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Nornicotine, a nicotine metabolite and tobacco alkaloid: desensitization of nicotinic receptor-stimulated dopamine release from rat striatum

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Abstract

Nornicotine, a major tobacco alkaloid and nicotine metabolite, accumulates in rat brain in pharmacologically relevant concentrations following repeated nicotine administration. Nornicotine-evoked striatal dopamine release is Ca^{2+}-dependent, stereoselective and sensitive to nicotinic receptor antagonists, indicating nicotinic receptor-mediation. The present study determined if *S*-(−)-nornicotine desensitizes nicotinic receptors and if cross-desensitization to *S*-(−)-nicotine occurs. *S*-(−)-Nicotine (10 and 100 nM) diminished [^{3}H]overflow from [^{3}H]dopamine-preloaded rat striatal slices following subsequent superfusion with 10 μM *S*-(−)-nicotine (46% and 74%, respectively) or 10 μM *S*-(−)-nornicotine (59% and 81%, respectively). *S*-(−)-Nornicotine (1 and 10 μM) diminished the response to subsequent superfusion with 10 μM *S*-(−)-nornicotine (85% and 97%, respectively) or 10 μM *S*-(−)-nicotine (82% and 88%, respectively). Thus, similar to *S*-(−)-nicotine, *S*-(−)-nornicotine desensitizes nicotinic receptors, but with ~12-fold lower potency. Cross-desensitization suggests involvement of common nicotinic receptor subtypes. Therefore, *S*-(−)-nicotine metabolites, such as nornicotine, have neuropharmacologically relevant effects.

Keywords: Nornicotine; Nicotine; Nicotinic receptor; Desensitization; Dopamine release; Striatum, rat

1. Introduction

S-(−)-Nicotine, the major alkaloid in tobacco, is generally believed to be responsible for maintenance of tobacco smoking behavior. Evidence indicates that dopaminergic systems in the central nervous system (CNS) mediate, at least in part, the reinforcing effects (Corrigall et al., 1992, 1994; Singer et al., 1982; Mansbach et al., 2000), the locomotor sensitization (Clarke et al., 1988) and discriminative stimulus effects (Reavill and Stolerman, 1987; Varvel et al., 1999) of *S*-(−)-nicotine in animal models. Similar to *S*-(−)-nicotine, metabolites of *S*-(−)-nicotine also have been reported to have behavioral effects following peripheral administration. For example, nornicotine has been reported to produce reinforcement (Bardo et al., 1999), stimulant-like locomotor sensitization (Stolerman et al., 1995; Dwoskin et al., 1999b; Dwoskin and Crooks, 2001) and discriminative stimulus effects (Bardo et al., 1997). Furthermore, nornicotine pretreatment decreases intravenous nicotine self-administration in rats (Green et al., 2000), indicating that nornicotine has similar reinforcing effects as nicotine.

S-(−)-Nicotine stimulates nicotinic receptors on dopaminergic striatal nerve terminals to facilitate dopamine release from superfused striatal slices (Giorguieff-Chesselet et al., 1979; Westfall et al., 1987; Harsing et al., 1992; Teng et al., 1997a,b) and synaptosomes (Rapier et al., 1988; Grady et al., 1992; Rowell and Hillebrand, 1994). *S*-(−)-Nicotine-evoked striatal dopamine release is Ca^{2+}-dependent, stereoselective and sensitive to nicotinic receptor antagonists, such as mecamylamine and dihydro-β-erythroidine (Rapier et al., 1988; Grady et al., 1992; Sacaan et al., 1995; Teng et al., 1997a), indicating mediation by nicotinic receptor(s). Moreover, exposure of striatal synaptosomes to *S*-(−)-nicotine (10–100 nM) results in an attenuation of *S*-(−)-nicotine-evoked [^{3}H]dopamine release in response to subsequent superfusion with higher concentrations (5–10 μM) of *S*-(−)-nicotine (Grady et al., 1994, 1997; Rowell and Hillebrand, 1994; Rowell and Li, 1997; Rowell and Duggan, 1998). The latter results clearly indicate that *S*-(−)-nicotine stimulates presynaptic nicotinic receptors to evoke dopamine release, with subsequent receptor desensitization.

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S-(−)-Nicotine metabolites and other tobacco alkaloids may also contribute to the neuropharmacological effects resulting from tobacco use (Crooks and Dwoskin, 1997). In neurochemical studies, nornicotine and cotinine have been shown to inhibit high affinity binding of [^{3}H]nicotine to rat brain membranes (Abood et al., 1981; Copeland et al., 1991; Zhang and Nordberg, 1993) and to evoke dopamine release from striatal slices in a concentration-dependent, Ca^{2+}-dependent, stereoselective, mecamylamine-sensitive and dihydro-β-erythroidine-sensitive manner (Dwoskin et al., 1993, 1999a; Teng et al., 1997a), suggesting a nicotinic receptor-mediated mechanism of action. Nornicotine is also a major tobacco alkaloid, constituting 15–20% of total alkaloid content (Bush et al., 1993; Curvall and Kazemi Vala, 1993). Cotinine, another alkaloidal constituent of tobacco, is also a major peripheral and central oxidative metabolite of nicotine in several animal species, including humans (Gorrod and Wahren, 1993; Benowitz et al., 1994; Crooks et al., 1997; Ghosheh et al., 1999). Although nornicotine is a minor nicotine metabolite in the periphery (Cundy and Crooks, 1984; Kyerematen et al., 1988; Kyerematen and Vesell, 1991; Benowitz et al., 1994), it is a major nicotine metabolite in the CNS (Crooks et al., 1995a, 1997; Crooks and Dwoskin, 1997). Furthermore, nornicotine and cotinine have significantly longer plasma half-lives (7.2–8.5 and 9.8–13.6 h, respectively) when compared to nicotine (0.7–1.4 h) (Kyerematen et al., 1990). Importantly, nornicotine, but not cotinine, has recently been shown to accumulate in rat CNS to pharmacologically relevant concentrations following repeated, peripheral administration of nicotine (Ghosheh et al., 2001). Therefore, as a result of direct exposure to nornicotine in tobacco, indirect exposure to nornicotine from nicotine biotransformation, and due to the comparatively longer brain residence time of nornicotine resulting in CNS accumulation, it is likely that nornicotine will reach pharmacologically relevant concentrations in the brain of tobacco smokers.

Due to the contributory role for nornicotine in the neuropharmacology of nicotine and/or tobacco smoke exposure, a more complete understanding of the effect of nornicotine on nicotinic receptors is warranted. Our initial experiments showed that *S*-(−)-nicotine and *S*-(−)-nornicotine evoked an increase in endogenous dihydroxyphenyacetic acid overflow from superfused rat striatal slices; however, desensitization was not apparent (Dwoskin et al., 1993). Subsequently, we showed that prolonged exposure (60-min duration) to *S*-(−)-nicotine, *S*-(−)-nornicotine or *S*-(−)-cotinine in the superfusion medium resulted in a peak increase in [^{3}H]dopamine overflow after 10–15 min of alkaloid exposure; subsequently, the response returned towards basal (i.e., apparent densensitization) despite the continued presence of the alkaloid in the superfusion buffer (Teng et al., 1997a,b; Dwoskin et al., 1999a). The present study determined if pre-exposure of rat striatal slices to *S*-(−)-nornicotine diminishes the dopaminergic response to a subsequent *S*-(−)-nornicotine exposure, thereby providing clear evidence for desensitization of nicotinic receptors responsible for stimulating dopamine release from presynaptic terminal stores. The ability of *S*-(−)-nicotine and *S*-(−)-nornicotine to cross-desensitize these nicotinic receptors was also determined to ascertain if common receptor subtypes are involved.

2. Materials and methods

2.1. Materials

S-(−)-Nicotine ditartrate and nomifensine maleate were purchased from Research Biochemicals (Natick, MA). *S*-(−)-Nornicotine was synthesized as the perchlorate salt and enantiomeric purity was determined by nuclear magnetic resonance spectroscopy and polarimetric analysis (Ravard and Crooks, 1996). [^{3}H]Dopamine (3,4-ethyl-2-[*N*-^{3}H]dihydroxyphenylethylamine; specific activity, 25.6 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Ascorbic acid, α-D-glucose, and pargyline hydrochloride were purchased from AnalaR (BHD, Poole, UK), Aldrich Chemical (Milwaukee, WI), and Sigma (St. Louis, MO), respectively. TS-2 Tissue solubilizer was purchased from Research Products International (Mount Prospect, IL). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

2.2. Subjects

Male Sprague–Dawley rats (200–250 g) were obtained from Harlan Laboratories (Indianapolis, IN) 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]Dopamine release assays

Drug effects on [^{3}H]overflow from rat striatal slices preloaded with [^{3}H]dopamine were determined using a previously published methodology (Dwoskin and Zahniser, 1986). Briefly, rat striatal slices (500 μm, 6–8 mg) 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 $NaHCO_3$, 0.11mM L-ascorbic acid, and 0.004 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4, saturated with 95% O_2/5% CO_2, at 34°C). Slices were then incubated for an additional 30 min in buffer containing 0.1 μM [^{3}H]dopamine. Each slice was transferred to a superfusion chamber and superfused (1 ml/min) for 60 min with Krebs' buffer containing nomifensine (10 μM), a dopamine uptake inhibitor, and

pargyline (10 μM), a monoamine oxidase inhibitor, such that the [^{3}H]overflow primarily represented [^{3}H]dopamine, rather than [^{3}H]metabolites (Cubeddu et al., 1979; Rapier et al., 1988). Two 5-min (5 ml) samples were collected to determine basal [^{3}H]outflow.

To determine the ability of *S*-(−)-nicotine and *S*-(−)-nornicotine to desensitize nicotinic receptors and to determine if cross-desensitization between *S*-(−)-nicotine and *S*-(−)-nornicotine occurs, striatal slices were superfused initially for a 60-min period with either buffer (no drug), *S*-(−)-nornicotine (0.1, 1 and 10 μM; Experiments 1 and 2) or *S*-(−)-nicotine (1, 10 and 100 nM; Experiments 3 and 4) followed by a second 60-min period of superfusion with 10 μM *S*-(−)-nicotine or 10 μM *S*-(−)-nornicotine. Experiments were performed using a repeated measures design, such that striatal slices from each rat were exposed to all combinations of treatment conditions within each experiment. For all experiments, slices from a given rat were randomly assigned to drug concentration and combination. Thus, depending upon the experimental design, five to seven striatal slices from each rat were utilized, and experiments were repeated using 6–10 rats.

For Experiment 1, five slices obtained from one rat were superfused for 60 min with either buffer (3 slices) or 0.1 μM *S*-(−)-nornicotine (2 slices). Subsequently, one slice from each pre-exposure condition was superfused for a second period with 10 μM *S*-(−)-nicotine or with 10 μM *S*-(−)-nornicotine. The fifth slice was superfused with buffer throughout the experiment in order to determine the stability of the fractional release in the absence of either drug. Experiment 2 determined the effect of pre-exposure to additional *S*-(−)-nornicotine concentrations. Seven slices from one rat were superfused for 60 min with buffer (3 slices), 1 (M (2) slices) or 10 μM (2 slices) *S*-(−)-nornicotine followed by a second period of superfusion with either 10 μM *S*-(−)-nicotine or 10 μM *S*-(−)-nornicotine. As in the previous experiment, the seventh slice was superfused with buffer throughout the experiment. For Experiment 3, seven slices from one rat were superfused for 60 min with buffer (3 slices), 1 nM (2 slices) or 10 nM (2 slices) *S*-(−)-nicotine followed by a second 60-min period of superfusion with 10 μM *S*-(−)-nicotine or 10 μM *S*-(−)-nornicotine. The seventh slice was superfused with buffer throughout the experiment. Experiment 4 determined the effect of pre-exposure to an additional *S*-(−)-nicotine concentration. Five slices from one rat were superfused for 60 min with buffer or 100 nM *S*-(−)-nicotine followed by a second 60-min period of superfusion with 10 μM *S*-(−)-nicotine or 10 μM *S*-(−)-nornicotine. The fifth slice was superfused with buffer throughout the experiment. Slices superfused with buffer for the first 60 min and subsequently with 10 μM *S*-(−)-nicotine or 10 μM *S*-(−)-nornicotine served as control in each experiment.

Experiment 5 was performed to determine if prolonged superfusion with *S*-(−)-nicotine resulted in dopamine depletion. After the collection of two basal superfusate samples, one striatal slice was superfused for 60 min with 0.1 μM *S*-(−)-nicotine followed by a second 60-min period of superfusion with 10 μM *S*-(−)-nicotine, a third 60-min period with buffer, and then an electrical field-stimulation (i.e., after the 3-h period of superfusion). Electrical field-stimulation was delivered by a Grass stimulator (model SD9, Quincy, MA) and consisted of trains of unipolar, rectangular pulses (1 Hz, 20 mA, 2 ms duration, for 1 min). Upon field-stimulation, six superfusate samples were collected, allowing fractional release to return to basal levels. Concurrently, electrical field-stimulation was applied to a second slice from the same rat after the collection of two basal samples and at a time corresponding to the start of superfusion of the first slice with 0.1 μM *S*-(−)-nicotine. Subsequently, the second slice was superfused with buffer for 3 h followed by a second electrical field-stimulation. Six superfusate samples were collected after the first and second electrical field-stimulations. The amount of total [^{3}H]overflow evoked by the second field-stimulation from the slice superfused for 3 h with buffer was compared to field-stimulation after 3 h superfusion and exposure to *S*-(−)-nicotine, to ascertain whether the releasable dopamine pools were depleted.

At the end of the experiment, each slice was solubilized with TS-2. Radioactivity in superfusate and tissue samples was determined by liquid scintillation counting (Packard model B1600 TR Scintillation Counter, Meriden, CT) with an efficiency of 59%. Fractional release for each sample was calculated by dividing the tritium collected in superfusate by the total tissue tritium at the time of collection, in order to normalize potential differences in radioactivity between slices of varying weight. Basal outflow was calculated from the average of tritium collected in the two samples just before drug addition. Evoked total [^{3}H]overflow was calculated by summing the increases in collected tritium resulting from drug exposure after subtracting the basal outflow for an equivalent period of drug exposure. Calculation of [^{3}H]overflow also takes into account differences among tissue weights. Units of total [^{3}H]overflow are expressed as a percentage.

2.4. Statistics

When the data were expressed as total [^{3}H]overflow, a one-way analysis of variance (ANOVA) was performed. EC_{50} values for *S*-(−)-nicotine and for *S*-(−)-nornicotine to induce desensitization and cross-desensitization were determined by an iterative nonlinear least-squares curve-fitting program (Prism: GraphPAD; San Diego, CA). Total [^{3}H]overflow evoked by a concentration of *S*-(−)-nicotine for the first 60 min (pre-exposure) from various slices from the same rat were averaged, and were considered as $n = 1$. Since the desensitization/cross-desensitization experiments were conducted by applying *S*-(−)-nicotine and *S*-(−)-nornicotine at different concentration levels across

two different time periods in each experiment, statistics were analyzed in two parts. The first analysis dealt with time of superfusion, up to and including 70 min, which consisted of the first two basal samples (no drug added) and 12 samples collected during the first period of superfusion with *S*-(−)-nicotine or *S*-(−)-nornicotine. Thus, the first statistical analysis determined the effect of the initial exposure to drug. The second analysis dealt with the second period of superfusion equal to or greater than 70 min, which was when the slices were exposed to a single, higher concentration of either *S*-(−)-nicotine or *S*-(−)-nornicotine. Thus, the second statistical analysis determined whether desensitization or cross-desensitization occurred.

When slices were superfused with only buffer for the entire superfusion period, the basal outflow was not significantly decreased over the first 70 min of the experiment, when compared with the basal outflow at the 10-min time point; and the basal outflow was also not significantly decreased across the second 60-min period of superfusion when compared with the basal outflow at the 70-min time point (data not shown). Therefore, it is appropriate to make comparisons between basal outflow at 10 min and the fractional release in response to the first 60-min period of superfusion with *S*-(−)-nicotine or *S*-(−)-nornicotine. Also, it is appropriate to make comparisons between the basal outflow at 70 min and the fractional release in response to the second period of superfusion with *S*-(−)-nicotine or *S*-(−)-nornicotine.

For each part of the statistical analysis, two-way repeated-measures analysis of covariance (ANCOVA) was performed. ANCOVA accounted for the variation between basal outflow prior to superfusion with *S*-(−)-nicotine or *S*-(−)-nornicotine. The two repeated-measures factors in the analysis were time and the absence or presence of *S*-(−)-nicotine or *S*-(−)-nornicotine. In the first analysis, the covariate was the response at time equal to 10 min when low concentrations of *S*-(−)-nicotine or *S*-(−)-nornicotine were introduced into the superfusion buffer. In the second analysis, the covariate was the response at time equal to 70 min when the higher concentration of *S*-(−)-nicotine or *S*-(−)-nornicotine was introduced. In both the first and second analyses, the covariate was significant for the majority (10 out of 12 cases, data not shown) of the data set. Therefore, all the data were analyzed using ANCOVA. The covariance model was performed using SAS in PROC MIXED to take advantage of the generality of the covariance structure permitted. Fishers' Least Significant Difference (LSD) post hoc comparisons were subsequently performed.

3. Results

3.1. Effect of S-(−)-nicotine

The effect of the pre-exposure to *S*-(−)-nicotine (1–100 nM) on total [^{3}H]overflow during the first 60-min period of sample collection was analyzed by one-way ANOVA, which revealed a significant effect of *S*-(−)-nicotine concentration ($F(3,40) = 32.28$, $P < 0.0001$). Table 1 shows that low concentrations (1–10 nM) of *S*-(−)-nicotine did not increase total [^{3}H]overflow, whereas a higher concentration (100 nM) significantly increased total [^{3}H]overflow during the first 60-min exposure period. Expression of the data as fractional release as a function of time revealed no effect of 1 nM *S*-(−)-nicotine (data not shown). However, an increase in fractional release was observed during 15–45 min of exposure to 10 nM *S*-(−)-nicotine (two-way ANCOVA, *S*-(−)-nicotine × time interaction, $F(15,15) = 1.71$, $P < 0.05$; data not shown). An increase in fractional release was also observed during 10–70 min of exposure to 100 nM *S*-(−)-nicotine (two-way ANCOVA, *S*-(−)-nicotine × time interaction, $F(15,15) = 25.54$, $P < 0.0001$, Fig. 1). Fractional release evoked by 100 nM *S*-(−)-nicotine peaked after 10 min and returned to basal levels, in spite of the continuous superfusion with *S*-(−)-nicotine. Thus, *S*-(−)-nicotine evoked [^{3}H]overflow in a concentration-dependent manner, and the time course illustrates that desensitization is apparent during the first exposure period.

3.2. S-(−)-Nicotine-induced desensitization

S-(−)-Nicotine-induced desensitization was determined by comparing total [^{3}H]overflow evoked by 10 μM *S*-

Table 1
S-(−)-Nicotine-induced desensitization of 10 μM *S*-(−)-nicotine-evoked total [^{3}H]overflow and cross-desensitization of 10 μM *S*-(−)-nornicotine-evoked total [^{3}H]overflow from rat striatal slices. Striatal slices were superfused for the first 60 min with different pre-exposure *S*-(−)-nicotine concentrations followed by a second period of superfusion with 10 μM *S*-(−)-nicotine or 10 μM *S*-(−)-nornicotine. Superfusion with buffer during the pre-exposure period serves as control for each treatment. Data are presented as mean ± SEM total [^{3}H]overflow ($n = 7$–10 rats)

Pre-exposure *S*-(−)-nicotine (nM)	Total [^{3}H]overflow evoked by pre-*S*-(−)-nicotine exposure	Total [^{3}H]overflow evoked by 10 μM *S*-(−)-nicotine	Total [^{3}H]overflow evoked by 10 μM *S*-(−)-nornicotine
0	0 ± 0	2.26 ± 0.26	2.69 ± 0.45
1	0.11 ± 0.06	2.23 ± 0.55	1.93 ± 0.51
10	0.17 ± 0.06	1.21 ± 0.31[a]	1.11 ± 0.42[a]
100	1.52 ± 0.16[a]	0.58 ± 0.36[a]	0.51 ± 0.23[a]

[a] $P < 0.05$ compared to the corresponding control, Fisher's LSD post hoc comparisons.

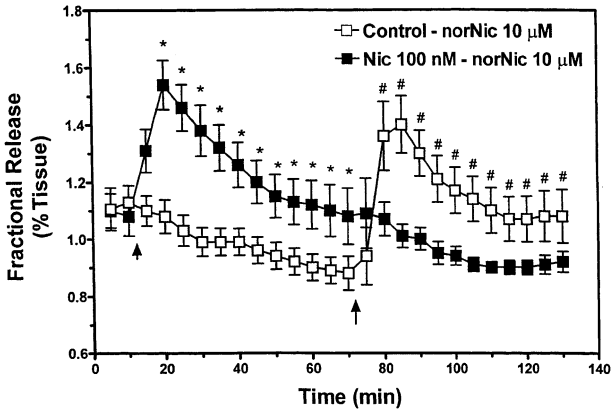

Fig. 1. Cross-desensitization of *S*-(−)-nicotine and *S*-(−)-nornicotine-evoked fractional release from rat striatal slices preloaded with [^{3}H]dopamine. During the first 60 min of sample collection, slices were superfused with buffer (control) or 100 nM *S*-(−)-nicotine (NIC). Subsequently, both control and *S*-(−)-nicotine-pretreated slices were superfused with 10 μM *S*-(−)-nornicotine (norNic) for 60 min. Data are expressed as mean ± standard error of the mean (SEM) fractional release, which was calculated by dividing the tritium collected in each superfusate sample by the total tissue tritium at the time of sample collection. Arrows indicate the addition of either *S*-(−)-nicotine or *S*-(−)-nornicotine to the superfusion buffer. $^{*}P < 0.05$, fractional release different from corresponding basal fractional release at the 10-min time point; $\#P < 0.05$, fractional release different from the corresponding basal fractional release at the 70-min time point; Fisher's LSD post hoc comparisons. $n = 7$ rats.

(−)-nicotine under control conditions (no drug pre-exposure) to that after pre-exposure to 1–100 nM *S*-(−)-nicotine; i.e., striatal slices that were superfused with buffer for the first 60 min followed by a second 60- or 80-min period of superfusion with 10 μM *S*-(−)-nicotine serving as control. Table 1 shows that superfusion with 1 nM *S*-(−)-nicotine did not attenuate the increase in total [^{3}H]overflow evoked by subsequent superfusion with 10 μM *S*-(−)-nicotine; however, superfusion with 10 or 100 nM *S*-(−)-nicotine significantly attenuated (46% and 74%, respectively) the increase in total [^{3}H]overflow in response to 10 μM *S*-(−)-nicotine. One-way ANOVA revealed a significant effect of *S*-(−)-nicotine concentration ($F(3,31) = 7.79$, $P < 0.001$), and the EC_{50} was 7.7 nM for nicotine-induced desensitization. Expression of the data as fractional release as a function of time similarly revealed that pre-exposure to 10 or 100 nM *S*-(−)-nicotine resulted in an attenuation of release evoked by 10 μM *S*-(−)-nicotine (data not shown). Repeated-measures, two-way ANCOVA revealed a significant main effect of 10 nM *S*-(−)-nicotine ($F(1,15) = 3.40$, $P < 0.05$) and of time ($F(15,15) = 16.47$, $P < 0.05$); however, the interaction was not significant. For 100 nM *S*-(−)-nicotine, a significant *S*-(−)-nicotine × time interaction ($F(15,15) = 13.85$, $P < 0.0001$) was found. Thus, a diminished response to 10 μM *S*-(−)-nicotine (desensitization) resulted from prior exposure to low concentrations of 10 or 100 nM *S*-(−)-nicotine, but not following 1 nM *S*-(−)-nicotine.

3.3. S-(−)-Nicotine-induced cross-desensitization to S-(−)-nornicotine

S-(−)-Nicotine-induced cross-desensitization to *S*-(−)-nornicotine was determined by comparing total [^{3}H]overflow evoked by 10 μM *S*-(−)-nornicotine under control conditions (no drug pre-exposure) to that after pre-exposure to 1–100 nM *S*-(−)-nicotine. Table 1 illustrates that superfusion with 1 nM *S*-(−)-nicotine did not attenuate the increase in total [^{3}H]overflow in response to the subsequent superfusion with 10 μM *S*-(−)-nornicotine; however, superfusion with 10 or 100 nM *S*-(−)-nicotine significantly attenuated (59% and 81%, respectively) the increase in total [^{3}H]overflow evoked by *S*-(−)-nornicotine. One-way ANOVA revealed a significant effect of *S*-(−)-nicotine concentration ($F(3,31) = 6.47$, $P < 0.001$), and the EC_{50} was 2.4 nM for cross-desensitization. Expression of the data as fractional release as a function of time revealed that pre-exposure to 1 nM *S*-(−)-nicotine did not attentuate the increase in release evoked by subsequent superfusion with 10 μM *S*-(−)-nornicotine (data not shown). Two-way ANCOVA revealed significant main effects of *S*-(−)-nicotine ($F(1,15) = 3.51$, $P < 0.05$) and time ($F(1,15) = 3.22$, $P < 0.05$), such that 10 nM *S*-(−)-nicotine significantly diminished the subsequent response to 10 μM *S*-(−)-nornicotine. Fig. 1 illustrates the time course of the effect of pre-exposure to 100 nM *S*-(−)-nicotine and the subsequent marked reduction in release evoked by 10 μM *S*-(−)-nornicotine. Two-way ANCOVA also revealed a significant *S*-(−)-nicotine × time interaction ($F(15,15) = 20.71$, $P < 0.0001$) for 100 nM *S*-(−)-nicotine to diminish the subsequent response to 10 μM *S*-(−)-nornicotine. Superfusion with 100 nM *S*-(−)-nicotine markedly reduced the response to subsequent superfusion with 10 μM *S*-(−)-nornicotine across the time course of exposure (Fig. 1). Thus, *S*-(−)-nicotine desensitized receptors that were stimulated by *S*-(−)-nornicotine to evoke dopamine release.

3.4. Effect of S-(−)-nornicotine

The effect of pre-exposure with *S*-(−)-nornicotine (0.1–10 μM) on total [^{3}H]overflow during the first 60-min period of sample collection was analyzed by one-way ANOVA, which revealed a significant effect of *S*-(−)-nornicotine concentration ($F(3,31) = 77.64$, $P < 0.0001$). Table 2 shows that superfusion with 0.1 μM *S*-(−)-nornicotine did not produce an increase in total [^{3}H]overflow; however, superfusion with higher concentrations (1 and 10 μM) increased total [^{3}H]overflow during the first 60-min period. Expression of the data as fractional release as a function of time revealed no effect of 0.1 μM *S*-(−)-nornicotine (data not shown). However, a significant increase in fractional release was observed during 10–70 min of exposure to 1 μM *S*-(−)-nornicotine (two-way ANCOVA, $F(15,15) = 7.54$, $P < 0.0001$; Fig. 2).

Table 2
S-(−)-Nornicotine-induced desensitization of 10 μM *S*-(−)-nornicotine-evoked total [^{3}H]overflow and cross-desensitization of 10 μM *S*-(−)-nicotine-evoked total [^{3}H]overflow from rat striatal slices. Striatal slices were superfused for the first 60 min with different pre-exposure *S*-(−)-nornicotine concentrations followed by a second period of superfusion with 10 μM *S*-(−)-nicotine or 10 μM *S*-(−)-nornicotine. Superfusion with buffer for pre-exposure serves as control for each treatment. Data are presented as mean ± SEM total [^{3}H]overflow (n = 7–10 rats)

Pre-exposure *S*-(−)-nornicotine (M)	Total [^{3}H]overflow evoked by pre-*S*-(−)-nornicotine exposure	Total [^{3}H]overflow evoked by 10 μM *S*-(−)-nicotine	Total [^{3}H]overflow evoked by 10 μM *S*-(−)-nornicotine
0	0 ± 0	3.54 ± 0.50	2.32 ± 0.32
0.1	0.53 ± 0.34	1.94 ± 0.68	2.00 ± 0.56
1	1.62 ± 0.20[a]	0.62 ± 0.21[a]	0.34 ± 0.17[a]
10	2.42 ± 0.34[a]	0.43 ± 0.19[a]	0.08 ± 0.06[a]

[a] $P < 0.05$ compared to the corresponding control, Fisher's LSD post hoc comparisons.

Fractional release of a greater magnitude was observed during 10–70 min of exposure to 10 μM *S*-(−)-nornicotine compared to 1 μM *S*-(−)-nornicotine (two-way ANCOVA, $F(15,15) = 1.37$, $P < 0.05$; data not shown). Fig. 2 illustrates that fractional release evoked by 1 μM *S*-(−)-nornicotine peaked after 10 min and returned to basal levels despite continuous superfusion with drug. Thus, *S*-(−)-nornicotine evoked [^{3}H]overflow in a concentration-dependent manner, and the time course illustrates that desensitization was apparent during the first exposure period.

3.5. S-(−)-Nornicotine-induced desensitization

S-(−)-Nornicotine-induced desensitization was determined by comparing total [^{3}H]overflow evoked by 10 μM *S*-(−)-nornicotine under control conditions (no drug pre-exposure) to that after pre-exposure to 0.1–10 μM *S*-(−)-nornicotine. Table 2 shows that superfusion with 0.1 μM *S*-(−)-nornicotine did not attenuate the increase in total [^{3}H]overflow evoked by subsequent superfusion with 10 μM *S*-(−)-nornicotine; however, superfusion with 1 or 10 μM *S*-(−)-nornicotine significantly attenuated (85% and 97%, respectively) the increase in total [^{3}H]overflow in response to 10 μM *S*-(−)-nornicotine (one-way ANOVA, *S*-(−)-nornicotine concentration, $F(3,40) = 9.51$, $P < 0.001$). The EC_{50} was 95 nM for *S*-(−)-nornicotine desensitization. Expression of the data as fractional release as a function of time similarly revealed that pre-exposure to 0.1 μM *S*-(−)-nornicotine did not diminish the subsequent fractional release evoked by 10 μM *S*-(−)-nornicotine (data not shown). Two-way ANCOVA revealed a significant *S*-(−)-nornicotine × time interaction for 1 μM *S*-(−)-nornicotine ($F(15,15) = 7.54$, $P < 0.0001$), and significant main effects of 10 μM *S*-(−)-nornicotine ($F(1,15) = 4.25$, $P < 0.05$) and time ($F(15,15) = 3.41$, $P < 0.01$), which diminished the subsequent response to 10 μM *S*-(−)-nornicotine. Fig. 2 illustrates the time course of the effect of pre-exposure to 1 μM *S*-(−)-nornicotine, and moreover, the subsequent attenuation of release evoked by 10 μM *S*-(−)-nornicotine. Thus, superfusion with 1 μM *S*-(−)-nornicotine (Fig. 2) and 10 μM *S*-(−)-nornicotine (data not shown) both markedly diminished the response to 10 μM *S*-(−)-nornicotine across the time course of exposure.

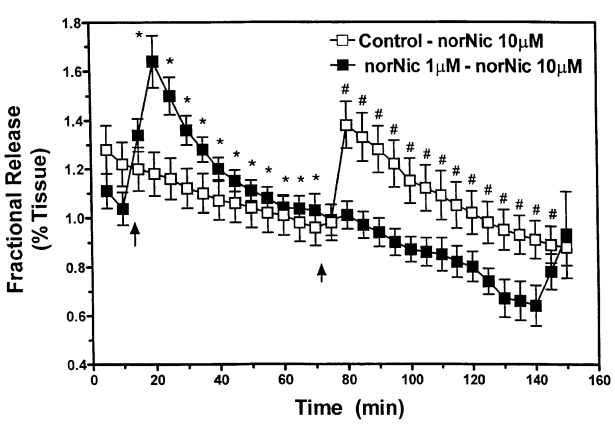

Fig. 2. *S*-(−)-Nornicotine-induced desensitization of fractional release from striatal slices preloaded with [^{3}H]dopamine. During the first 60 min of sample collection, slices were superfused with buffer (control) or 1 μM *S*-(−)-nornicotine (norNic). Subsequently, both control and *S*-(−)-nornicotine-pretreated slices were superfused with 10 μM *S*-(−)-nornicotine for 80 min. Data are expressed as mean ± SEM fractional release. Arrows indicate the addition of *S*-(−)-nornicotine to the superfusion buffer. $^*P < 0.05$, fractional release different from the corresponding basal fractional release at the 10 min time point; $\#P < 0.05$, fractional release different from the corresponding basal fractional release at the 70-min time point; Fisher's LSD post hoc comparisons. $n = 10$ rats.

3.6. S-(−)-Nornicotine-induced cross-desensitization to S-(−)-nicotine

S-(−)-Nornicotine-induced cross-desensitization was determined by comparing total [^{3}H]overflow evoked by 10 μM *S*-(−)-nicotine under control conditions (no drug pre-exposure) to that after pre-exposure to 0.1–10 μM *S*-(−)-nornicotine. Table 2 shows that superfusion with 0.1 μM *S*-(−)-nornicotine did not attenuate the increase in total [^{3}H]overflow evoked by subsequent superfusion with 10 μM *S*-(−)-nicotine. However, superfusion with 1 or 10 μM *S*-(−)-nornicotine significantly attenuated (82% and 88%, respectively) the increase in total [^{3}H]overflow in response to 10 μM *S*-(−)-nicotine (one-way, *S*-(−)-

nornicotine concentration–effect, $F(3,40) = 7.79$, $P < 0.001$; Table 2). The EC_{50} was 330 nM for cross-desensitization. Expression of the data as fractional release as a function of time revealed that pre-exposure to 0.1 μM *S*-(−)-nornicotine did not attentuate the increase in release evoked by subsequent superfusion with 10 μM *S*-(−)-nicotine (data not shown). However, two-way ANCOVA revealed that 1 μM *S*-(−)-nornicotine diminished the subsequent response to 10 μM *S*-(−)-nicotine (significant *S*-(−)-nornicotine × time interaction, $F(15,15) = 11.59$, $P < 0.001$). Fig. 3 illustrates the time course of the effect of pre-exposure to 1 μM *S*-(−)-nornicotine, and moreover, the subsequent attenuation of release evoked by 10 μM *S*-(−)-nicotine across time. Two-way ANCOVA also revealed a significant *S*-(−)-nornicotine × time interaction ($F(15,15) = 8.11$, $P < 0.0001$) for 10 μM *S*-(−)-nornicotine pre-exposure, which also markedly diminished the subsequent response to 10 μM *S*-(−)-nornicotine (data not shown). Thus, *S*-(−)-nornicotine desensitized receptors which were stimulated by *S*-(−)-nicotine to evoke dopamine release.

3.7. Lack of dopamine depletion

To determine if the diminished response to 10 μM *S*-(−)-nicotine following prolonged superfusion and pre-exposure to *S*-(−)-nicotine was the result of depletion of releasable stores of dopamine, rat striatal slices were field-stimulated following consecutive superfusion for 60 min with 100 nM *S*-(−)-nicotine, for 60 min with 10 μM *S*-(−)-nicotine, and for 60 min with Krebs' buffer. Field-stimulation of these striatal slices evoked total [^{3}H]overflow which was not different from slices superfused with Krebs' buffer for an equivalent period of time ($F(1,9) = 0.56$, $P > 0.05$; Table 3). Thus, the diminished response to 10 μM *S*-(−)-nicotine following prior exposure to *S*-(−)-nicotine was not due to depletion of the releasable dopamine pools.

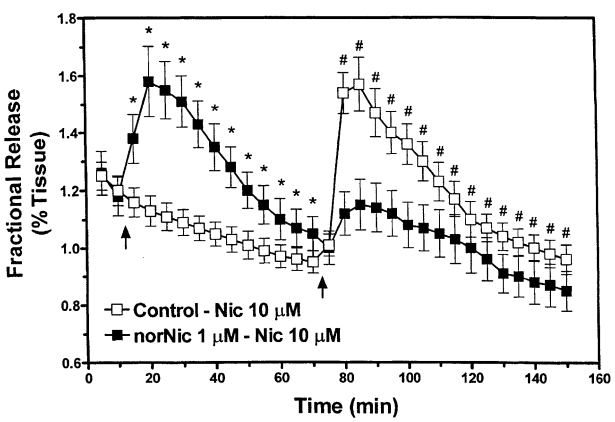

Fig. 3. Cross-desensitization of *S*-(−)-nornicotine and *S*-(−)-nicotine-evoked fractional release from rat striatal slices preloaded with [^{3}H]dopamine. During the first 60 min of sample collection, slices were superfused with buffer (control) or 1 μM *S*-(−)-nornicotine (norNic). Subsequently, both control and *S*-(−)-nornicotine-pretreated slices were superfused with 10 μM *S*-(−)-nicotine (Nic) for 60 min. Data are expressed as mean ± SEM fractional release. Arrows indicate the addition of either *S*-(−)-nornicotine or *S*-(−)-nicotine to the superfusion buffer. * $P < 0.05$, fractional release different from corresponding basal fractional release at the 10 min time point; # $P < 0.05$, fractional release different from the corresponding basal fractional release at the 70-min time point; Fisher's LSD post hoc comparisons. $n = 10$ rats.

Table 3
Total [^{3}H]overflow from striatal slices in response to superfusion with *S*-(−)-nicotine and to electrical field stimulation. Slices in treatment 1 were superfused with 0.1 μM *S*-(−)-nicotine (stimulus 1) for 60 min followed by another 60 min superfusion with 10 μM *S*-(−)-nicotine (stimulus 2). Slices were then superfused with buffer for 60 min until the application of the electrical field stimulation (stimulus 3). Slices in treatment 2 were stimulated electrically concurrent with stimulus 1 and 3, but were not superfused with drug. Data are presented as mean ± SEM total [^{3}H]overflow. ND indicates not determined ($n = 6$ rats)

Stimulus	Treatment 1	Treatment 2
1	*S*-(−)-nicotine 0.1 μM (60 min) 1.19 ± 0.34	Electrical stimulus (1 min) 1.10 ± 0.17
2	*S*-(−)-nicotine 10 μM (60 min) 0.39 ± 0.02	Superfusion with buffer (180 min) ND
3	Electrical stimulus (1 min) 1.01 ± 0.32	Electrical stimulus (1 min) 0.81 ± 0.22

4. Discussion

The present study demonstrates that neuronal nicotinic receptors are desensitized following exposure to *S*-(−)-nicotine or *S*-(−)-nornicotine; and moreover, cross-desensitization occurs between these drugs, suggesting the involvement of common nicotinic receptor subtypes. Thus, the present findings are an extension of our previous work (Dwoskin et al., 1993; Teng et al., 1997a,b) and further support the hypothesis that *S*-(−)-nornicotine and *S*-(−)-nicotine act at common nicotinic receptor subtypes to evoke dopamine release in striatum. Desensitization was apparent during the first presentation of either drug, since fractional release returned towards basal levels, despite continued superfusion with drug. Desensitization occurred 10–20 min after the initial drug exposure, and persisted for at least 40–50 min using the superfused striatal slice preparation. Superfusion with a low concentration (1 nM) of *S*-(−)-nicotine did not evoke fractional release, and moreover, did not diminish the response to the subsequent superfusion with 10 μM *S*-(−)-nicotine or 10 μM *S*-(−)-nornicotine. However, superfusion with higher concentrations (10 and 100 nM) of *S*-(−)-nicotine diminished the response to the subsequent superfusion with *S*-(−)-nicotine or *S*-(−)-nornicotine. EC_{50} values of 7.7 and 2.4 nM were obtained for *S*-(−)-nicotine to induce desensitization and cross-desensitization to *S*-(−)-nornicotine, respectively. *S*-(−)-Nornicotine also induced desensitization and cross-desensitization to *S*-(−)-nicotine, but with a

lower potency (12- and 165-foldless, respectively) compared to that obtained for *S*-(−)-nicotine. Only those concentrations of *S*-(−)-nornicotine which were found to evoke total [^{3}H]overflow subsequently diminished the response to a higher concentration of either *S*-(−)-nicotine or *S*-(−)-nornicotine; whereas a concentration (10 nM) of *S*-(−)-nicotine, which did not significantly evoke [^{3}H] overflow, produced both desensitization and cross-desensitization to *S*-(−)-nornicotine. Furthermore, desensitization to *S*-(−)-nicotine was not the result of depletion of the releasable [^{3}H]dopamine pool.

The present results are consistent with previous reports of nicotine-induced desensitization of native nicotinic receptors which evoke dopamine release from mouse or rat striatal synaptosomes (Grady et al., 1994; Rowell and Hillebrand, 1994). In these latter studies, desensitization occurred after a 2–10-min period of superfusion with *S*-(−)-nicotine at low concentrations (10–20 nM). These low concentrations of *S*-(−)-nicotine similarly did not evoke an increase in [^{3}H]dopamine release from the preloaded striatal synaptosomes. In this respect, the concentration range of *S*-(−)-nicotine (10 and 100 nM) which induced desensitization in the present study is in good agreement with previously published results. Following superfusion with higher concentrations of *S*-(−)-nicotine (>0.3 μM), presynaptic nicotinic receptors undergo a long-lasting inactivation (Grady et al., 1994, 1997; Rowell and Hillebrand, 1994; Rowell and Duggan, 1998). Thus, it is likely that following 60 min superfusion with 10 μM *S*-(−)-nicotine, presynaptic nicotinic receptors were similarly inactivated in the present study. Despite the probable inactivation of presynaptic nicotinic receptors in superfused striatal slices exposed to 10 μM *S*-(−)-nicotine, physiological mechanisms of dopamine release appear to be intact, since field-stimulated [^{3}H]dopamine release was not diminshed. The vesicular pool of presynaptic dopamine is generally accepted to be that from which nicotine stimulates dopamine release via a nicotinic receptor-mediated calcium-dependent mechanism. However, nicotinic agonists have also been reported to release dopamine and other neurotransmitters from a second presynaptic pool via a calcium-independent, transporter-mediated, non-exocytotic mechanism (Westfall et al., 1987; Harsing et al., 1992; Kiss et al., 1996; Lendvai et al., 1996; Vizi, 1998). Furthermore, nicotine has been reported to potentiate amphetamine-stimulated [^{3}H]dopamine release via nicotinic receptor modulation of the dopamine transporter (Drew et al., 2000). However, using the current methodology, we have previously shown that both *S*-(−)-nicotine and *S*-(−)-nornicotine evoke the release of [^{3}H]dopamine in a nicotinic-receptor mediated and calcium-dependent manner (Dwoskin et al., 1993; Teng et al., 1997a,b; Green et al., 2001), indicative of release from the vesicular dopamine pool in striatum.

Cross-desensitization between nicotine and nornicotine was also demonstrated in the current study, suggesting the involvement of common nicotinic receptor subtypes. However, the specific subunit combinations of native nicotinic receptors, which when stimulated results in dopamine release and subsequent desensitization, have not yet been elucidated. Putative nicotinic receptors containing α3 and β2 subunits have been implicated based on sensitivity to receptor antagonists, including neuronal bungarotoxin (Schulz and Zigmond, 1989; Grady et al., 1992), *N*-octylnicotinium iodide (NONI; Crooks et al., 1995b; Dwoskin and Crooks, 2001), and α-conotoxin-MII (Cartier et al., 1996; Kaiser et al., 1998). Results from in vivo and in vitro studies utilizing β2 knockout mice (Picciotto et al., 1998) also implicate β2-containing receptors in nicotine-evoked striatal dopamine release. The observation that α-conotoxin-MII only partially inhibits nicotinic receptor agonist-evoked dopamine release (Kulak et al., 1997; Kaiser et al., 1998) suggests heterogeneity of presynaptic nicotinic receptors that mediate striatal dopamine release. Additionally, other evidence suggests the involvement of α4-, β2- and β4-containing nicotinic receptors in this response (Rapier et al., 1990; Grady et al., 1992; Sharples et al., 2000). Furthermore, rat substantia nigra pars compacta neurons express mRNA for α3, α4, α5, α6, α7, β2, β3 and β4 subunits (Wada et al., 1989, 1990; Deneris et al., 1989; Dineley-Miller and Patrick, 1992; Charpantier et al., 1998; Arroyo-Jimenez et al., 1999). Thus, many receptor subtypes may be involved in nicotine-evoked dopamine release in striatum. Moreover, high levels of expression of α6 and β3 mRNA have been observed in substantia nigra (Deneris et al., 1989; LeNovere et al., 1996; Goldner et al., 1997; Charpantier et al., 1998), suggesting that α6 and β3 subunits are likely candidates for combination with α3 and β2 subunits in modulating nicotine-evoked dopamine release and desensitization in striatum. Therefore, nicotinic receptors in dopaminergic terminal fields in striatum may express various combinations of subunits, and a single neuron may express more than one receptor subtype of varying subunit combination.

Neuronal nicotinic receptor desensitization has also been demonstrated in cloned nicotinic receptors expressed in *Xenopus* oocytes. The sensitivity to nicotine, the rate of receptor desensitization, as well as the recovery from desensitization of heterologously expressed receptors, varies with subunit composition. For example, α7 receptors desensitize more rapidly than α3β2 receptors, which desensitize more rapidly than α4β2 receptors (Couturier et al., 1990; Seguela et al., 1993; Gross et al., 1991; Amar et al., 1995; Vibat et al., 1995; Fenster et al., 1997). Also, β-subunits play a role in the rate of desensitization. For example, β2 subunits reach near maximal desensitization more rapidly than β4-containing receptors when exposed to low concentrations of nicotine (Fenster et al., 1997). Furthermore, inclusion of α5 subunits into recombinant receptors containing α3 or α4 subunits produced increases in sensitivity, rate and magnitude of desensitization (Ramirez-Latorre et al., 1996; Gerzanich et al., 1998).

The ability of nicotine to induce receptor desensitization may underlie certain aspects of tobacco use. Habitual tobacco smokers intermittently self-administer both nicotine and nornicotine, as tobacco alkaloids. Smokers have been reported to regulate their plasma levels of nicotine to maintain concentrations between 0.1 and 1.0 μM (Benowitz et al., 1990). These concentrations are able to stimulate dopamine release and subsequently desensitize nicotinic receptors. The period of abstinence imposed by sleep may allow a large proportion of the desensitized receptors to change their conformation and return to the activated state. The pattern of intermittent dosing during tobacco smoking may optimize the trade-off between receptor activation and desensitization. Habitual smokers report that the first cigarette of the day is generally the most satisfying (Surgeon General's Report, 1988).

The present results suggest that nornicotine in the CNS also contributes to the ratio of activated and desensitized nicotinic receptors. Nornicotine has been detected in rat brain at concentrations of 0.04 and 0.1 μM after administration of either 0.3 or 0.8 mg/kg, respectively, behaviorally relevant *S*-(−)-nicotine doses (Ghosheh et al., 1999). Exposure to the upper limit of this nornicotine concentration range produced marginal nicotinic receptor desensitization in the present study. Moreover, following repeated, intermittent, peripheral administration of *S*-(−)-nicotine (0.3 mg/kg, 10 doses, 30 min inter-injection interval), nornicotine was observed to accumulate in brain to ~10-fold higher concentrations than after a single dose (Ghosheh et al., 2001). Also, the present results on nornicotine-induced nicotinic receptor desensitization suggest that the concentration of nornicotine in brain resulting from this intermittent nicotine administration may maintain desensitization for a greater time period than might be predicted from the pharmacokinetics of nicotine alone, due to the long CNS half-life of nornicotine (Ghosheh et al., 1999).

In behavioral models examining nicotine self-administration, in which rats repeatedly and intermittently self-administer *S*-(−)-nicotine (Corrigall et al., 1992; 1994; Singer et al., 1982; Mansbach et al., 2000), there is a high likelihood that nornicotine is accumulating in brain at pharmacologically active concentrations, and that these concentrations are maintained for prolonged periods due to the long CNS half-life of nornicotine relative to nicotine. The present results, showing that nornicotine both releases DA and produces nicotinic receptor desensitization, suggest that nornicotine may be contributing to this behavioral pharmacological effect, i.e., the maintenance of self-administration behavior. Furthermore, our previous research has demonstrated that nornicotine itself maintains self-administration (Bardo et al., 1999), probably through activation and/or desensitization of nicotinic receptors. Thus, it is possible that nicotine self-administration behavior in both animal models and smokers is maintained, at least in part, by prolonged nicotinic receptor stimulation by nornicotine even after nicotine concentrations have diminished below the pharmacologically active range. In summary, the present study further supports our hypothesis that the nicotine metabolite and tobacco alkaloid, nornicotine, via activation and desensitization of neuronal nicotinic receptors, contributes to the neuropharmacological effects of nicotine exposure.

Acknowledgements

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