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Contributory role for nornicotine in nicotine neuropharmacology: nornicotine-evoked [^{3}H]dopamine overflow from rat nucleus accumbens slices

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Abstract

Nornicotine is a tobacco alkaloid and an active nicotine metabolite, which accumulates in brain to pharmacologically relevant concentrations following repeated nicotine administration to rats. Furthermore, nornicotine is self-administered by rats, indicating that it has reinforcing efficacy and may contribute to nicotine dependence. Since drugs of abuse activate the mesolimbic dopamine (DA) system to produce rewarding effects, the present study tested the hypothesis that nornicotine evokes DA release from nucleus accumbens in a nicotinic receptor-mediated manner. Rat nucleus accumbens slices were preloaded with [^{3}H]DA and superfused for 60 min in the absence and presence of a range of alkaloid concentrations. Superfusate samples were collected and alkaloid-evoked [^{3}H]overflow was determined. *S*(–)-Nornicotine (EC_{50} value = 3.0 μM), *R*(+)-nornicotine (EC_{50} value = 0.48 μM), and *S*(–)-nicotine (EC_{50} value = 70 nM) evoked [^{3}H]overflow in a concentration-dependent manner. For each nornicotine enantiomer, 0.3 μM was the lowest concentration to evoke significant [^{3}H]overflow. Dihydro-β-erythroidine (DHβE, 10 μM), a classical nicotinic receptor antagonist, inhibited the *S*(–)-nornicotine-evoked [^{3}H]overflow, indicating the involvement of nicotinic receptors. Furthermore, the effect of *S*(–)-nornicotine was calcium-dependent, consistent with a nicotinic receptor-mediated mechanism. Whereas *S*(–)-nornicotine was found previously to be more potent in the striatum, *R*(+)-nornicotine was more potent than its enantiomer in nucleus accumbens, suggesting the involvement of different nicotinic receptor subtypes in these brain regions. Thus, the results of the current study indicate that nornicotine stimulated DA release from nucleus accumbens in a nicotinic receptor-mediated manner, further supporting the hypothesis that nornicotine contributes to tobacco dependence.

Keywords: Nicotine; Nornicotine; Dopamine release; Nucleus accumbens; Tobacco smoking

1. Introduction

Nicotine, the primary alkaloid in tobacco, is generally believed to be responsible for the neuropharmacological effects of tobacco smoking. The reinforcing effect of nicotine has been demonstrated in animal models using the intravenous self-administration paradigm [1–4]. Many drugs of abuse, including nicotine, activate the mesolimbic DA pathway to produce their reinforcing properties [5–8]. Nicotinic receptors, which mediate the reinforcing effect of nicotine, are localized on mesolimbic DA cell bodies and terminals [6,9–11]. Nicotine stimulates DA release from nucleus accumbens *in vivo* [12–14] and *in vitro* [15,16]. Thus, the reinforcing effects of nicotine appear to be due to activation of the mesolimbic DA system, resulting in an increased DA release in the nucleus accumbens.

The *N*-demethylated nicotine metabolite nornicotine is present in the brain after peripheral exposure to nicotine, is pharmacologically active, and may contribute to the etiology and maintenance of tobacco dependence [17]. After acute peripheral administration of nicotine to rats, nornicotine was found in rat brain [18–20]. Moreover, the half-life of nornicotine in the brain is 3-fold longer than that of nicotine, indicating that its effects persist long after nicotine is cleared from the brain. Furthermore, after repeated intermittent peripheral administration of nicotine (0.3 mg/kg, s.c., 10 doses, 30 min inter-dose interval), nornicotine ac-

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Abbreviations: DA, dopamine; and DHβE, dihydro-β-erythroidine.

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cumulated in the brain to a concentration 10-fold higher than that obtained after acute nicotine administration [21]. The presence of nornicotine in the brain and its accumulation after repeated nicotine exposure may be of additional relevance to tobacco smoking, because nornicotine also is a major alkaloidal constituent of *Nicotiana tabacum* [22]. Thus, accumulation of nornicotine in the brain of a smoker may arise both from nicotine metabolism and from exposure to alkaloidal nornicotine present in tobacco.

Similar to nicotine, nornicotine is active in both behavioral and neurochemical studies. Nornicotine induces locomotor stimulant effects and maintains intravenous self-administration in rats, indicating reinforcing efficacy [23,24]. Results from *in vitro* studies show that nornicotine has a high affinity for nicotinic receptors assessed in [^{3}H]nicotine binding assays [25–27], and it also activates nicotinic receptors in the striatum to release DA [28–30]. Since drugs of abuse activate the mesolimbic DA pathway to produce their reinforcing effects, the present study investigated whether nornicotine was able to evoke DA release from nucleus accumbens *in vitro.*

2. Materials and methods

2.1. Materials

S(–)-Nicotine ditartrate, nomifensine maleate, and DHβE were purchased from Research Biochemicals, Inc. *S*(–)-Nornicotine and *R*(+)-nornicotine were synthesized and crystallized as perchlorate salts, and their enantiomeric purities were determined by nuclear magnetic resonance spectroscopy and polarimetric analysis [31]. [^{3}H]DA (3,4-ethyl-2[N-^{3}H]-dihydroxyphenylethylamine; specific activity 25.6 Ci/mmol) was purchased from New England Nuclear. Ascorbic acid, α-D-glucose, and pargyline hydrochloride were purchased from AnalaR (BHD Ltd.), the Aldrich Chemical Co., and the Sigma Chemical Co., respectively. TS-2 tissue solubilizer was purchased from Research Products International. All other chemicals were purchased from Fisher Scientific.

2.2. Animals

Male Sprague–Dawley rats (200–250 g) were obtained from Harlan Laboratories and were housed two per cage with free access to food and water in the Division of Lab Animal Resources at the College of Pharmacy, University of Kentucky. Experimental protocols involving the animals were in strict accordance with the NIH "Guide for the Care and Use of Laboratory Animals" and were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

2.3. [^{3}H]DA release assays

Dissection of nucleus accumbens was carried out according to a previously published method [32]. Briefly, the brain was removed and inverted to obtain a 2-mm thick coronal section through the olfactory tubercles, which was placed flat on a chilled dissection plate. Three cuts were made to isolate each nucleus accumbens. A horizontal cut removed the olfactory tubercles, and a vertical cut removed the medial septal region. Finally, a diagonal cut separated the nucleus accumbens from the striatum. Then the nucleus accumbens was cut into slices (500 μm, 3–4 mg) with a McIlwain tissue chopper. Drug effects on [^{3}H]overflow from rat brain slices preloaded with [^{3}H]DA also were determined as described previously [33]. Briefly, rat nucleus accumbens slices were incubated for 30 min in Krebs buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 1.3 mM $CaCl_2$, 11.1 mM glucose, 25 mM $NaHCO_3$, 0.11 mM L-ascorbic acid, and 0.004 mM ethylenediaminetetraacetic acid, pH 7.4, saturated with 95% O_2/5% CO_2, at 34°). Then slices were incubated for an additional 30 min in buffer containing 0.1 μM [^{3}H]DA. Slices were transferred to superfusion chambers and superfused (1 mL/min) for 60 min with Krebs buffer containing 10 μM nomifensine (a DA uptake inhibitor) and 10 μM pargyline (a monoamine oxidase inhibitor), to ensure that the [^{3}H]overflow primarily represented [^{3}H]DA, rather than [^{3}H]metabolites [34–36]. When basal outflow was stabilized after 60 min superfusion, three 5-min (5-mL) samples were collected to determine basal [^{3}H]outflow followed by superfusion with different concentrations of drug. For each experiment, nucleus accumbens slices pooled from two rats were assigned randomly (one or two slices per chamber) to all drug concentrations.

For concentration–response and determination of stereoselectivity, each chamber was exposed to only one concentration of either *S*(–)-nornicotine (0.1 to 100 μM) or *R*(+)-nornicotine (0.1 to 100 μM). For comparison, the effect of S(–)-nicotine (0.1 nM to 10 μM) was also determined. Drugs were added to the superfusion buffer after collection of the third basal superfusate sample and remained in the buffer for the 60-min sampling period. In each experiment, one chamber was superfused in the absence of drug, and served as the buffer control.

To determine if the [^{3}H]overflow evoked by *S*(–)-nornicotine (0.1 to 100 μM) was mediated by nicotinic receptors, [^{3}H]DA-preloaded slices were superfused in the absence or presence of 10 μM DHβE, a competitive nicotinic receptor antagonist [37], for 60 min prior to the addition of *S*(–)-nornicotine. This concentration of DHβE was chosen because it maximally inhibited *S*(–)-nornicotine-evoked [^{3}H]overflow from striatal slices [30]. Basal [^{3}H]outflow was determined prior to DHβE exposure and during the last 15 min of DHβE exposure. Superfusion continued for another 60 min in the presence of *S*(–)-nornicotine with or without DHβE. Slices superfused in the absence of DHβE

constituted the *S*(–)-nornicotine control condition. In each experiment, an additional chamber was superfused in the absence of exposure to any drug, and is referred to as the buffer control condition.

A series of experiments was performed to determine whether the effect of *S*(–)-nornicotine was dependent upon extracellular calcium. Within each experiment, slices were superfused in the absence or presence of *S*(–)-nornicotine (1 or 10 μM), either under control conditions (Krebs buffer) or with a low-calcium buffer. For the low-calcium buffer, 0.5 mM ethyleneglycol-*bis*-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid was added, and $CaCl_2$ was omitted from the Krebs buffer.

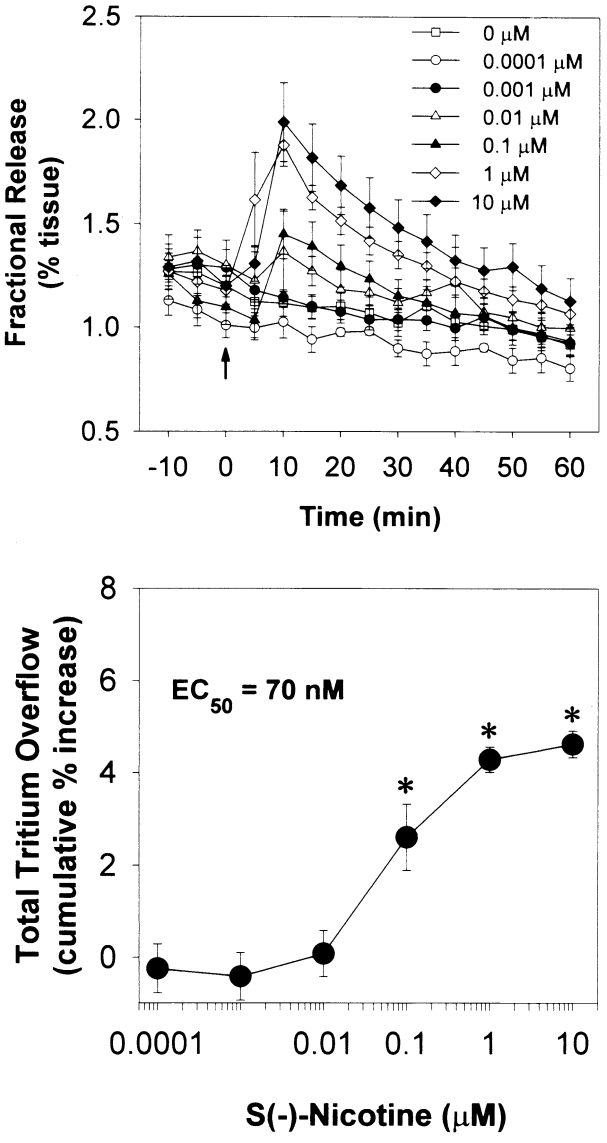

Fig. 1. Concentration-dependence of *S*(–)-nicotine-evoked [^{3}H]overflow from rat nucleus accumbens slices preloaded with [^{3}H]DA. The top panel illustrates the time course of the response to 60 min superfusion with *S*(–)-nicotine (0.1 nM to 10 μM). In each experiment, one chamber was superfused in the absence of *S*(–)-nicotine, constituting the control condition. Data are expressed as mean ± SEM fractional release. The bottom panel illustrates the concentration-dependence of *S*(–)-nicotine-evoked [^{3}H]overflow. Data are expressed as mean ± SEM total [^{3}H]overflow as a cumulative percent increase above baseline. Key: (*) $P < 0.05$, significantly different from control; N = 6.

At the end of the experiment, each nucleus accumbens slice was solubilized with TS-2. Radioactivity in the superfusate and tissue samples was determined by liquid scintillation counting (Packard model B1600 TR Scintillation Counter). To normalize potential differences in radioactivity between slices of varying weight, fractional release for each sample was calculated by dividing the tritium in the superfusate by the total tissue tritium at the time of collection, and was expressed as a percentage of tissue tritium. Fractional release as a function of time provides the duration and time course of the effect of the drug. Basal outflow was calculated as the average of the fractional release in the three samples immediately before the addition of drug. Data from the control slice were used to calculate outflow (baseline) across the duration of the session for comparison with slices exposed to drug. Total [^{3}H]overflow was determined as the cumulative percent increase above basal outflow.

2.4. Statistical analyses

One-way ANOVAs were used to analyze the concentration-dependence of *S*(–)-nornicotine- and *S*(–)-nicotine-evoked [^{3}H]overflow from nucleus accumbens slices. The EC_{50} values for *S*(–)- and *R*(+)-nornicotine, as well as for *S*(–)-nicotine, were determined using an iterative, nonlinear, least-squares, curve-fitting program (GraphPad Prism). Repeated measures two-way ANOVAs were performed to analyze the time course of the drug-induced increase in fractional release. Furthermore, repeated measures two-way ANOVAs also were used to analyze the calcium dependence, DHβE inhibition, and the stereoselectivity of the nornicotine-induced increase in [^{3}H]overflow. *Post hoc* analysis was performed using Fisher's least significant difference (LSD). Results were considered statistically significant when $P < 0.05$.

3. Results

3.1. Effect of S(–)-nicotine on [^{3}H]overflow from superfused rat nucleus accumbens slices preloaded with [^{3}H]DA

S(–)-Nicotine increased [^{3}H]overflow from rat nucleus accumbens slices preloaded with [^{3}H]DA in a concentration-dependent manner (Fig. 1). Analysis of the time course revealed significant main effects of concentration ($F_{(6,24)} = 4.42$, $P < 0.01$) and time ($F_{(14,56)} = 54.06$, $P < 0.001$), and a significant concentration × time interaction ($F_{(84,336)} = 6.29$, $P < 0.001$). Fractional release peaked 10–15 min after addition of *S*(–)-nicotine to the buffer and declined toward basal levels, despite the presence of *S*(–)-nicotine through-

out the remainder of the superfusion period. Analysis of cumulated evoked [^{3}H]overflow revealed a significant effect of *S*(–)-nicotine concentration ($F_{(6,24)} = 32.22$, $P < 0.001$), with an EC_{50} value of 70 nM (confidence intervals, 45–113 nM). A plateau in the concentration–effect curve was observed at 1–10 μM *S*(–)-nicotine.

3.2. Effect of S(–)-nornicotine on [^{3}H]overflow from superfused rat nucleus accumbens slices preloaded with [^{3}H]DA

S(–)-Nornicotine evoked an increase in [^{3}H]overflow from rat nucleus accumbens slices preloaded with [^{3}H]DA in a concentration-dependent manner (Fig. 2). Analysis of the time course revealed a significant main effect of concentration ($F_{(6,30)} = 4.47$, $P < 0.01$) and of time ($F_{(14,70)} = 40.82$, $P < 0.001$), and a significant concentration × time interaction ($F_{(84,420)} = 4.42$, $P < 0.001$). Fractional release peaked 10–15 min after the addition of *S*(–)-nornicotine to the buffer and declined toward basal levels, despite the presence of *S*(–)-nornicotine throughout the remainder of the superfusion period. Examination of cumulated evoked [^{3}H]overflow revealed a significant effect of concentration ($F_{(6,30)} = 12.02$, $P < 0.001$), with an EC_{50} value of 3.0 μM (confidence intervals, 1.2 to 8.6 μM). Furthermore, a plateau in the concentration–response curve was observed at high concentrations (30 and 100 μM) of *S*(–)-nornicotine.

3.3. DHβE antagonism of S(–)-nornicotine-evoked [^{3}H]overflow from rat nucleus accumbens slices preloaded with [^{3}H]DA

DHβE (10 μM) inhibited S(–)-nornicotine-evoked [^{3}H]overflow from rat nucleus accumbens slices, except at the highest concentration (100 μM) of *S*(–)-nornicotine examined (Fig. 3). Analysis did not reveal a significant main effect of DHβE ($F_{(1,6)} = 4.73$, $P = 0.07$). However, a significant main effect of *S*(–)-nornicotine concentration ($F_{(6,36)} = 25.92$, $P < 0.001$) and, moreover, a significant DHβE × concentration interaction ($F_{(6,36)} = 2.97$, $P < 0.05$) were found. *Post hoc* analyses revealed that DHβE (10 μM) inhibited the response to *S*(–)-nornicotine at concentrations ranging from 0.3 to 30 μM.

3.4. Calcium dependence of S(–)-nornicotine-evoked [^{3}H]overflow from rat nucleus accumbens slices preloaded with [^{3}H]DA

S(–)-Nornicotine (1 and 10 μM)-evoked [^{3}H]overflow was calcium-dependent (Table 1). Analysis revealed significant main effects of calcium ($F_{(1,3)} = 47.99$, $P < 0.01$) and concentration ($F_{(1,3)} = 20.55$, $P < 0.05$); however, the interaction was not significant ($F_{(1,3)} = 0.22$, $P > 0.05$). *Post hoc* analyses showed that 1 and 10 μM *S*(–)-nornicotine evoked [^{3}H]overflow under control conditions, but not during superfusion with the low-calcium buffer.

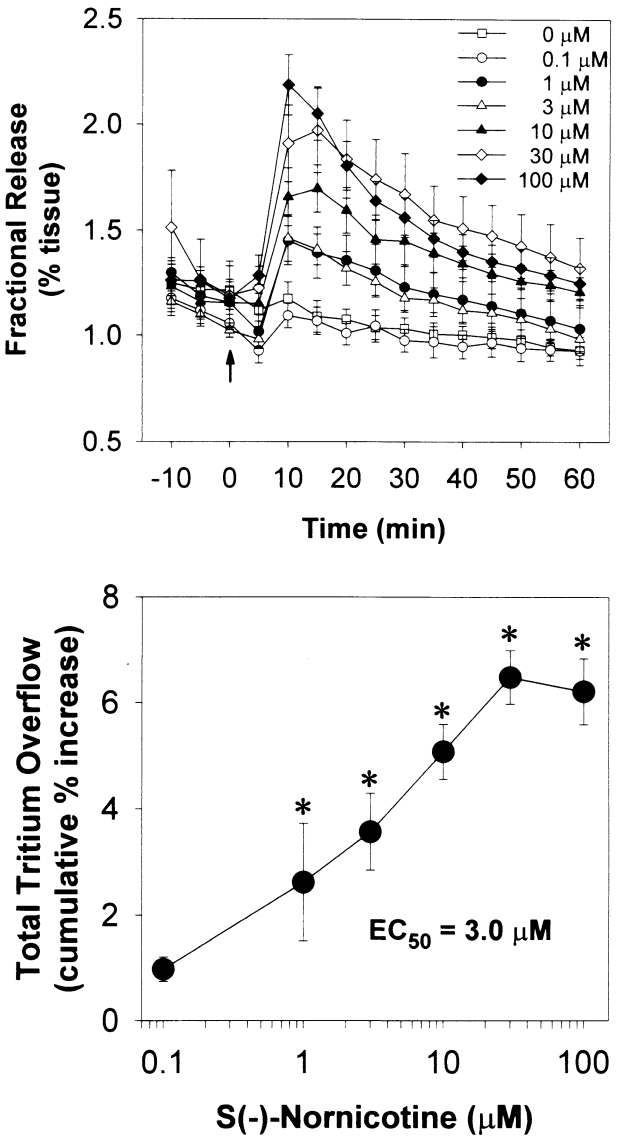

Fig. 2. Concentration-dependence of *S*(–)-nornicotine-evoked [^{3}H]overflow from rat nucleus accumbens slices preloaded with [^{3}H]DA. The top panel illustrates the time course of the response to 60 min superfusion with *S*(–)-nornicotine (0.1 to 100 μM). In each experiment, one chamber was superfused in the absence of drug, constituting the control condition. Data are expressed as mean ± SEM fractional release. The bottom panel illustrates the concentration-dependence of *S*(–)-nornicotine-evoked [^{3}H]overflow. Data are expressed as mean ± SEM total [^{3}H]overflow as a cumulative percent increase above baseline. Key: (*) $P < 0.05$, significantly different from control; N = 6.

3.5. Stereoselectivity of nornicotine-evoked [^{3}H]overflow from rat nucleus accumbens slices preloaded with [^{3}H]DA

When analysis of the concentration–effect of *R*(+)- and *S*(–)-nornicotine was performed concurrently, a significant main effect of enantiomer ($F_{(1,7)} = 1.94$, $P = 0.21$) was not found; however, a significant main effect of concentration ($F_{(5,35)} = 20.03$, $P < 0.001$) and, moreover, an enantio-

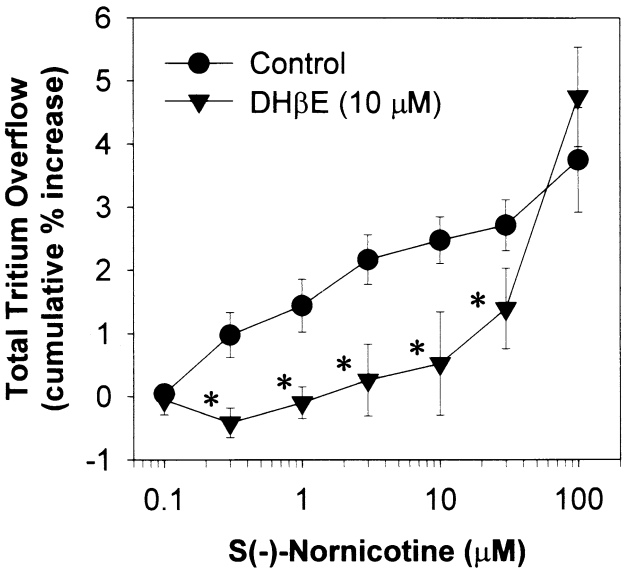

Fig. 3. DHβE inhibition of *S*(–)-nornicotine-evoked [^{3}H]overflow from rat nucleus accumbens slices preloaded with [^{3}H]DA. Slices were superfused in the absence or presence of DHβE (10 μM) for 60 min prior to the addition of *S*(–)-nornicotine (0.1 to 100 μM) to the buffer for an additional 60 min. Data are presented as mean ± SEM total [^{3}H]overflow as a cumulative percent increase above baseline. Key: (*) $P < 0.05$, significantly different from control; N = 7.

mer × concentration interaction ($F_{(5,35)} = 4.45$, $P < 0.005$) were found. *Post hoc* comparisons revealed that only the highest concentration (100 μM) showed significant differences between the enantiomers (Table 2). ANOVA for each enantiomer revealed that 0.3 μM was the lowest concentration to evoke significant ($P < 0.05$) [^{3}H]overflow for both *S*(–)- and *R*(+)-nornicotine. Analysis of the EC_{50} values showed that *R*(+)-nornicotine was significantly more potent than *S*(–)-nornicotine (EC_{50} values, 0.48 μM vs 3.0 μM, respectively; $F_{(1,7)} = 5.77$, $P < 0.05$).

4. Discussion

The present study demonstrated that *S*(–)-nornicotine releases DA from superfused nucleus accumbens slices in a concentration-dependent, calcium-dependent, and nicotinic receptor-mediated manner. As with *S*(–)-nicotine, the time course of the response to *S*(–)-nornicotine revealed that DA release returns toward basal levels despite the continual presence of *S*(–)-nornicotine in the superfusion buffer, indicative of receptor desensitization. Furthermore, the response to *S*(–)-nornicotine at concentrations less than or equal to 30 μM was inhibited completely by the classical nicotinic receptor antagonist DHβE, and was dependent upon the presence of calcium. These results are consistent with a nicotinic receptor mechanism for nornicotine in the nucleus accumbens.

Relatively little information is available concerning the concentration of nornicotine in brain arising from nicotine metabolism. Five minutes after an acute bolus injection of nicotine (0.8 mg/kg, free base), nornicotine was not observed in rat brain; after 1 hr, the concentration of nornicotine was ~10% of the nicotine concentration in brain [20]. However, the half-life of nornicotine is more than 3-fold that of nicotine (brain $T_{1/2}$ = 52 min), suggesting that accumulation of nornicotine may result following repeated nicotine administration. In a subsequent study [21], acute administration of nicotine (0.3 mg/kg, free base) to rats resulted in brain nicotine and nornicotine concentrations of 1.1 and 0.04 μM, respectively, 30 min following administration, whereas after 10 intermittent injections of nicotine (0.3 mg/kg, free base, 30 min inter-injection interval), brain concentrations increased to 2.1 and 0.34 μM, respectively. Thus, the nicotine and nornicotine concentrations in brain increased 2- and 10-fold upon repeated intermittent administration of nicotine. Therefore, following repeated nicotine administration, a combination of metabolism and nornicotine accumulation in brain affords concentrations of nornicotine that approach the pharmacologically active concentration range (i.e. evokes DA release from presynaptic terminals in nucleus accumbens and striatum [present study and Ref. 30]). Furthermore, nicotinic receptor desensitiza-

Table 1
Calcium dependence of *S*(−)-nornicotine-evoked [^{3}H]overflow from rat nucleus accumbens slices

	S(−)-Nornicotine concentration (μM)	[^{3}H]Overflow[a]
Control	1	1.31 ± 0.28
	10	2.45 ± 0.58
Low-calcium	1	−0.48 ± 0.19[b,*]
	10	0.50 ± 0.33*

[a] Data (mean ± SEM, N = 4) represent total [^{3}H]overflow as a cumulative percent increase above baseline.
[b] Number indicates that release was lower than that predicted by baseline.
* $P < 0.05$, compared with control.

Table 2
Stereoselectivity of nornicotine-evoked [^{3}H]overflow from rat nucleus accumbens slices

	Concentration (μM)	[^{3}H]Overflow[a]
S(−)-Nornicotine	0.1	0.21 ± 0.27
	0.3	1.47 ± 0.42
	1	1.99 ± 0.36
	3	3.09 ± 0.35
	10	3.39 ± 0.36
	100	5.56 ± 0.95
R(+)-Nornicotine	0.1	1.04 ± 0.47
	0.3	1.45 ± 0.35
	1	1.98 ± 0.38
	3	2.66 ± 0.45
	10	2.90 ± 0.48
	100	3.41 ± 0.52*

[a] Data (mean ± SEM, N = 8) represent total [^{3}H]overflow as a cumulative percent increase from baseline.
* $P < 0.05$, compared with 100 μM *S*(−)-nornicotine.

tion may be maintained by even lower concentrations of nornicotine across time periods during which nicotine is no longer present in the brain.

Human tobacco smokers are exposed to nornicotine both as a nicotine biotransformation product and as an alkaloidal constituent of commercial tobacco (nornicotine constitutes 15–20% of the total alkaloid content) [38]. An important pharmacokinetic characteristic of nornicotine is that its half-life in rat brain (166 min) is 3-fold longer than that of nicotine [20]. Therefore, as a result of the direct exposure to nornicotine in tobacco, the indirect exposure to nornicotine from nicotine biotransformation, and the comparatively longer brain-residence time of nornicotine, it is likely that nornicotine will accumulate in the brain of chronic smokers and reach pharmacologically relevant concentrations.

The contribution of alkaloidal nornicotine to the concentration of nornicotine in the brain of smokers is difficult to assess, since no information is available on either the ability of nornicotine to penetrate the blood–brain barrier or the metabolic fate of this tobacco alkaloid. The relatively higher brain concentration of nornicotine may be due, at least in part, to local oxidative metabolism of nicotine to nornicotine in brain. In this regard, a recent study has reported that repeated administration of nicotine (0.1 to 1.0 mg/kg, for 7 days) to rats resulted in induction of a specific cytochrome P450 isozyme in a cell-specific and brain region-specific manner, without altering hepatic levels of the enzyme [39]. Thus, highly localized enzyme induction in brain may produce regionally discrete increases in levels of nicotine metabolites, such as nornicotine.

Although a similar nornicotine concentration range evoked DA release from nucleus accumbens (present study) and striatum [30], differential stereoselectivity was observed between these brain regions. Whereas *S*(–)-nornicotine was more potent in releasing DA from superfused striatal slices [30], *R*(+)-nornicotine was the more potent enantiomer in nucleus accumbens (present study). These results suggest that nornicotine is acting at different nicotinic receptor subtypes in the striatum and nucleus accumbens. The optical purity and specific chirality of nornicotine resulting from *S*(-)-nicotine metabolism have not been determined; thus, it is not known if a smoker is exposed indirectly to both or only one of the nornicotine enantiomers. However, it is important to note that nornicotine in tobacco is known to be a racemic mixture (i.e. equal amounts of *R*(+) and *S*(–)-enantiomers) [40]. Thus, smokers are exposed directly to both of these optical isomers of nornicotine.

Release of DA in the nucleus accumbens is thought to be responsible for the reinforcing properties of nicotine [6,41, 42]. Importantly, racemic nornicotine has been shown to be self-administered intravenously in rats [23], indicating that nornicotine has reinforcing properties. The present study demonstrates mesolimbic dopaminergic activation by both *S*(–)- and *R*(+)-enantiomers of nornicotine, suggesting a likely neurochemical mechanism for nornicotine reinforcement. Thus, the current findings begin to link a neurochemical mechanism involving the mesolimbic DA system to the demonstrated reinforcing properties of nornicotine in rats, and, more importantly, provide further evidence of a contributory role for nornicotine in tobacco dependence.

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