fan motors provided ventilation and masking noise for each chamber. House lights were illuminated during training and testing sessions. A computer with Logic "1" interface and MED-PC software (MED Associates) was used to control schedule contingencies and to record data.

Procedures. *Lever press training.* Each mouse was placed in a standard operant chamber and trained to lever press according to a fixed ratio (FR)-1 schedule of reinforcement. Milk reinforcement was delivered after every lever press. The FR value was gradually increased to the final FR-10 schedule of reinforcement in which 10 consecutive responses were required for delivery of milk reinforcement. After mice were trained on one lever, contingency requirements for milk delivery were switched to the other lever. Lever press training at this second lever proceeded identically to that at the first lever. When responding on the second lever under an FR-10 schedule was acquired, discrimination training began.

Discrimination training. Mice were trained to press one lever after administration of 0.8 mg/kg nicotine and to press the other lever after saline administration according to an FR-10 schedule of milk reinforcement. Each response on the incorrect lever reset the response requirement on the correct lever. Daily injections were administered on a double alternation sequence of nicotine and saline (e.g., drug, drug, vehicle, vehicle). Daily 15-min training sessions were held Monday to Friday until the mice had met two criteria during eight of 10 consecutive sessions: 1) the first completed FR-10 (e.g., consecutive correct responses >10) and 2) $\geq 80\%$ of the total responding occurred on the correct lever. When these two criteria were met, acquisition of the discrimination was established and substitution testing began.

Substitution tests. After successful acquisition of the discrimination, stimulus substitution tests were conducted on Tuesdays and Fridays during 15-min test sessions. Training continued on Mondays, Wednesdays, and Thursdays. During test sessions, responses on either lever delivered reinforcement according to an FR-10 schedule. To be tested, mice must have completed the first FR-10 on the correct lever and $\geq 80\%$ of the total responding must have occurred

on the correct lever on the preceding day. In addition, the mouse must have met these same criteria during previous training sessions with the alternate training compound (training drug or vehicle). Before substitution tests generalization curve for nicotine were generated with all mice. Then, substitution tests were conducted with bupropion, (2S,3S)-hydroxybupropion and (2R,3R)-hydroxybupropion. To ensure that nicotine's discriminative stimulus effects were effectively maintained through out these studies, control tests with the training dose of nicotine and with vehicle were redetermined before conducting all substitution tests.

Statistical Analysis

For the drug discrimination studies, acquisition indices were the percentage of animals that pressed the first FR on the correct lever and achieved $\geq 80\%$ of the total responding on the correct lever during the course of the session. For each test session, percentage of responses on the drug lever and response rate (responses/sec) was calculated. Because mice that responded less than 10 times during a test session did not press either lever a sufficient number of times to earn a reinforcer, their data were excluded from analysis of nicotine lever selection, but their response rate data were included. Response-rate suppression (relative to rates after vehicle administration) was determined by separate analyses of variance (ANOVA) using GB-STAT statistical software (Dynamic Microsystems, Silver Spring, MD). Significant ANOVAs were further analyzed with Dunnett's post hoc tests ($\alpha = 0.05$) to specify differences between means. For all other behavioral studies, statistical analyses were performed using two-way analysis of variance test with nicotine and bupropion treatments as the between-subject factors. p values < 0.05 were considered to be statistically significant. Significant results were further analyzed using the Bonferroni post hoc test. ED_{50} values were calculated for percentage of responses on the nicotine lever using least-squares linear regression analysis followed by calculation of 95% confidence limits (Tallarida and Murray, 1987).

Results

Effects of Bupropion, (2S,3S)-Hydroxybupropion, or (2R,3R)-Hydroxybupropion on the Induction of Nicotine CPP in Mice. As shown in Fig. 1, nicotine (0.5 mg/kg s.c.) produced a robust and significant CPP in mice, and bupropion pretreatment altered its development. Comparison of the different treatments reveals significant effects of nicotine and bupropion groups (nicotine: $F_{1,63} = 29$, $p < 0.001$; bupropion: $F_{3,63} = 13.67$, $p < 0.001$; and nicotine $\times$ bupropion interaction: $F_{3,63} = 12.02$, $p < 0.001$). Pretreatment with bupropion (0.1, 0.5, or 1 mg/kg s.c.) significantly

Fig. 1. Effects of bupropion on the acquisition of the nicotine-induced CPP. Nicotine (0.5 mg/kg s.c.) elicited significant place preference (shaded bar) that was blocked by administration of bupropion at different doses (0.1, 0.5, and 1 mg/kg) during the conditioning phase. Each bar represents the mean ($n = 8-10$ /group) $\pm$ S.E.M. of preference score in seconds. *, $p < 0.05$ versus the vehicle control groups.

Fig. 2. Effects of (2*S*,3*S*)-hydroxybupropion on the acquisition of the nicotine-induced CPP. Nicotine (0.5 mg/kg s.c.) elicited significant place preference (shaded bar) that was blocked by administration of (2*S*,3*S*)-hydroxybupropion at different doses (0.05, 0.1, or 0.5 mg/kg) during the conditioning phase. Each bar represents the mean ($n = 8-10/\text{group}$) $\pm$ S.E.M. of preference score in seconds. *, $p < 0.05$ versus the vehicle control groups.

TABLE 1

Summary of antagonistic potency of bupropion and its hydroxymetabolites toward $\alpha_4\beta_2^*$ and $\alpha_3\beta_4^*$ nAChR subtypes and DA and NE transporters uptake

Estimated EC₅₀ values in the CPP, somatic signs of withdrawal, and drug discrimination tests also are shown.

	$\alpha_3\beta_4^*$, IC ₅₀ ^a	$\alpha_4\beta_2^*$, IC ₅₀ ^a	[³ H]DA, IC ₅₀ ^a	[³ H]NE, IC ₅₀ ^a	CPP, EC ₅₀	Somatic Signs, EC ₅₀	Drug Discrimination, EC ₅₀
	μM		nM			mg/kg	
Bupropion	1.8	12	550	1900	0.35	9	45
(2 <i>S</i> ,3 <i>S</i>)-OH Bupropion	10	3.3	790	520	0.1	1.8	24
(2 <i>R</i> ,3 <i>R</i>)-OH Bupropion	6.5	31	>10,000	>10,000	10% at 1	30% at 10	6% at 60

^a Data from Damaj et al. (2004).

Fig. 3. Effects of (2*R*,3*R*)-hydroxybupropion on the acquisition of the nicotine-induced CPP. Nicotine (0.5 mg/kg s.c.) elicited significant place preference (shaded bar) that was not blocked by administration of (2*R*,3*R*)-hydroxybupropion at 1 mg/kg during the conditioning phase. Each bar represents the mean ($n = 8-10/\text{group}$) $\pm$ S.E.M. of preference score in seconds. *, $p < 0.05$ versus the vehicle control groups.

decreased the development of nicotine CPP in mice conditioned with 0.5 mg/kg nicotine, with the dose of 1 mg/kg completely blocking the response of nicotine. In a separate group of mice, bupropion alone at the doses tested (0.1, 0.5, or 1 mg/kg s.c.) did not produce a significant response in mice conditioned with saline. However, at higher doses of 5 and 10 mg/kg, bupropion dose-dependently induced a significant CPP in mice [vehicle, 15 ± 7 s; bupropion (5 mg/kg), 39 ± 14 s; and bupropion (10 mg/kg), 75 ± 9]. However, only the 10-mg/kg dose was significantly higher than the vehicle treatment.

Likewise, the (2*S*,3*S*)-hydroxybupropion metabolite significantly decreased the development of nicotine CPP in mice in a dose-dependent manner [nicotine: $F_{1,62} = 26$, $p < 0.001$; (2*S*,3*S*)-hydroxybupropion: $F_{3,62} = 14.71$, $p < 0.001$; and nicotine $\times$ (2*S*,3*S*)-hydroxybupropion interaction: $F_{3,62} = 13.64$, $p < 0.001$] (Fig. 2) and was more potent than bupropion (Table 1). Indeed, a dose of 0.1 mg/kg of the metabolite significantly reduced nicotine CPP, whereas the same dose of bupropion did not significantly block the effects of nicotine

(Fig. 1). In a separate group of mice, (2*S*,3*S*)-hydroxybupropion alone at the doses tested (0.05, 0.1, or 0.5 mg/kg s.c.) did not produce a significant response in mice conditioned with saline. However, a higher dose of 2 mg/kg (2*S*,3*S*)-hydroxybupropion induced a significant CPP response in mice [vehicle, 6.7 ± 7.1 s; and (2*S*,3*S*)-hydroxybupropion (2 mg/kg), 64 ± 15 s].

In contrast to the effects of (2*S*,3*S*)-hydroxybupropion (Table 1), the (2*R*,3*R*)-isomer at the relatively high dose of 1 mg/kg failed to block nicotine-induced CPP in mice [nicotine: $F_{1,28} = 11.9$, $p < 0.0017$; (2*R*,3*R*)-hydroxybupropion: $F_{1,28} = 0.006$, $p < 0.93$; and nicotine $\times$ (2*R*,3*R*)-hydroxybupropion interaction: $F_{1,28} = 0.365$, $p < 0.55$] (Fig. 3). Higher doses of (2*R*,3*R*)-hydroxybupropion were not tested.

Effects of Bupropion, (2*S*,3*S*)-Hydroxybupropion, or (2*R*,3*R*)-Hydroxybupropion on the Expression of Nicotine Withdrawal Signs in Mice. Anxiety-related behavior (affective sign), somatic signs, and hyperalgesia (physical sign) were measured in mice 18 to 24 h after withdrawal from chronic nicotine. As shown in Fig. 4, mice exhibited a significant anxiety-related response (nicotine: $F_{1,66} = 17.9$, $p <$

Fig. 4. Effects of bupropion on affective and somatic signs of nicotine withdrawal in mice. Mice chronically infused with saline (0 mg/kg/day nicotine) or with nicotine (36 mg/kg/day) for 14 days had minipumps removed for 18 to 24 h and were tested on the elevated plus maze (time in seconds spent in open arms; top right), monitored for somatic signs (number of signs; top left), and assessed for hyperalgesia (hindpaw withdrawal latency, seconds; bottom) starting 30 min after receiving subcutaneous vehicle or bupropion (1, 10, or 15 mg/kg s.c.). Pretreatment with bupropion dose-dependently attenuated expression of both the somatic and affective nicotine withdrawal response in mice. Each point represents the mean $\pm$ S.E.M. of six to eight mice per group. *, $p < 0.05$ versus the saline group. #, $p < 0.05$ nicotine control groups.

0.001; bupropion: $F_{3,66} = 2.89$, $p < 0.04$; and nicotine $\times$ bupropion interaction: $F_{3,66} = 1.56$, $p < 0.2$), an increase in total somatic signs (nicotine: $F_{1,66} = 88.8$, $p < 0.0001$; bupropion: $F(3,66) = 7.09$, $p < 0.0003$; and nicotine $\times$ bupropion interaction: $F_{3,63} = 7.34$, $p < 0.0003$), and a decrease in paw withdrawal latency after nicotine withdrawal (nicotine: $F_{1,66} = 21.79$, $p < 0.001$; bupropion: $F_{3,66} = 5.59$, $p < 0.0018$; and nicotine $\times$ bupropion interaction: $F_{3,66} = 1.3$, $p < 0.28$). Acute pretreatment with bupropion dose dependently reversed both physical and affective nicotine withdrawal signs. Whereas the lowest dose of 1 mg/kg failed to significantly block nicotine withdrawal signs, the highest dose (15 mg/kg) reversed them all.

Likewise, the (2S,3S)-hydroxybupropion metabolite dose-dependently reversed somatic [nicotine: $F_{1,56} = 74.7$, $p < 0.0001$; (2S,3S)-hydroxybupropion, $F_{3,56} = 19.8$, $p < 0.0001$; and nicotine $\times$ (2S,3S)-hydroxybupropion interaction: $F_{3,56} = 22.73$, $p < 0.001$], anxiety-like [nicotine: $F_{1,56} = 18.25$, $p < 0.0015$; (2S,3S)-hydroxybupropion: $F_{3,56} = 22.2$, $p < 0.0013$; and nicotine $\times$ (2S,3S)-hydroxybupropion interaction: $F_{3,56} = 3.84$, $p < 0.014$], and hyperalgesia [nicotine: $F_{1,56} = 6.3$, $p < 0.014$; (2S,3S)-hydroxybupropion: $F_{3,56} = 3.51$, $p < 0.01$; and nicotine $\times$ (2S,3S)-hydroxybupropion interaction: $F_{3,56} = 2.61$, $p < 0.05$] signs after nicotine withdrawal (Fig. 5). However, effects on nicotine withdrawal of the (2S,3S)-hydroxy isomer were manifest at 2, 5, and 10 mg/kg for somatic signs and in

Fig. 5. Effects of (2S,3S)-hydroxybupropion on affective and somatic signs of nicotine withdrawal in mice. Mice chronically infused with saline (0 mg/kg/day nicotine) or with nicotine (36 mg/kg/day) for 14 days had minipumps removed for 18 to 24 h and were tested on the elevated plus maze (time in seconds spent in open arms; top right), monitored for somatic signs (number of signs; top left), and assessed for hyperalgesia (hindpaw withdrawal latency, seconds; bottom) starting 30 min after receiving subcutaneous vehicle or (2S,3S)-hydroxybupropion (2, 5, or 10 mg/kg s.c.). Pretreatment with (2S,3S)-hydroxybupropion dose-dependently attenuated expression of both the somatic and affective nicotine withdrawal response in mice. Each point represents the mean $\pm$ S.E.M. of six to eight mice per group. *, $p < 0.05$ versus the saline group. #, $p < 0.05$ versus nicotine control groups.

Fig. 6. Effects of (2*R*,3*R*)-hydroxybupropion on affective and somatic signs of nicotine withdrawal in mice. Mice chronically infused with saline (0 mg/kg/day nicotine) or with nicotine (36 mg/kg/day) for 14 days had minipumps removed for 18 to 24 h and were tested on the elevated plus maze (time in seconds spent in open arms; top right), monitored for somatic signs (number of signs; top left), and assessed for hyperalgesia (hindpaw withdrawal latency, seconds; bottom) starting 30 min after receiving subcutaneous vehicle or (2*R*,3*R*)-hydroxybupropion (2, 5, or 10 mg/kg s.c.). Pretreatment with (2*R*,3*R*)-hydroxybupropion dose-dependently attenuated expression of both the somatic and affective nicotine withdrawal response in mice. Each point represents the mean $\pm$ S.E.M. of six to eight mice per group. *, $p < 0.05$ versus the saline group. #, $p < 0.05$ versus nicotine control groups.

the plus maze test and at 5 and 10 mg/kg in the hyperalgesia assay, indicating that it has higher potency than bupropion in reversing nicotine withdrawal (Table 1).

It is interesting to note that treatment with the (2*R*,3*R*)-hydroxy isomer blocked nicotine withdrawal in a dose-related manner (Fig. 6), with the dose of 10 mg/kg statistically reversing somatic [nicotine: $F_{1,43} = 66.6$, $p < 0.0001$; (2*R*,3*R*)-hydroxybupropion: $F_{2,43} = 3.81$, $p < 0.01$; and nicotine $\times$ (2*R*,3*R*)-hydroxybupropion interaction: $F_{3,56} = 5.42$, $p < 0.0079$], anxiety-like [nicotine: $F_{1,43} = 19.8$, $p < 0.0001$; (2*R*,3*R*)-hydroxybupropion: $F_{2,43} = 5.31$, $p < 0.0086$; and nicotine $\times$ (2*R*,3*R*)-hydroxybupropion interaction: $F_{2,43} = 3.84$, $p < 0.02$], and hyperalgesia [nicotine: $F_{1,43} = 18.05$, $p < 0.0001$; (2*R*,3*R*)-hydroxybupropion: $F_{2,43} = 3.71$, $p < 0.01$; and nicotine $\times$ (2*R*,3*R*)-hydroxybupropion interaction: $F_{3,43} = 0.63$, $p < 0.53$] signs. Nevertheless, the (2*R*,3*R*)-metabolite had lower potency in each assay than (2*S*,3*S*)-hydroxybupropion (Table 1).

The doses of bupropion and its metabolites used in this study did not significantly affect behavioral responses in any withdrawal test, nor did they precipitate withdrawal signs in nicotine-dependent mice (data not shown).

Effects of Bupropion, (2*S*,3*S*)-Hydroxybupropion, or (2*R*,3*R*)-Hydroxybupropion on Nicotine Discrimination in Mice. Nicotine fully and dose-dependently substituted for itself (Fig. 7A), with an ED₅₀ value of 0.26 mg/kg (95% confidence limits, 0.21–0.33). Repeated measures ANOVA conducted on the response rate data from the nicotine dose-effect curves resulted in significant differences as a function of dose ($F_{5,36} = 5.7$; $p < 0.05$). Compared with responding after vehicle injections, response rates were significantly decreased only at 1.2 mg/kg nicotine ($p < 0.05$) in both groups. No other significant changes in response rates for nicotine-treated mice were observed.

In substitution tests, bupropion (Fig. 7B) at 56 mg/kg produced partial substitution with percentage maximal drug lever of 69.6%. Repeated measures ANOVA conducted on the

response rate data from bupropion revealed significant differences as a function of dose ($F_{5,48} = 10.5$; $p < 0.05$). Post hoc tests revealed that bupropion significantly decreased response rates compared with vehicle at 40, 56, and 65 mg/kg ($p < 0.05$; Fig. 7C).

Similarly, in substitution tests (2*S*,3*S*)-hydroxybupropion (Fig. 7B) produced partial substitution with percentage maximal drug lever of 70.9% at 30 mg/kg. Repeated measures ANOVA conducted on the response rate data from (2*R*,3*R*)-hydroxybupropion revealed significant differences as a function of dose ($F_{5,30} = 8.9$; $p < 0.05$). Post hoc tests revealed that (2*S*,3*S*)-hydroxybupropion significantly decreased response rates compared with vehicle at 30 mg/kg ($p < 0.05$; Fig. 7C). In contrast (2*R*,3*R*)-hydroxybupropion did not exhibit nicotine-like responding, producing a maximum of only 6.0% nicotine-lever responding at doses up to 30 mg/kg (Fig. 7B). Repeated measures ANOVA conducted on the response rate data from (2*R*,3*R*)-hydroxybupropion revealed significant differences as a function of dose ($F_{4,24} = 6.6$; $p < 0.05$). Post hoc tests revealed that (2*R*,3*R*)-hydroxybupropion significantly decreased response rates compared with vehicle at 30 mg/kg ($p < 0.05$; Fig. 7C).

Discussion

Bupropion is one of the effective antismoking medications currently available. Metabolism studies indicated that bupropion metabolism is closer in humans and mice than in humans and rats. In mice, bupropion is predominantly metabolized to hydroxybupropion, similarly to what occurs in humans (Suckow et al., 1986). We have reported previously that (2*S*,3*S*)-hydroxybupropion displays the same or better activity than the parent compound, at endpoints associated with the drug actions at DA transporters, NE transporters, and $\alpha_4\beta_2$ nAChRs (Damaj et al., 2004). That bupropion is extensively converted to biologically active metabolites raises the possibility that the latter may contribute to the mecha-

Fig. 7. Effects of bupropion and its hydroxymetabolites on nicotine drug discrimination in mice. A, effects of nicotine on percentage of nicotine lever responding (left-hand axis) and response rates (right-hand axis) after subcutaneous administration in mice trained to discriminate 0.8 mg/kg nicotine from vehicle. Points above vehicle (VEH) and nicotine (NIC) represent the results of control tests with vehicle and 0.8 mg/kg nicotine conducted before each dose-effect determination. Values represent the mean $\pm$ S.E.M. *, $p < 0.05$, significant decrease in response rates compared with mean rates of responding occurring during the VEH tests. B, effects of bupropion, (2*R*,3*R*)-hydroxybupropion (*R*-OH bupropion), and (2*S*,3*S*)-hydroxybupropion (*S*-OH bupropion) on percentage of nicotine lever in mice trained to discriminate 0.8 mg/kg nicotine from vehicle. Points above VEH and NIC represent the results of control tests with vehicle and 0.8 mg/kg nicotine conducted before each dose-effect determination. Values represent the mean $\pm$ S.E.M. C, effects of bupropion, *R*-OH bupropion, and *S*-OH bupropion on response rates in mice trained to discriminate 0.8 mg/kg nicotine from vehicle. Points above VEH and NIC represent the results of control tests with vehicle and 0.8 mg/kg nicotine conducted before each dose-effect determination. Values represent the mean $\pm$ S.E.M. *, $p < 0.05$, significant decrease in response rates compared with mean rates of responding occurring during the VEH tests.

nism of action of bupropion as a smoking cessation agent. Our data support a role for the (2*S*,3*S*) isomer in the actions of bupropion in nicotine dependence, because this isomer was the most potent compound in altering the behavioral effects of nicotine in reward, discrimination, and withdrawal endpoints measured in mice.

Recent evidence from our groups and others suggested that bupropion, its hydroxymetabolites, or a combination may act as a functional antagonist at neuronal nicotinic acetylcholine receptors (Slemmer et al., 2000; Miller et al., 2002; Damaj et al., 2004). Our data showing blockade of the acute rewarding effects of nicotine in the CPP model by bupropion and its (2*S*,3*S*)-isomer at doses that were ineffective on their own support this hypothesis. At higher doses, the independent effects of bupropion and (2*S*,3*S*)-hydroxybupropion in inducing preference are observed, making it difficult to separate their potential nicotinic antagonist effects. Our CPP data support a role for the (2*S*,3*S*)-isomer in the actions of bupropion, because this isomer was the most potent compound in blocking the behavioral effects of nicotine. Indeed, the (2*R*,3*R*)-isomer failed to significantly to block nicotine reward at a dose of 1 mg/kg, whereas 0.5 and 1 mg/kg (2*S*,3*S*)-hydroxybupropion or 1 mg/kg bupropion completely block the effect of nicotine in mice. These results suggest that (2*S*,3*S*)-hydroxybupropion may act acutely to attenuate the rewarding effects of nicotine, thus increasing the likelihood of cessation. Indeed, recent clinical studies provided evidence that bupropion attenuated the rewarding effects of nicotine in

smokers (Jorenby et al., 2006; West et al., 2008). Our findings in mice are at odds with those reported by Dwoskin et al. (2006), who found that bupropion augmented nicotine CPP when coadministered with nicotine in rats. This discrepancy is probably due to species differences. As indicated above, rats and mice metabolize bupropion differently, with such differences leading to different behavioral effects (Suckow et al., 1986).

Several clinical and preclinical studies have provided evidence for amelioration of nicotine withdrawal as being an essential part in the helpful effects of bupropion on smoking cessation rates (for review, see Paterson, 2009). Our results show that bupropion and its hydroxymetabolite isomers reversed both the physical and negative affective aspects of nicotine withdrawal that are expressed as hyperalgesia, anxiety-like behavior, and somatic signs of withdrawal. As seen with the CPP data, (2*S*,3*S*)-hydroxybupropion was relatively more potent in reversing all signs of withdrawal compared with bupropion and (2*R*,3*R*)-hydroxybupropion. It is interesting that the doses needed in ameliorating withdrawal signs are much higher than the doses observed in blocking the rewarding effects of nicotine in the CPP test. The effect of bupropion on somatic signs of withdrawal is consistent with previous studies reporting that bupropion reversed somatic signs of nicotine withdrawal in rats (Cryan et al., 2003; Malin et al., 2006; Wing and Shoaib, 2007). The reversal of the affective aspects of withdrawal (anxiety-like effects) by bupropion complements previous studies in rats where bupro-

pion was shown to ameliorates nicotine withdrawal-associated deficits in contextual fear conditioning (Portugal and Gould, 2007), aversion (Malin et al., 2006), and anhedonia as tested in the intracranial self-stimulation paradigm (Cryan et al., 2003).

The 5-fold or >10-fold higher potency for (2*S*,3*S*)-hydroxybupropion in the CPP assay relative to bupropion and the (2*R*,3*R*)-hydroxy enantiomer, respectively, is more consistent with the selectivity of the (2*S*,3*S*)-isomer in vitro over the other agents in inhibition of NET (9- or 50-fold) and $\alpha_4\beta_2$ nAChR (4- or 10-fold) than with the comparable activities of bupropion and the (2*S*,3*S*)-isomer and the inactivity of (2*R*,3*R*)-hydroxybupropion at DAT, just as is the case for effects of these drugs on acute actions of nicotine on antinociception, locomotor activity, and body temperature (Damaj et al., 2004). A similar explanation can be advanced as to why (2*S*,3*S*)-hydroxybupropion has 2- to 5-fold more potency than the other agents in blocking withdrawal signs; its has higher potency as an inhibitor of NET and $\alpha_4\beta_2$ -nAChR. However, it is difficult to implicate the exact mechanisms by which bupropion and its (2*R*,3*R*)-hydroxymetabolite reverse nicotine withdrawal-associated signs. The disconnect between the lack of inhibition of DAT and NET by (2*R*,3*R*)-hydroxybupropion relative to bupropion and the comparable effectiveness of these agents in reversing withdrawal signs, suggest the involvement of additional targets in the effects of (2*R*,3*R*)-hydroxybupropion on nicotine withdrawal. One possibility is that bupropion and its metabolites may ameliorate nicotine withdrawal through their effects on other neuronal nAChR subtypes. For example, relative potencies of (2*S*,3*S*)- and (2*R*,3*R*)-hydroxybupropion are reversed for $\alpha_4\beta_2$ and $\alpha_3\beta_4$ nAChR subtype actions (Damaj et al., 2004), both of which are known to mediate affective and somatic signs of withdrawal (Jackson et al., 2008; Salas et al., 2009).

Nicotine discrimination studies in rodents provide a measure of the subjective-like effects of nicotine. It is interesting to note that bupropion and (2*S*,3*S*)-hydroxybupropion partially generalize (70% at 56 and 30 mg/kg, respectively) to nicotine in the mouse drug discrimination paradigm. However, the (2*R*,3*R*)-isomer failed to do so. Our results in mice are similar to those in rats where bupropion was shown to share discriminative stimulus effects with nicotine (Wiley et al., 2002; Young and Glennon, 2002) (Bondarev et al., 2003). The comparable potencies of bupropion and (2*S*,3*S*)-hydroxybupropion in inhibition of DAT as well as the lack by the inhibition of (2*R*,3*R*)-isomer at the same target (Damaj et al., 2004) suggest the possible dominance of dopaminergic mechanisms in the mouse discrimination response. This is consistent with the rat nicotine discrimination data, because the effects of bupropion were blocked by dopamine D₁ and D₂ receptor antagonists (Terry and Katz, 1997) but were unaffected by the nicotinic antagonist mecamylamine (Wiley et al., 2002; Young and Glennon, 2002).

The differences in bupropion and its metabolites in the various behavioral tests are noticeable. For example, the doses of bupropion and (2*S*,3*S*)-hydroxybupropion that reduce nicotine-induced CPP are very low compared with doses that ameliorate the affective and somatic symptoms of nicotine withdrawal. These doses, in turn, are much lower than the doses that are required to substitute for the discriminative stimulus effects of nicotine in the discrimination procedure (for summary, see Table 1). These dif-

ferences suggest that both bupropion and its hydroxymetabolites act on different neuropharmacological systems and that the dose selectivity of effects observed in these behavioral responses might reflect different affinities for these compounds for their various targets such as nAChRs and neurotransmitters (Table 1). In addition, the behaviors measured in each test may have differing sensitivities to bupropion and its hydroxymetabolites; so differences in potency of the analogs that produce such effects may depend on the specific behavioral response measured after repeated or chronic exposure to nicotine.

Collectively, our findings support the hypothesis that the utility of bupropion as a pharmacological treatment of nicotine dependence reflects actions of bupropion, its hydroxymetabolites, or a combination on a mixture of targets, including DAT, NET, and neuronal nAChR subtypes. Finally, our data suggest that (2*S*,3*S*)-hydroxybupropion may be a better drug candidate for smoking cessation than bupropion because of its higher potency at the relevant targets and higher behavioral activity in relevant nicotine dependence models.

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