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Repeated Nicotine Administration Robustly Increases bPiDDB Inhibitory Potency at $\alpha 6\beta 2$ -containing Nicotinic Receptors Mediating Nicotine-evoked Dopamine Release

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Abstract

The novel nicotinic receptor (nAChR) antagonist, *N,N*-dodecane-1,12-diyl-*bis*-3-picolinium dibromide (bPiDDB), and its chemically-reduced analog, r-bPiDDB, potentially inhibit nicotine-evoked dopamine (DA) release from rat striatal slices. Since tobacco smokers self-administer nicotine repeatedly, animal models incorporating repeated nicotine treatment allow for mechanistic evaluation of therapeutic candidates following neuroadaptive changes. The current study determined the ability of bPiDDB, r-bPiDDB and α -conotoxin MII (α -CtxMII), a peptide antagonist selective for $\alpha 6\beta 2$ -containing nAChRs, to inhibit nicotine-evoked [³H]DA release from striatal slices from rats repeatedly administered nicotine (0.4 mg/kg for 10 days) or saline (control). Concomitant exposure to maximally effective concentrations of r-bPiDDB (1 nM) and α -CtxMII (1 nM) resulted in inhibition of nicotine-evoked [³H]DA release no greater than that produced by either antagonist alone, suggesting that r-bPiDDB inhibits $\alpha 6\beta 2$ -containing nAChRs. Repeated nicotine treatment increased locomotor activity, demonstrating behavioral sensitization. Concentration-response curves for nicotine-evoked [³H]DA release were not different between nicotine-treated and control groups. Maximal inhibition for α -CtxMII was greater following repeated nicotine compared to control ($I_{\max}$ =90% vs. 62%), with no change in potency. bPiDDB was 3-orders of magnitude more potent in inhibiting nicotine-evoked [³H]DA release in nicotine-treated rats compared to control rats (IC_{50} =5 pM vs 6 nM), with no change in maximal inhibition. Neither a shift to left in the concentration response nor a change in maximal inhibition was observed for r-bPiDDB following repeated nicotine. Thus, repeated nicotine treatment may differentially regulate the stoichiometry, conformation and/or composition of $\alpha 6\beta 2$ -containing

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nAChRs mediating nicotine-evoked striatal DA release. Therefore, bPiDDB and r-bPiDDB appear to target different $\alpha 6 \beta 2$ -containing nAChR subtypes.

Keywords

smoking cessation; nicotine; nicotinic acetylcholine receptor; dopamine release; nicotinic receptor antagonist

1. INTRODUCTION

Tobacco smoking is a chronic, relapsing disorder [1,2], and nicotine plays a major role in continued tobacco use through its intrinsic reinforcing properties [3,4]. Nicotine is self-administered intravenously in animal models [5,6,7] and by smokers that have been tobacco deprived [8]. Nicotine self-administration is decreased (>60%) by the nonselective nAChR antagonist, mecamylamine [9,10], indicating mediation by nAChRs. Nicotine activation of nAChRs leads to increased extracellular dopamine (DA) concentrations, which produces reward and ultimately tobacco addiction [11,12]. Although the exact subunit composition and stoichiometry of native nicotinic receptors has not been elucidated conclusively, based on research using transgenic mice and on α -conotoxin MII (α -CtxMII)-sensitivity, six different nAChR subtypes appear to contribute to nicotine-evoked DA release from mouse striatal synaptosomes, including: $\alpha 6 \beta 2$ -, $\alpha 6 \beta 2 \gamma 3$ -, $\alpha 6 \beta 4 \gamma 2$ - and $\alpha 6 \beta 4 \gamma 2 \delta 3$ -containing receptors, which are α -CtxMII-sensitive, and $\alpha 4 \beta 2$ - and $\alpha 4 \beta 5 \gamma 2$ -containing receptors, which are α -CtxMII-insensitive [13,14]. Furthermore, the $\alpha 4 \beta 6 \gamma 2 \delta 3$ -containing receptor subtype, which constitutes ~50% of $\beta 6$ -containing nAChRs on DA terminals in striatum from wild-type mice, has the highest sensitivity to nicotine [14,15], strongly implicating this subtype in nicotine-evoked DA release and reward.

Neuroadaptations in response to repeated nicotine treatment are thought to play an important role in the maintenance of nicotine addiction [2]. In recombinant receptor systems, incubation with nicotine (1 μ M - 1 mM for 24 hr) alters nAChR subtype, stoichiometry, function, and post-translational maturation, i.e., the process of subunits leaving the endoplasmic reticulum and the subsequent incorporation into functional nAChRs [16-18]. Incubation with nicotine (10 μ M for 24 hr) also influences nAChR conformation (i.e., subunit folding) in cell expression systems [19]. Recombinant nAChR systems allow the study of single subtypes in isolation from other subtypes and from cellular factors that could complicate or obscure receptor responses. However, this approach is not capable of capturing the anatomical and multi-transmitter complexity inherent in brain tissue, and thus, translation of findings in these studies to smokers is limited inherently.

Early studies using animal models report increases in [3 H]nicotine and [3 H]cytisine binding in both rats and mice repeatedly treated with nicotine, indicating an increased number of nAChR binding sites [20,21]. Subsequent studies using fluorescently tagged $\alpha 4$ subunits have demonstrated that chronic nicotine increases expression of nAChRs containing $\alpha 4$ subunits in the ventral tegmental area and substantia nigra pars compacta [22]. Deletion of the $\beta 2$ subunit in mice eliminates increases in [125 I]epibatidine binding observed after continuous nicotine administration (1-4 mg/kg/hr for ten days) [23]. Collectively, these studies indicate that chronic nicotine administration results in up-regulation of $\alpha 4$ - and $\beta 2$ -containing nAChR subtypes. Recently, binding studies using [125 I] α -CtxMII to selectively label $\beta 6$ -containing nAChRs showed a decrease in receptor number in rat and mouse striatum following chronic nicotine treatment [24-26]. Immunoprecipitation studies have demonstrated that the presence of $\beta 5$ and $\gamma 3$ subunits in $\alpha 4 \beta 5 \gamma 2$ - and $\alpha 6 \beta 2 \gamma 3$ -containing nAChR subtypes, respectively, prevents the alterations (both up and down regulation,

respectively) in subtype expression following chronic nicotine treatment [24,27]. Thus, expression of specific subunits in a given subtype can prevent observed neuroadaptations in response to repeated nicotine treatment. Therefore, pharmacological history is a critical consideration when investigating the role of different nAChR subtypes mediating the CNS response to nicotine.

The novel nAChR antagonist, *N,N'*-dodecane-1,12-diyl-*bis*-3-picolinium dibromide (bPiDDB; Fig. 1), potently ($IC_{50} = 2$ nM) inhibits nicotine-evoked striatal [3H]DA release through an interaction with $\alpha 6$ -containing nAChRs [28,29] and inhibits nicotine-evoked DA release from nucleus accumbens in *in vivo* microdialysis studies [30]. Further, peripherally-administered bPiDDB decreases intravenous nicotine self-administration in rats [31]. In addition, as part of the optimization to improve drug-like properties of bPiDDB, a structural analog was designed in which the two 3-picolinium moieties were reduced chemically to tertiary amino moieties (3-methyl-1,2,5,6-tetrahydropyridyl), thus affording r-bPiDDB (Fig. 1). r-bPiDDB is 10-fold more potent ($IC_{50} = 0.3$ nM) at inhibiting nicotine-evoked [3H]DA release than is bPiDDB, and r-bPiDDB decreases nicotine self-administration [32]. The purpose of the current study was to determine if bPiDDB and/or r-bPiDDB inhibits nicotine-evoked [3H]DA release from striatal slices from rats repeatedly administered nicotine. Also, studies were performed to determine whether r-bPiDDB inhibits $\alpha 6$ -containing nAChR subtypes in rats administered nicotine or saline repeatedly.

2. METHODS

2.1. Materials

[3H]DA (dihydroxyphenylethylamine, 3,4-[ring-2,5,6- 3H]-; specific activity 28.0 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences, Inc. (Boston, MA). S(-)Nicotine ditartrate (nicotine), nomifensine maleate and pargyline hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO). TS-2 tissue solubilizer and scintillation cocktail were purchased from Research Products International Corp (Mt. Prospect, IL). All other chemicals used in the *in vitro* assay buffers were purchased from Thermo Fisher Scientific (Waltham, MA). -CtxMII and bPiDDB were synthesized as described previously [33,34]. r-bPiDDB was prepared by chemical reduction of the parent *bis*-quaternary ammonium analog, bPiDDB [35]. Chemical structures of bPiDDB and r-bPiDDB (Fig. 1) were verified by 1H - and ^{13}C -NMR spectroscopy, mass spectrometry and X-ray crystallography.

2.2. Animals

Adult male Sprague-Dawley rats (200–225 g) were obtained from Harlan Laboratories, Inc. (Indianapolis, IN) and housed two per cage with *ad libitum* access to food and water in the Division of Laboratory Animal Resources (University of Kentucky, Lexington, KY). All experimental animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Kentucky. Groups of rats were administered nicotine (0.4 mg/kg; free base, sc) or saline once daily for 10 consecutive days. Immediately following each injection, locomotor activity was measured for 60 min. All injections were administered in a volume of 1 ml/kg body weight. Striatal slices were obtained from non-, saline-, and nicotine-injected rats 24 hr after the last injection.

2.3. [3H]DA Overflow Assay

Nicotine-evoked [3H]DA overflow was determined using superfused rat striatal slices preloaded with [3H]DA [36]. Briefly, coronal slices of striata (500 μ m, 5–7 mg) were incubated for 30 min in Krebs' buffer (in mM; 118 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1 NaH_2PO_4 , 1.3 $CaCl_2$, 11.1 $-D$ -glucose, 25 $NaHCO_3$, 0.11 L-ascorbic acid and 0.004 disodium EDTA,

pH 7.4, saturated with 95% O₂/5% CO₂) at 34°C, and then incubated with 0.1 μM [³H]DA (final concentration) for 30 min. After rinsing in fresh buffer, slices were transferred to a Brandel 2500 Suprafusion system (Biomedical Research and Development Laboratories, Inc.; Gaithersburg, MD) and superfused (0.6 ml/min) for 60 min with Krebs' buffer at 34°C. Superfusion buffer contained nomifensine (10 μM), a DA transporter inhibitor, and pargyline (10 μM), a monoamine oxidase inhibitor, to prevent reuptake and metabolism of [³H]DA, respectively, and to assure that the [³H] collected in superfusate primarily represented parent neurotransmitter. Following an initial 60 min period of superfusion, two 4-min samples (2.4 ml/sample) were collected to determine basal [³H]DA outflow.

To determine the concentration-dependent effect of nicotine to evoke [³H]DA release from striatal slices obtained from rats injected with nicotine or saline repeatedly, a series of experiments was conducted in which each striatal slice from an individual rat was superfused for 36 min in the absence (buffer control) or presence of a single concentration of nicotine (0.1 – 100 μM). Nicotine remained in the buffer throughout the experiment and samples were collected every 4 min until the end of the experiment. Based on the results from the concentration response, 10 μM nicotine was chosen as appropriate for assessing antagonist-induced inhibition.

The inhibitory potency of bPiDDB, r-bPiDDB and -CtxMII was determined in rats administered nicotine or saline repeatedly. To determine if these inhibitors evoked [³H]DA overflow (intrinsic activity), each striatal slice from an individual rat was superfused for 36 min in either the absence or presence of a single concentration of bPiDDB (1 nM – 10 μM), r-bPiDDB (10 pM – 1 μM) or -CtxMII (1 pM – 10 nM); each antagonist remained in the buffer throughout the experiment. Concentration ranges were chosen from previous studies [29,32]. Subsequently, nicotine (10 μM) was added to the buffer of each superfusion chamber for 36 min. Antagonist-induced inhibition of nicotine-evoked [³H]DA overflow was determined. At the end of each experiment, slices were solubilized, and the [³H]-content of the tissue and superfusate samples was determined using a Tri-Carb 2900 TR liquid scintillation counter (Perkin Elmer, Inc., Waltham MA).

To determine if r-bPiDDB interacts with -CtxMII-sensitive nAChRs, maximal inhibitory concentrations (1 nM) of -CtxMII, r-bPiDDB, and -CtxMII plus r-bPiDDB concurrently were superfused for 36 min in duplicate slices from either repeated nicotine-injected or non-injected rats. Then, nicotine (10 μM) was added to the buffer of all chambers and superfusion continued for an additional 36 min. Duplicate slices in each experiment were superfused for 36 min in the absence of antagonist, followed by superfusion with 10 μM nicotine (nicotine control). Duplicate slices also were superfused with maximally inhibitory concentrations of mecamylamine (10 μM), a nAChR antagonist at all known receptor subtypes, or dihydro- β -erythroidine (DH E; 10 μM), an α 4 β 2 antagonist; [37, 38] as positive controls.

2.4. Data analysis

To determine if repeated nicotine resulted in behavioral sensitization, a two-way repeated measures analysis of variance (ANOVA) was used to assess the effect of repeated nicotine on total distance travelled (cm), with nicotine treatment as a between-group factor and time as a within-subject factor. Significant interactions were assessed using one-way ANOVAs, and Tukey's *post hoc* analyses were used to make pairwise comparisons.

Fractional release was calculated by dividing the amount of [³H] in each 4-min sample by the total tissue-[³H] at the time of sample collection. Basal [³H]DA outflow was calculated as the average fractional release in the two samples just before addition of antagonist to the superfusion buffer. Antagonist- or nicotine-evoked total [³H]DA overflow was calculated by

summing the increases in fractional release above basal for an equivalent period of exposure. Nicotine and antagonist concentration response data were fit by nonlinear least-squares regression using a variable slope, sigmoidal function. IC_{50} and percent maximal inhibition (I_{max}) values were determined using Prism 5.0 (GraphPad Software Inc, San Diego, CA). Statistical analyses were conducted using SPSS (version 17.0; SPSS Inc, Chicago, IL). A two-way repeated measures ANOVA analyzed the effect of repeated nicotine on the *in vitro* nicotine concentration response in the [3 H]DA overflow assay, with repeated nicotine treatment as a between-group factor and *in vitro* nicotine concentration as a within-subject factor. Two-way repeated measures ANOVAs were also used to analyze the effect of repeated nicotine on the concentration response curves for bPiDDB-, r-bPiDDB- and -CtxMII-induced inhibition of nicotine-evoked [3 H]DA overflow, with repeated nicotine as a between-group factor and antagonist concentration as a within-subject factor. When appropriate, Tukey's *post hoc* analysis determined significant differences between the repeated nicotine and control groups across each antagonist concentration. To determine if nicotine treatment altered the parameters of antagonist-induced inhibition, Student's t-tests compared either log IC_{50} values or arithmetic I_{max} values between the nicotine treatment group and the control group.

To determine if r-bPiDDB acts at -CtxMII-sensitive nAChR subtypes, the inhibitory effect of concomitant r-bPiDDB and -CtxMII exposure was compared to r-bPiDDB alone and to -CtxMII alone using a one-way ANOVA with antagonist as a within-subject factor. When appropriate, Tukey's *post hoc* analyses were used to make pairwise comparisons. Statistical significance was declared at $p < 0.05$.

3. RESULTS

3.1. Inhibition produced by r-bPiDDB and α -CtxMII is not additive

Concomitant exposure to two antagonists acting at the same receptor would be expected to produce inhibition not different from either antagonist alone when maximally inhibitory concentrations are evaluated. To determine if r-bPiDDB interacts with -CtxMII-sensitive nAChRs, inhibition produced by concomitant exposure to maximally inhibitory concentrations (1 nM) of r-bPiDDB and -CtxMII were compared to inhibition produced by each antagonist alone. A one-way ANOVA revealed a significant effect of antagonist ($F_{4,17} = 23.8$, $p < 0.01$; Fig. 2). *Post hoc* analysis revealed that the nicotine-evoked [3 H]DA overflow in the presence of each antagonist was different from nicotine-evoked [3 H]DA overflow in the absence of antagonist. Mecamylamine inhibited almost completely ($93 \pm 3\%$) nicotine-evoked [3 H]DA overflow and produced greater inhibition than r-bPiDDB, -CtxMII or the combination. In separate experiments, striatal slices were exposed concomitantly to maximally inhibitory concentrations of -CtxMII (1 nM) plus DH E (10 μ M), an $\alpha 2$ antagonist, both alone and in combination (Fig. 3). One-way ANOVA revealed a significant effect of antagonist ($F_{4,25} = 11.88$; $p < 0.0001$). *Post hoc* analysis revealed that inhibition ($\sim 80\%$) produced by concomitant exposure to -CtxMII and DH E was greater than that produced by either antagonist alone. Further, concomitant exposure to -CtxMII and DH E was not different from inhibition produced by the nonselective antagonist mecamylamine. Thus, the nearly complete inhibition of nicotine-evoked [3 H]DA overflow produced by mecamylamine and by co-exposure to DH E and -CtxMII demonstrate that a floor effect is not responsible for the observation with r-bPiDDB and -CtxMII. Therefore, r-bPiDDB interacts with -CtxMII-sensitive nAChRs.

3.2. Repeated nicotine administration produces locomotor sensitization, but does not alter the concentration response for nicotine to evoke [³H]DA overflow from superfused rat striatal slices

A two-way repeated measures ANOVA revealed main effects of repeated nicotine treatment ($F_{1,198} = 5.80$, $p < 0.05$; Fig. 4) and time ($F_{1,198} = 5.44$, $p < 0.0001$) and a significant treatment x time interaction ($F_{9,198} = 8.86$, $p < 0.0001$) on locomotor activity. Simple effect analyses revealed that repeated nicotine-treated rats ($F_{9,110} = 3.34$, $p < 0.01$), but not repeated saline-treated rats, exhibited greater locomotor activity on days 7-10 compared to the first day of testing and that rats administered nicotine repeatedly exhibited greater locomotor activity on days 7-10 compared to saline control group. Thus, behavioral sensitization to nicotine was observed.

Striatal slices from rats repeatedly treated with nicotine were superfused with nicotine (0.1 – 100 μ M) and evoked [³H]DA overflow determined to assess the effects of repeated nicotine treatment on the *in vitro* nicotine concentration-response curve. Two-way repeated measures ANOVA revealed no main effect of treatment and no treatment x time interaction, but a main effect of nicotine concentration ($F_{6,78} = 26.0$, $p < 0.0001$; Fig. 5) was observed. Thus, repeated nicotine treatment did not alter the *in vitro* concentration response to nicotine. A concentration of 10 μ M nicotine was chosen for subsequent experiments determining antagonist-induced inhibition of nicotine-evoked [³H]DA overflow in rats repeatedly treated with nicotine.

3.3. r-bPiDDB interacts with α -CtxMII-sensitive nAChRs in nicotine-sensitized rats

To determine if repeated nicotine treatment alters the interaction of r-bPiDDB with α -6 2-containing nAChRs, inhibition produced by concomitant exposure to maximally inhibitory concentrations (1 nM) of r-bPiDDB and α -CtxMII were compared to inhibition produced by each antagonist alone using striatal slices from nicotine-sensitized rats. Similar to the findings in non-injected control rats, one-way ANOVA revealed a significant effect of antagonist ($F_{4,23} = 86.9$, $p < 0.0001$; Fig. 6), and *post hoc* analysis revealed that the nicotine-evoked [³H]DA overflow in the presence of antagonist was different from nicotine-evoked [³H]DA overflow in the absence of either antagonist. Mecamylamine inhibited nicotine-evoked [³H]DA overflow almost completely ($90 \pm 1\%$) and produced greater inhibition than either r-bPiDDB, α -CtxMII or the combination. However, concomitant exposure to r-bPiDDB and α -CtxMII resulted in inhibition of nicotine-evoked [³H]DA overflow not different from either antagonist alone. Thus, r-bPiDDB acts at the same receptors subtypes as α -CtxMII (i.e., α -6 2-containing), including in animals repeatedly treated with nicotine.

3.4. Repeated nicotine administration increases the potency for bPiDDB, but not for r-bPiDDB or α -CtxMII, to inhibit nicotine-evoked [³H]DA overflow from striatal slices

α -CtxMII, bPiDDB and r-bPiDDB inhibition of nicotine-evoked [³H]DA overflow from striatal slices obtained from rats treated repeatedly with nicotine or saline was analyzed using separate two-way repeated measures ANOVA. Initially, the α -6-containing nAChR subtype-selective antagonist α -CtxMII was evaluated. A main effect of α -CtxMII concentration ($F_{6,60} = 14.59$, $p < 0.0001$; Fig. 7, top) was found; however, no main effect of nicotine treatment or treatment x concentration interaction was observed. Using nonlinear regression, a sigmoidal function for α -CtxMII was revealed with IC_{50} values for nicotine-treated and control groups of 31.4 ± 15.1 and 26.1 ± 10.1 pM, respectively ($p > 0.05$). However, repeated nicotine significantly increased ($t_8 = 2.70$, $p < 0.05$) α -CtxMII-induced maximal inhibition ($90 \pm 7\%$) compared to control ($62 \pm 7\%$). Thus, while repeated nicotine treatment did not alter α -CtxMII potency, a greater percent of the response to nicotine *in vitro* was mediated by α -CtxMII-sensitive nAChRs.

For the *bis*-azaaromatic quaternary ammonium compound bPiDDB, a main effect of both nicotine treatment ($F_{1,60} = 5.44$, $p < 0.05$; Fig 7, middle) and bPiDDB concentration ($F_{6,60} = 21.0$, $p < 0.0001$) was observed; however, the interaction was not significant. Tukey's *post hoc* analysis revealed significant differences between the repeated nicotine treated and control groups at the 0.1 and 1 nM bPiDDB concentrations. Nonlinear regression revealed a sigmoidal function for bPiDDB in both nicotine-treated and control groups; the IC_{50} value in the nicotine-treated group was 3-orders of magnitude lower than in the saline control group ($IC_{50} = 5.27 \pm 1.51$ pM and 6.17 ± 2.24 nM, respectively; $t_6 = 2.75$, $p < 0.05$). No differences in I_{max} values ($76 \pm 3\%$ and $76 \pm 8\%$) were observed. Thus, repeated nicotine treatment robustly increased the potency of bPiDDB to inhibit nicotine-evoked striatal [3H]DA release from striatum.

For the tertiary amine analog of bPiDDB, r-bPiDDB, a main effect of concentration ($F_{6,60} = 15.16$, $p < 0.0001$; Fig. 7, bottom) was observed, but neither the main effect of nicotine treatment, nor the treatment $\times$ concentration interaction were significant. Nonlinear regression revealed a sigmoidal function for r-bPiDDB with IC_{50} values for nicotine-treated and control groups of 0.08 ± 0.03 and 0.15 ± 0.07 nM, respectively ($p > 0.05$). I_{max} values ($64 \pm 10\%$ and $67 \pm 13\%$) were not different between the groups. Thus, repeated nicotine treatment did not alter the concentration response for r-bPiDDB to inhibit nicotine-evoked [3H]DA overflow.

4. DISCUSSION

Our previous studies showed that bPiDDB, a novel *bis*-azaaromatic quaternary ammonium analog, potently ($IC_{50} = 2$ nM) inhibits nicotine-evoked [3H]DA release from rat striatal slices via an interaction with $\alpha 6$ 2-containing nAChRs [29]. Peripheral administration of bPiDDB to rats was shown to inhibit nicotine-evoked DA release in nucleus accumbens using *in vivo* microdialysis [30] and to decrease intravenous nicotine self-administration [31]. bPiDDB is brain bioavailable following peripheral administration and is transported actively by the blood-brain barrier choline transporter into the central compartment [39]. After subcutaneous administration of behaviorally relevant doses of bPiDDB to rats, bPiDDB has been detected in brain as shown by both mass spectrometry and HPLC with radiometric analysis [40]. Nevertheless, bPiDDB is a polar, cationic compound with limited oral bioavailability; thus, the drug-like properties of bPiDDB as defined in the literature [41] remain an issue with respect to its therapeutic utility.

To improve the drug-like properties of bPiDDB, a simple chemical reduction was performed to convert the two quaternary ammonium moieties (i.e., 3-picolinium headgroups) into tertiary amino moieties (i.e., 3-methyl-1,2,5,6-tetrahydropyridines), yielding r-bPiDDB. r-bPiDDB is a highly lipophilic molecule with predicted physicochemical properties of a logP value of 5, pKa of 9 and a MW of 434 Daltons, suggesting good oral bioavailability [42]. Similar to bPiDDB, r-bPiDDB inhibits nicotine-evoked [3H]DA release from superfused rat striatal slices, but with a 10-fold higher potency ($IC_{50} = 0.3$ nM; [32]). The current results demonstrate that inhibition of nicotine evoked [3H]DA release produced by a maximally inhibitory concentration of r-bPiDDB is not different from that produced by concomitant exposure to maximally inhibitory concentrations of r-bPiDDB and α -CtxMII, indicating that r-bPiDDB interacts with $\alpha 6$ 2-containing nAChRs. Thus, r-bPiDDB appears to retain the pharmacological properties of the parent compound, bPiDDB.

Importantly, the nonselective, noncompetitive nAChR antagonist mecamylamine inhibited nicotine-evoked DA release by $\sim 90\%$. Thus, the incomplete inhibition produced by r-bPiDDB plus α -CtxMII was not the result of a "floor" effect. Additionally, the $\sim 80\%$ inhibition produced by co-exposure to maximally inhibitory concentrations of DH E and α -

CtxMII provides evidence that nearly complete inhibition of the effect of nicotine is obtained when an $\alpha 4 \beta 2$ antagonist and an $\alpha 6 \beta 2$ antagonist act in concert. Thus, the current results support the suggestion that r-bPiDDB is selective for $\alpha 6 \beta 2$ -containing nAChR subtypes.

Individuals undergoing treatment for smoking cessation will have been exposed to nicotine repeatedly prior to the initiation of cessation treatment. Taking this into account, the current study determined both the ability of nicotine to evoke [3 H]DA release and the inhibitory effects of the nAChR antagonists using striatum from rats repeatedly administered nicotine (0.4 mg/kg daily for 10 days). The concentration response for nicotine to evoke [3 H]DA release from striatal slices obtained from rats injected with nicotine repeatedly was not different from that generated following repeated saline. Thus, the *in vitro* response to nicotine was not altered in striatum despite the dynamic neuronal adaptation that occurred following repeated nicotine treatment, as evidenced by the expression of behavioral sensitization. These findings are in agreement with earlier studies demonstrating that nicotine-evoked striatal DA release, either *in vitro* or *in vivo*, is not altered following chronic nicotine (0.125 – 1.0 mg/kg/hr) administered via osmotic minipump for 10 or 14 days [43,44]. However, these results are not consistent with studies showing that nicotine-evoked DA release is either increased [25,45] or decreased [23,25,46] following chronic nicotine administration. These discrepant findings are likely due to differences in experimental design and the parameters used (e.g., length of nicotine administration, animal models employed, tissue preparation utilized, brain region assessed). These discrepancies could be resolved through parametric analyses of the effect of repeated nicotine administration on subsequent nicotine-evoked DA release. For example, parametric analyses would require the evaluation of the same repeated nicotine dose and route of administration, employment of the same brain region and tissue preparation in several species, followed by determination of the *in vitro* concentration-response for nicotine-evoked DA release.

In the current study, maximum inhibition produced by $\alpha 6 \beta 2$ -CtxMII was significantly greater in repeated nicotine-treated rats compared to control rats ($I_{\max} = 90\%$ and 62% , respectively). This increase in $I_{\max}$ for $\alpha 6 \beta 2$ -CtxMII between the repeated nicotine group and the control group was observed in different series of experiments using nicotine-sensitized and non-injected control rats evaluated at different times, as well as when striata from nicotine-sensitized and saline-control rats were evaluated contemporaneously. In contrast, maximum inhibition produced by either r-bPiDDB or by the concurrent administration of r-bPiDDB and $\alpha 6 \beta 2$ -CtxMII was not different between the repeated-nicotine group and the non-injected controls. The increase in $\alpha 6 \beta 2$ -CtxMII $I_{\max}$ may be attributed to an up-regulation of nAChRs. In contrast to this interpretation, binding studies using [125 I] $\alpha 6 \beta 2$ -CtxMII to label $\alpha 6 \beta 2$ -containing nAChRs consistently report decreased (36-50%) receptor number in striatum following repeated nicotine administration [24,25,47]. However, [125 I] $\alpha 6 \beta 2$ -CtxMII probes multiple subtypes of $\alpha 6 \beta 2$ -containing nAChRs, i.e., $\alpha 6 \beta 2$ -, $\alpha 6 \beta 2 \gamma$ -, $\alpha 6 \beta 4 \gamma$ - and $\alpha 6 \beta 4 \gamma 2$ -containing subtypes [13,14]. To determine if specific $\alpha 6 \beta 2$ -containing subtypes are regulated by repeated nicotine administration, the $\alpha 6 \beta 4 \gamma 2$ -CtxMII analog E11A, which is an $\alpha 6 \beta 4 \gamma 2$ subtype-selective antagonist, was used to differentiate between subtypes containing both $\alpha 6$ and $\alpha 4$ subunits (i.e., $\alpha 6 \beta 4 \gamma 2$ and $\alpha 6 \beta 4 \gamma 2 \delta$) and those $\alpha 6 \beta 2$ -containing subtypes which do not contain $\alpha 4$ subunits (i.e., $\alpha 6 \beta 2$ and $\alpha 6 \beta 2 \gamma$) [25]. Repeated nicotine was reported to down regulate $\alpha 6 \beta 4 \gamma 2$ -containing nAChRs by 50% and up-regulate $\alpha 6$ (non- $\alpha 4$) $\beta 2$ -containing nAChRs by ~25%. Thus, the relative increase (~25%) in $\alpha 6$ (non- $\alpha 4$) $\beta 2$ -containing nAChRs [25] is consistent with the observed increase (28%) in $\alpha 6 \beta 2$ -CtxMII inhibition of nicotine-evoked [3 H]DA release from striatum from nicotine-sensitized rats. The observed increase in $I_{\max}$ occurred without a change in IC_{50} , suggesting that the affinity of $\alpha 6 \beta 2$ -CtxMII for these $\alpha 6$ (non- $\alpha 4$) $\beta 2$ receptors is not changed.

In contrast to the results with α -CtxMII, the concentration response for bPiDDB to inhibit nicotine-evoked [3 H]DA release was shifted to the left by three-orders of magnitude ($IC_{50} = 5.27$ pM) in the nicotine sensitized rats compared to control ($IC_{50} = 6.17$ nM), with no change in I_{max} . With r-bPiDDB, neither the IC_{50} nor the I_{max} were altered following repeated nicotine treatment. Of note, r-bPiDDB has high potency in both the nicotine sensitized and control conditions. Thus, unlike α -CtxMII, neither bPiDDB nor r-bPiDDB showed an increase in I_{max} following repeated nicotine treatment. One potential interpretation is that bPiDDB and r-bPiDDB do not act at $\alpha 6$ (non- $\alpha 4$) 2 -containing nAChRs, since I_{max} was not increased after repeated nicotine. Furthermore, previous studies have shown that subtypes incorporating $\alpha 3$ subunits are resistant to regulation of receptor number by chronic nicotine treatment [24]. Since the concentration response for r-bPiDDB was not altered following repeated nicotine, this antagonist may act at the $\alpha 6 \alpha 4 \alpha 2 \alpha 3$ -containing subtype, which has been suggested to comprise ~50% of nAChRs on DA terminals in striatum and to be the most sensitive of the nAChR subtypes to nicotine [14,15].

With respect to bPiDDB and the observed 3-orders of magnitude shift in the concentration response following repeated nicotine treatment, several interpretations of these results are possible. Repeated nicotine treatment alters nAChR stoichiometry, conformation and composition [19], and long term exposure to nicotine alters nAChR maturation by increasing nAChR subunit oligomerization and folding [17,48]. In addition, nicotine alters protein kinase A- or C-induced phosphorylation of specific amino acid residues on the nAChR complex, an effect that modulates the sensitivity of nAChRs to ligands [49,50]. Thus, one interpretation is that bPiDDB acts more potently at the particular stoichiometry, conformation, composition and/or phosphorylated state of nAChR(s) that mediate nicotine-evoked DA release and that predominate following repeated nicotine administration. Alternatively, repeated nicotine treatment is thought to initiate the transformation of nAChRs through different receptor states, and large shifts in ligand affinity can accompany these changes in receptor state [49-52]. Thus, rather than interacting with an alternate subtype composition following repeated nicotine, another interpretation is that bPiDDB may stabilize or “lock” the specific receptor subtype(s) into a high affinity state of the receptor that remains “activatable”. Although not clearly defined, such a mechanism would be consistent with the large magnitude of the shift in potency for bPiDDB when evaluated following repeated nicotine.

In summary, bPiDDB and r-bPiDDB represent novel small molecules that potently inhibit nicotine-evoked DA release and appear to discriminate between subpopulations of $\alpha 6 \alpha 2$ -containing nAChR subtypes. Importantly, both compounds have been shown to decrease nicotine self-administration in an animal model, suggesting that these compounds are not only leads in the search for novel smoking cessation agents, but may represent novel pharmacological tools for unraveling the complexity and diversity of nAChR effects.

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Nonstandard Abbreviations

nAChR	nicotinic acetylcholine receptor
DA	dopamine
NIC	nicotine

SAL	saline
MEC	mecamylamine
DH E	dihydro- α -erythroidine
-CtxMII	-conotoxin MII
bPiDDB	<i>N,N'</i> -dodecane-1,12-diyl- <i>bis</i> -3-picolinium dibromide
r-bPiDDB	1,12- <i>bis</i> (3-methyl-1,2,5,6-tetrahydropyridinyl)dodecane
ANOVA	analysis of variance

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α -CtxMII

Gly-Cys-Cys-Ser-Asn-Pro-Val-Cys-His-Leu-Glu-His-Ser-Asn-Leu-Cys

bPiDDB **r-bPiDDB**

Fig. 1.

The amino acid sequence for α -conotoxin MII (α -CtxMII; top) and the structures of *N,N'*-dodecane-1,12-diyl-*bis*-picolinium dibromide (*bPiDDB*; middle) and reduced-*bPiDDB* (*r-bPiDDB*; bottom)

Fig. 2. Concomitant exposure to r-bPiDDB and α-CtxMII inhibits nicotine-evoked [³H]DA overflow compared to control, but is not different from either antagonist alone
Control nicotine-evoked [³H]DA overflow (in the absence of antagonist) was 1.06 ± 0.29 total [³H]DA overflow (mean $\pm$ SEM). * $p < 0.05$, indicates mecamylamine (10 μ M) different from inhibition produced by all antagonists; $n = 4$.

Fig. 3. Inhibition of nicotine (10 μ M)-evoked [³H]DA release produced by concomitant exposure to α -CtxMII (1 nM) and DH β E (10 μ M) was greater than that produced by either drug alone. Control nicotine-evoked [³H]DA overflow (in the absence of antagonist) was 1.69 ± 0.31 total [³H]DA overflow (mean $\pm$ SEM). * $p < .05$, indicates different from inhibition produced by α -CtxMII and DH β E alone; $n = 6$.

Fig. 4. Repeated nicotine treatment produces behavioral sensitization

Rats were injected with nicotine (0.4 mg/kg/day; sc) or saline for 10 days. Activity was measured as total distance traveled (cm) measured during a 60-min session immediately following each injection. Data are mean $\pm$ SEM total distance traveled (cm). * $p < 0.05$, indicates different from activity on the first day of testing, and different from the control group on the indicated day; $n = 12$ /group.

Fig. 5. Concentration response for nicotine-evoked $[^3\text{H}]\text{DA}$ overflow from striatal slices taken from rats treated repeatedly with nicotine or saline

Rats were injected (sc) with 0.4 mg/kg/day of nicotine or saline for 10 days. Twenty-four hr after the last injection, striata were obtained and nicotine concentration response was determined. Buffer represents $[^3\text{H}]\text{DA}$ overflow in the absence of nicotine. Data are mean $\pm$ SEM total $[^3\text{H}]\text{DA}$ overflow; $n = 8$ and 7 rats for repeated nicotine-treated and control groups, respectively. Concentration response curves were generated using nonlinear regression.

Fig. 6. In repeated nicotine-treated rats, concomitant exposure of r-bPiDDB and α-CtxMII inhibits nicotine-evoked [³H]DA overflow compared to control, but is not different from either antagonist alone

Rats were injected with nicotine (0.4 mg/kg/day, sc) or saline for 10 days and striata obtained 24 hr after the last injection. Control nicotine-evoked [³H]DA overflow (in the absence of antagonist) was 0.73 ± 0.16 (mean $\pm$ SEM). * $p < 0.05$, indicates mecamylamine (10 μ M) different from inhibition produced by all antagonists; $n = 5$.

Fig. 7. Concentration response for α -CtxMII (top), bPiDDB (middle) and r-bPiDDB (bottom) to inhibit nicotine-evoked $[^3\text{H}]\text{DA}$ overflow from striatal slices obtained from rats repeatedly treated with nicotine or saline

Rats were injected (sc) with nicotine (0.4 mg/kg/day) or saline for 10 days and striata obtained 24 hr after the last injection. Control represents $[^3\text{H}]\text{DA}$ overflow in response to 10 μM nicotine (total $[^3\text{H}]\text{DA}$ overflow as a percentage of tissue $[^3\text{H}]$ -content, mean $\pm$ S.E.M.). Data are expressed as percentage of control; $n = 6/\text{group}$. For nicotine- and saline-treated groups, nicotine control response *in vitro* (in the absence of antagonist) was 0.71 ± 0.09 and 0.66 ± 0.12 total $[^3\text{H}]\text{DA}$ overflow in α -CtxMII experiments, 1.16 ± 0.14 and 1.08 ± 0.20 total $[^3\text{H}]\text{DA}$ overflow, respectively in bPiDDB experiments, and 0.61 ± 0.12 and 0.68 ± 0.16 total $[^3\text{H}]\text{DA}$ overflow in r-bPiDDB experiments. No significant differences between

control responses within or between experiments were observed. Concentration response curves were generated by nonlinear regression.