N-n-Alkylpyridinium Analogs, A Novel Class of Nicotinic Receptor Antagonists:
Selective Inhibition of Nicotine-Evoked [³H]Dopamine Overflow
from Superfused Rat Striatal Slices

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Abbreviations:
DA, dopamine; nAChR, neuronal nicotinic acetylcholine receptor; NBuPI, N-n-butylpyridinium iodide; NDNI, N-n-decynicotinium iodide; NDDNI, N-n-dodecynicotinium iodide; NDPI, N-n-decylpyridinium iodide; NDDPI, N-n-dodecylpyridinium iodide; NEcPB, N-n-eicosylpyridinium bromide; NEPI, N-ethylpyridinium iodide; NHPI, N-n-heptylpyridinium iodide; NHxPI, N-n-hexylpyridinium iodide; NMPI, N-methylpyridinium iodide; NNPI, N-n-nonylpyridinium iodide; NOPI, N-n-octylpyridinium iodide; NPDPB, N-n-pentadecylpyridinium bromide; NPI, N-n-pentylpyridinium iodide; NPrPI, N-n-propylpyridinium iodide; NUPI, N-n-undecylpyridinium iodide; ANOVA, analysis of variance.

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ABSTRACT

Structural simplification of $N$-$n$-alkylnicotinium analogs, antagonists at neuronal nicotinic acetylcholine receptors (nAChRs), was achieved by removal of the $N$-methylpyrrolidino moiety affording $N$-$n$-alkylpyridinium analogs with carbon chain lengths of C$_1$ to C$_{20}$. $N$-$n$-Alkylpyridinium analog inhibition of [$^3$H]nicotine and [$^3$H]methyllycaconitine binding to rat brain membranes assessed interaction with $\alpha 4\beta 2^*$ and $\alpha 7^*$ nAChRs, respectively; whereas, inhibition of nicotine-evoked [$^3$H]overflow from [$^3$H]dopamine ([$^3$H]DA)-preloaded rat striatal slices assessed antagonist action at nAChR subtypes mediating nicotine-evoked DA release. No inhibition of [$^3$H]methyllycaconitine binding was observed, although $N$-$n$-alkylpyridinium analogs had low affinity for [$^3$H]nicotine binding sites, i.e., 1-3 orders of magnitude lower than that of the respective $N$-$n$-alkylnicotinium analogs. These results indicate that the $N$-methylpyrrolidino moiety in the $N$-$n$-alkylnicotinium analogs is a structural requirement for potent inhibition of $\alpha 4\beta 2^*$ nAChRs. Importantly, $N$-$n$-alkylpyridinium analogs with $n$-alkyl chains $< C_{10}$ did not inhibit nicotine-evoked [$^3$H]DA overflow; whereas, analogs with $n$-alkyl chains ranging from C$_{10}$-C$_{20}$ potently and completely inhibited nicotine-evoked [$^3$H]DA overflow (IC$_{50}$= 0.12-0.49 µM), with the exceptions of $N$-$n$-pentadecylpyridinium bromide (C$_{15}$) and $N$-$n$-eicosylpyridinium bromide (C$_{20}$), which exhibited maximal inhibition of ~50%. The mechanism of inhibition of a representative analog of this structural series, $N$-$n$-dodecylpyridinium iodide, was determined by Schild analysis. Linear Schild regression with slope not different from unity indicated competitive antagonism at nAChRs mediating nicotine-evoked [$^3$H]DA overflow and a $K_B$ value of 0.17 µM. Thus, the simplified $N$-$n$-alkylpyridinium analogs are potent, selective and competitive antagonists of nAChRs mediating nicotine-evoked [$^3$H]DA overflow, indicating that the $N$-methylpyrrolidino moiety is not a structural requirement for interaction with nAChR subtypes mediating nicotine-evoked DA release.
INTRODUCTION

Nicotine (Fig. 1, Structure 1) activates neuronal nicotinic acetylcholine receptors (nAChRs), which are members of a ligand-gated ion channel family, consisting of transmembrane pentameric proteins with diverse composition (Le Novère et al., 2002). Twelve genes encode α2-α10 and β2-β4 nAChR subunits, and in situ hybridization reveals their discrete, but overlapping, CNS distribution (Wada et al., 1989; Dineley-Miller and Patrick, 1992). Individual neurons elaborate multiple nAChR subtypes, and more than two different subunits can assemble forming functional nAChRs (Forsayeth and Kobrin, 1997; Zoli et al., 2002), greatly increasing the complexity of nAChR pharmacology. Thus, nAChR subtype diversity originates from differences in amino acid sequences of subunit proteins and from multiple combinations of subunit assemblies forming functional nAChRs. The exact subunit composition, stoichiometry and arrangement of native nAChRs remain to be elucidated (Lukas et al., 1999).

Although predominance does not necessarily reflect functional importance, the α4β2* subtype, probed by high affinity [3H]nicotine binding predominates in brain. Greater than 90% of [3H]nicotine binding sites are immunoprecipitated with anti-β2 antibody (Whiting and Lindstrom, 1987; Flores et al., 1992), and β2-knockout mice do not exhibit high affinity [3H]nicotine binding (Zoli et al., 1998). Homomeric α7 nAChRs, probed by [3H]methyllycaconitine ([3H]MLA, Fig. 1, Structure 4) binding (Davies et al., 1999), are also abundant in brain (Wada et al., 1989; Flores et al., 1992).

nAChRs are preferentially located presynaptically and modulate neurotransmitter release (McGehee and Role, 1995; Wonnacott, 1997). nAChRs are located on the soma and terminals of substantia nigra dopamine (DA) neurons (Wonnacott, 1997), and nicotine evokes DA release in striatum (Teng et al., 1997). Subtype assignment of native nAChRs mediating nicotine-evoked DA release is
based on several experimental approaches, including inhibition of agonist-induced response by subtype-selective nAChR antagonists, which are defined by inhibitory activity in cell systems expressing nAChR subunits of known composition, by results from studies using nAChR-subunit knockout mice, and by in situ hybridization and single cell PCR of mRNA in nigral neurons. Results from these current experimental approaches have generated considerable controversy regarding the exact subunit composition of nAChR subtypes mediating nicotine-evoked DA release (Klink et al., 2001; Azam et al., 2002; Champtiaux et al., 2002; Whiteaker et al., 2002; Zoli et al., 2002), and great effort is focused currently on elucidating the specific nAChR subtypes involved in this response.

Increased understanding of the structural and functional diversity of nAChRs has stimulated great interest in the development of subtype-selective nAChR agonists; however, less attention has focused on development of subtype-selective nAChR antagonists (Dwoskin et al., 2000; Dwoskin and Crooks, 2001). Pharmacophore geometries for antagonist binding sites are ill-defined, and very few subtype-selective nAChR antagonists are available currently. Notwithstanding the complexity and controversy concerning the composition of nAChR subtypes mediating nicotine-evoked DA release, the development of subtype-selective antagonists and the identification of antagonist pharmacophores at these nAChRs will provide an arsenal of pharmacological agents to further our understanding of subtype compositions mediating nicotine-evoked DA release, as well as unraveling the complexity of the effect of nicotine on this response. Additionally, these subtype-selective antagonists may find utility as therapeutic agents in the treatment of neurological diseases associated with cholinergic modulation of dopaminergic neurotransmission.

Our previous studies show that structural modification of the nicotine molecule affords a series of N-n-alkylnicotinium analogs (Fig. 1, Structure 2), exhibiting high affinity and selectivity as competitive antagonists at nAChRs. N-n-Octylnicotinium iodide (NONI; Fig. 1, Structure 2a) competitively inhibits nicotine-evoked [3H]DA overflow from rat striatal slices, without inhibiting [3H]nicotine binding to
striatal membranes (Wilkins et al., 2002, 2003a). *N*-n-Decylnicotinium iodide (NDNI; Fig 1, Structure 2b) is a potent and competitive inhibitor of [³H]nicotine binding and ⁸⁶Rb⁺ efflux; however, NDNI does not inhibit nicotine-evoked [³H]DA release (Wilkins et al., 2002, 2003a, 2003b). Furthermore, these analogs do not exhibit affinity for α7* nAChRs, assessed using [³H]MLA binding. Thus, NONI and NDNI are relatively selective and potent nAChR antagonists, but they act at different nAChR subtypes.

The purpose of the present study was to begin to elucidate the structural requirements for this class of *N*-n-alkynicotinium nAChR antagonists. The initial strategy was structural simplification by removal of the *N*-methylpyrrolidino moiety to afford *N*-n-alkylpyridinium analogs (Fig. 1, Structure 3), which were assessed for their activity at α4β2* and α7* nAChR subtypes, and at nAChR subtypes mediating nicotine-evoked DA release.
MATERIALS AND METHODS

Chemicals. *N*-*n*-Alkylpyridinium analogs were synthesized *via* direct *N*-alkylation of pyridine utilizing the method of Ayers et al., (2002). Structures of these *N*-*n*-alkylpyridinium analogs are shown in Fig. 1. Iodide salts of analogs with *n*-alkyl chain lengths varying from C1 to C12 were prepared, whereas C15 and C20 analogs were prepared as bromide salts: *N*-methylpyridinium iodide (C1, NMPI; Fig 1, Structure 3a), *N*-ethylpyridinium iodide (C2, NEPI; Structure 3b), *N*-propylpyridinium iodide (C3, NPrPI; Structure 3c), *N*-butylpyridinium iodide (C4, NBuPI; Structure 3d), *N*-pentylpyridinium iodide (C5, NPPI; Structure 3e), *N*-hexylpyridinium iodide (C6, NHxPI; Structure 3f), *N*-heptylpyridinium iodide (C7, NHPI; Structure 3g), *N*-octylpyridinium iodide (C8, NOPI; Structure 3h), *N*-nonylpyridinium iodide (C9, NNPI; Structure 3i), *N*-decylpyridinium iodide (C10, NDPI; Structure 3j), *N*-undecylpyridinium iodide (C11, NUPI; Structure 3k), *N*-dodecylpyridinium iodide (C12, NDDPI; Structure 3l), *N*-pentadecylpyridinium bromide (C15, NPDPB; Structure 3m) and *N*-eicosylpyridinium bromide (C20, NEcPB; Structure 3n). Structures of compounds were confirmed by 1H- and 13C- nuclear magnetic resonance and fast atom bombardment mass spectrometry. Analogs were also characterized by elemental analysis and afforded values that did not deviate by ± 0.4% of theoretical values.

[3H]Nicotine (S-(−)-[methyl-3H]-nicotine; specific activity, 81.5 Ci/mmol) and [3H]DA (3,4-ethyl-2-[N-3H]-dihydroxyphenylethylamine; specific activity, 27.1 Ci/mmol) were purchased from PerkinElmer Life Science (Boston, MA). [3H]Methyllycaconitine ([1α,4(S),6β,14α,16β]-20-ethyl-1,6,14,16-tetramethoxy-4-[[2-[[3-3H]-methyl-2,5-dioxo-1-pyrrolidinyl]benzoyloxy]methyl]aconitane-7,8-diol; [3H]MLA; specific activity, 25.4 Ci/mmol) and cold MLA were purchased from Tocris Cookson Ltd. (Bristol, UK). S-(−)-Nicotine di–d-tartrate and nomifensine maleate were purchased from Sigma/RBI (Natick, MA). Pargyline HCl, α-D-glucose, N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid] (HEPES), tris-[hydroxymethyl]aminomethane hydrochloride (Trizma HCl), tris-
[hydroxymethyl]aminoethane (Trizma base), polyethylenimine (PEI) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). TS-2 tissue solubilizer was purchased from Research Products International (Mount Prospect, IL). Other chemical components used in the preparation of the binding and release assay buffers were purchased from Fisher Scientific (Pittsburgh, PA). Chemicals and solvents used in the synthetic procedures were obtained from Aldrich Chemical Co. (Milwaukee, WI).

Subjects. Male Sprague-Dawley rats (220-250 g) were obtained from Harlan Laboratories (Indianapolis, IN) and were maintained on a 12/12-hr light/dark cycle with two rats per cage and free access to food and water in the Division of Lab Animal Resources at the College of Pharmacy at the University of Kentucky. Experimental protocols involving the animals were in accordance with the National Institutes of Health 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.

[^3H]Nicotine Competition Binding Assay. The procedures for the binding assay were a modification of a previously described method (Wilkins et al., 2003a). Striatum was dissected, frozen and stored at -70°C. Striata from two rats were pooled and homogenized with a Tekmar polytron homogenizer (setting 40; Tekmar, Cincinnati, OH), in 10 volumes of ice-cold 20 mM Krebs-HEPES buffer, pH 7.5, containing 118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl₂ and 1.2 mM MgSO₄. Homogenate was incubated at 37°C for 5 min, then cooled to 4°C, and subsequently centrifuged (25,000g for 20 min at 4°C). The pellet was resuspended in 10 volumes ice-cold Milli-Q water, incubated at 37°C for 5 min and centrifuged (25,000g for 20 min at 4°C). The previous incubation and centrifugation steps were repeated twice using 10 volumes of ice-cold 10% Krebs-HEPES buffer. The final pellet was stored in 10% Krebs-HEPES buffer at -70°C until assay. Upon assay, the final pellet was resuspended in 2.0 ml ice-cold Milli-Q water to obtain a final protein concentration of 150-200 µg/100 µl of membrane suspension,
determined using the Bradford dye-binding method (Bradford, 1976) using bovine gamma globulin as the standard.

Competition binding experiments were performed at 4°C in a final volume of 200 µl of incubation buffer, containing 20 mM Krebs-HEPES buffer and 200 mM Tris buffer (pH 7.5). Reactions were initiated by addition of 100 µl of membrane suspension to duplicate tubes containing one of nine concentrations of analog (1.0 nM – 1.0 mM, final concentration) and one concentration of [3H]nicotine (3 nM, final concentration), which was based on the observed Kd value (1.3 nM) for [3H]nicotine from preliminary experiments. Nonspecific binding was determined in the presence of 10 µM nicotine. The reaction was terminated after a 90-min incubation period by dilution of the samples with 3 ml of ice-cold 20 mM Krebs-HEPES buffer followed by immediate filtration through glass fiber filters (Schleicher & Schuell grade #32, Keene, NH) presoaked in 0.5% PEI using a Brandel harvester (Biomedical Research and Development Lab. Inc., Gaithersburg, MD). Filters were rinsed 3 times with 3 ml of ice-cold 20 mM Krebs-HEPES buffer, transferred to vials and scintillation cocktail (4 ml) added. Radioactivity was determined using a Tri-Carb 2100 TR Liquid Scintillation Analyzer (Packard Instrument Co., Meriden, CT).

[3H]MLA Binding Assay. The [3H]MLA binding assay was performed using previously described methods (Xu et al., 2002; Wilkins et al., 2003a). Briefly, whole rat brain (minus cortex, striatum and cerebellum) was homogenized in 20 volumes of ice-cold hypotonic buffer containing 2 mM HEPES, 14.4 mM NaCl, 0.15 mM KCl, 0.2 mM CaCl2 and 0.1 mM MgSO4 (pH 7.5). Homogenate was incubated at 37°C for 10 min and centrifuged (25,000g for 15 min at 4°C). Pellet was washed 3 times by resuspension in 20 volumes of the same buffer and centrifuged using the above parameters. Final pellet was resuspended in the incubation buffer to yield ~150 µg protein/100 µl membrane suspension. Protein concentration was determined as previously described.
Binding assays were performed in duplicate, in a final volume of 250 µl of incubation buffer, containing 20 mM HEPES, 144 mM NaCl, 1.5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄ and 0.05% bovine serum albumin (pH 7.5). Binding assays were initiated by the addition of 100 µl of membrane suspension (~150 µg protein) to samples containing one of seven concentrations of analog (1.0 nM – 1.0 mM, final concentration) and [³H]MLA (2.5 nM, final concentration), and incubated for 2 h at room temperature. Nonspecific binding was determined in the presence of 10 µM MLA. Binding assays were terminated and radioactivity determined as previously described for the [³H]nicotine binding assay.

[³H]DA Overflow Assay. [³H]DA overflow from superfused rat striatal slices was determined using previously published methods (Dwoskin and Zahniser, 1986; Teng et al., 1997; Wilkins et al., 2002). Briefly, coronal striatal slices (500 µm thickness, 6-8 mg) were incubated in Krebs’ buffer (containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl, 1.3 mM CaCl₂, 1.0 mM NaH₂PO₄, 11.1 mM glucose, 25 mM NaHCO₃, 0.11 mM L-ascorbic acid and 0.004 mM EDTA, pH 7.4, saturated with 95% O₂/5% CO₂) in a metabolic shaker for 30 min at 34°C. Then, slices (6-8 slices/3 ml) were incubated with fresh buffer containing [³H]DA (0.1 µM, final concentration) for an additional 30 min. After rinsing in fresh buffer, each slice was transferred to a superfusion chamber and superfused at a flow rate of 1 ml/min with Krebs’ buffer saturated with 95% O₂/5% CO₂. Superfusion buffer contained 10 µM nomifensine (a DA uptake inhibitor) and 10 µM pargyline (a monoamine oxidase inhibitor). After 60 min of superfusion, three 5-min samples (5 ml/sample) were collected to determine basal [³H]outflow. After collection of the third basal sample, each striatal slice from an individual rat was superfused for 60 min in either the absence or presence of one of six concentrations of analog (1 nM – 0.1 mM) to determine analog-induced intrinsic activity (i.e., ability of analog to evoke [³H]overflow). Each slice was exposed to only one concentration of analog, which remained in the buffer throughout the experiment. Following 60 min of superfusion in the absence or presence of analog, two 5-min samples were collected to assess
basal [3H]outflow. Subsequently, nicotine (10 µM) was added to the buffer, and 5-min superfusate samples were collected for a period of 60 min to determine analog-induced inhibition of nicotine-evoked [3H]DA overflow. A control striatal slice in each experiment was superfused for 60 min in the absence of analog, followed by superfusion with nicotine (10 µM) to determine nicotine-evoked total [3H]DA overflow. Furthermore, each striatal slice was exposed to only one concentration of analog. Thus, a repeated measures design was utilized to determine analog-induced intrinsic activity and analog-induced inhibition of nicotine-evoked [3H]DA overflow using striatal slices from a single rat; the effect of each analog was determined using a group of 5-6 rats. At the end of the experiment, slices were solubilized in 1.0 ml TS-2 tissue solubilizer, and the pH and volume of the solubilized samples were adjusted to those of the superfusate samples. Radioactivity was determined by liquid scintillation spectroscopy.

Another series of experiments determined the ability of mecamylamine (10 µM) to inhibit the intrinsic activity produced by a representative analog of this structural series, NDDPI, at a concentration (10 µM) found to produce significant intrinsic activity in the above experiments. Experiments were performed as described above except that after collection of the third basal sample, each striatal slice from an individual rat was superfused for 60 min in either the absence or presence of mecamylamine, which remained in the buffer throughout the experiment. Following 60 min of superfusion in the absence or presence of mecamylamine, NDDPI (10 µM) was added to the buffer, and 5-min superfusate samples were collected for a period of 60 min to determine mecamylamine-induced inhibition of NDDPI-evoked [3H]DA overflow. A control striatal slice in each experiment was superfused for 60 min in the absence of mecamylamine, followed by superfusion with NDDPI (10 µM) to determine NDDPI-evoked total [3H]DA overflow. Another control slice was superfused for the entire period in the absence of either mecamylamine or NDDPI. Thus, a repeated measures design was used, and the effect of NDDPI was determined using a group of 6 rats.
The mechanism of inhibition of NDDPI was determined by Schild analysis. In each experiment, the concentration-response for nicotine (1 nM – 100 µM) was determined in the absence and presence of a single concentration of NDDPI using striatal slices from a single rat. NDDPI inhibition of the effect of nicotine was determined at 3 concentrations of NDDPI (0.1, 0.3 or 0.6 µM), based on the previously determined IC$_{50}$ value for NDDPI-induced inhibition of nicotine (10 µM)-evoked $[3^H]$overflow. Slices were superfused for 60 min with buffer containing pargyline and nomifensine. Subsequently, slices were superfused in the absence or presence of a single concentration of NDDPI, which remained in the buffer throughout the experiment. After 60 min of superfusion in the absence or presence of NDDPI, one of six concentrations of nicotine (1 nM – 100 µM) was added to the buffer and superfusion continued for an additional 60 min. Each slice from a single rat was exposed to only one concentration of nicotine and one concentration of NDDPI. These experiments utilized a repeated measures design, such that the concentration-response for nicotine was determined using striatum from a single rat; and NDDPI concentration was a between-group factor. Tissue and superfusate samples were processed as previously described.

To assess the selectivity of NDDPI-induced inhibition of the effect of nicotine, the ability of NDDPI (1 nM – 1 µM) to inhibit electrical field stimulation-evoked $[3^H]$overflow was determined. Striatal slices were preloaded with $[3^H]$DA, transferred to superfusion chambers and superfused as previously described. After 60 min of superfusion, three 5-min samples were collected to determine basal $[3^H]$outflow, and subsequently, slices were superfused for 60 min in the absence or presence of NDDPI, which remained in the buffer until the end of the experiment. Subsequently, electrical field stimulation was applied and consisted of a train of unipolar, rectangular pulses (1 Hz; 2-ms duration for 2 min; 120 pulses; model SD9 stimulator, Grass Instruments, Quincy, MA). The number of pulses was chosen to provide $[3^H]$overflow equivalent to that evoked by superfusion with 10 µM nicotine. Superfusate samples were collected for an additional 60-min period. Each slice was exposed to only one concentration of
NDDPI. One striatal slice in each experiment was superfused in the absence of NDDPI and stimulated with 120-pulse electrical field stimulation, serving as the control condition.

**Data Analysis.** For the \[^3\text{H}\]nicotine competition binding assay, the concentration (IC\(_{50}\)) of \(N-n\)-alkylpyridinium analog that inhibited specific \[^3\text{H}\]nicotine binding by 50% was determined by nonlinear regression fitting of the data to a one-site model. For each analog, the one-site model provided the best fit compared with the two-site model (F-test). Pseudo Hill slopes were determined by nonlinear regression fit of the data to a sigmoidal dose-response equation (variable slope): \%

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\text{binding} = \text{Bottom} + \left(\text{Top} - \text{Bottom}\right) \left[1 + 10^{\left(\log \text{IC}_{50} - X\right) \cdot n}\right],
\]

where \(X\) is the logarithm of inhibitor concentration, \(n\) is the pseudo Hill slope, and Bottom is fixed to zero. The inhibition constant (\(K_i\)) for each analog was calculated from IC\(_{50}\) values using the Cheng-Prusoff equation \([K_i = \text{IC}_{50}/(1 + \text{ligand}/K_d)]\). One-way ANOVA followed by Tukey’s post hoc test \((p < 0.05)\) was used to determine significant differences among log \(K_i\) values. Linear regression was used to determine the relationship between log \(K_i\) values and alkyl chain length.

For the \[^3\text{H}\]DA overflow assay, fractional release for each superfusion sample was calculated by dividing the \[^3\text{H}\] in each 5-min sample by the total \[^3\text{H}\] present in the tissue at the time of sample collection; and these values were expressed as a percentage of basal \[^3\text{H}\]outflow. Basal \[^3\text{H}\]outflow was calculated from the average fractional release in the three 5 min samples just prior to addition of analog to the superfusion buffer. Fractional release data were analyzed by repeated measures two-way ANOVA with time and concentration as repeated measures factors. Total \[^3\text{H}\]DA overflow was calculated as the sum of the increase in fractional release above basal \[^3\text{H}\]outflow during superfusion with analog or nicotine. Analog-induced intrinsic activity was analyzed by one-way repeated measures ANOVA followed by Dunnnett’s post hoc test \((p < 0.05)\). Analog-induced inhibition of nicotine-evoked \[^3\text{H}\]overflow was expressed as a % of total \[^3\text{H}\]DA overflow in the absence of analog (i.e., % control). Percent inhibition data were analyzed by one-way repeated measures ANOVA followed by Dunnnett’s post hoc test \((p < 0.05)\). IC\(_{50}\) values were determined by nonlinear regression fit of the percent inhibition.
data to a sigmoid dose-response equation: response = Bottom + (Top – Bottom)/(1 + 10^{logIC50 – X}), where X is the logarithm of analog concentration.

The mechanism by which NDDPI inhibited nicotine-evoked [3H]DA overflow was determined using Schild analysis. Nicotine concentration-response curves in the absence and presence of NDDPI were generated by nonlinear regression fit of the data to a sigmoid dose-response equation (variable slope): response = Bottom + (Top – Bottom)/(1 + 10^{logEC50 – X \cdot n}), where X is the logarithm of the nicotine concentration and n is the Hill Slope. For each experiment, the dose ratio (dr) for each concentration of NDDPI was calculated as that producing an equivalent response in the absence and presence of NDDPI, such that total [3H]DA overflow was determined at a value of 1% of tissue tritium content. The log of the dose ratio - 1 was plotted as a function of log of the NDDPI concentration to provide the Schild regression. Data were fit by linear regression, the slope determined and linearity assessed. The ability of NDDPI to inhibit electrically evoked [3H]overflow was analyzed by one-way repeated measures ANOVA, with NDDPI concentration as a within-subjects factor. All data analyses were performed using the commercially available programs GraphPAD-PRIZM (GraphPAD, San Diego, CA) and SPSS standard version 9.0 (SPSS, Inc., Chicago, IL).
N-n-Alkylpyridinium-Induced Inhibition of [3H]Nicotine and [3H]MLA Binding to Rat Brain Membranes. N-n-Alkylpyridinium analogs with chain lengths ranging from C_1 to C_20 inhibited [3H]nicotine binding to rats striatal membranes (Fig. 2). With the exception of NEcPB (C_20 analog) which inhibited binding by ~25%, this series of analogs completely inhibited [3H]nicotine binding at high concentrations (0.1-1.0 mM). The values for K_i and pseudo Hill slope (n_H) of the competition curves are presented in Table 1. Analogs with C_4–C_11 chain length had the highest affinity (K_i values from 9 to 20 µM) for the [3H]nicotine binding site relative to the other analogs in this series. However, these analogs had relatively low affinity for the [3H]nicotine binding site compared to nicotine (K_i = 1.34 nM; CI, 1.2 – 1.64 nM). The C_12 and C_20 analogs, NDDPI and NEcPB, respectively, had the lowest affinity (K_i > 100 µM) of the series for the [3H]nicotine binding site. A significant linear relationship was not found between N-n-alkylpyridinium affinity for the [3H]nicotine binding site and number of carbons in the n-alkyl chain (r^2 = 0.324). With respect to [3H]MLA binding, none of the analogs in the series inhibited [3H]MLA binding (data not shown), and thus, had no detectable affinity for this site.

N-n-Alkylpyridinium-induced Inhibition of Nicotine-evoked [3H]DA Overflow from Superfused Striatal Slices. In an initial series of experiments, inhibition of nicotine (10 µM)-evoked [3H]DA overflow by the N-n-alkylpyridinium analogs (C_1-C_12) was determined at two concentrations (0.1 and 1.0 µM). Analogs with chain lengths from C_1 to C_9 did not inhibit nicotine-evoked [3H]DA overflow, and thus, were not evaluated further. However, the C_10 (NDPI), C_11 (NUPI) and C_12 (NDDPI) analogs significantly inhibited nicotine-evoked [3H]DA overflow (F_{2,12} = 46.35; p < 0.0001), such that a 1.0 µM concentration of analog inhibited nicotine-evoked [3H]overflow by ~70-90%. Subsequently, the complete concentration response (0.01-100 µM) for the C_{10}-C_{12} analogs was determined. To further evaluate the
role of n-alkyl chain length on inhibition of nicotine-evoked [$^3$H]DA overflow, the effect of C_{15} and C_{20} analogs was also determined. Intrinsic activity of the C_{10}-C_{20} N-n-alkylpyridinium analogs was assessed during the 60-min period of superfusion with each analog prior to addition of nicotine to the superfusion buffer (Table 2). Intrinsic activity was not observed for any of the analogs at concentrations $\leq$ 1.0 $\mu$M. NDPI (C_{10}) and NEcPB (C_{20}) also showed no intrinsic activity at 10 $\mu$M. However, NUPI (C_{11}), NDDPI (C_{12}) and NPDPB (C_{15}) significantly increased [$^3$H]DA overflow at 10 $\mu$M, whereas NDPI (C_{10}) and NEcPB (C_{20}) increased [$^3$H]DA overflow at 100 $\mu$M (Table 2; repeated measures one-way ANOVAs: NDPI (C_{10}), F_{5.35} = 27.19, $p < 0.0001$; NUPI (C_{11}), F_{5.35} = 55.53, $p < 0.0001$; NDDPI (C_{12}), F_{5.35} = 19.16, $p < 0.0001$; NPDPB (C_{15}), F_{5.20} = 36.784, $p < 0.001$; NEcPB (C_{20}), F_{5.19} = 4.830, $p < 0.01$). Thus, at high concentrations (10-100 $\mu$M), intrinsic activity was observed for these C_{10}-C_{20} N-n-alkylpyridinium analogs.

The ability of mecamylamine (10 $\mu$M) to inhibit NDDPI-induced intrinsic activity was determined. NDDPI (10 $\mu$M) evoked 20.9 $\pm$ 1.68 total [$^3$H]overflow during the 60 min superfusion period. Inclusion of mecamylamine in the superfusion buffer for 60 min prior to addition of NDDPI to the buffer resulted in 17.2 $\pm$ 1.91 total [$^3$H]overflow. At the concentration used, mecamylamine did not increase [$^3$H]overflow above that observed for control slices superfused with buffer only. Thus, mecamylamine did not inhibit intrinsic activity induced by the representative analog, NDDPI.

The ability of the N-n-alkylpyridinium analogs to inhibit nicotine-evoked [$^3$H]overflow is illustrated in Fig. 3. High concentrations of analogs that produced intrinsic activity were not included in the analysis of inhibition of the effect of nicotine. For each analog, repeated measures one-way ANOVA revealed significant concentration-dependent inhibition of nicotine-evoked [$^3$H]DA overflow: NDPI (C_{10}), F_{5.35} = 29.44, $p < 0.0001$; NUPI (C_{11}), F_{4.29} = 24.58, $p < 0.0001$; NDDPI (C_{12}), F_{3.23} = 11.07, $p < 0.001$; NPDPB (C_{15}), F_{5.18} = 24.31, $p < 0.001$; NEcPB (C_{20}), F_{5.15} = 9.29, $p < 0.001$. IC_{50} values derived from the
nonlinear sigmoid curve fits ranged from 0.12 - 0.49 µM (Table 3). NDPI (C10), NUPI (C11) and NDDPI (C12) completely inhibited nicotine-evoked [3H]DA overflow (Fig. 3). Interestingly, the longer chain analogs, NPDPB (C15) and NEcPB (C20), inhibited nicotine-evoked [3H]DA overflow by a maximum of 45-55%.

The time course of the NDDPI-induced inhibition of nicotine-evoked [3H]DA overflow is also shown in Fig. 3. Repeated measures two-way ANOVA revealed significant main effects of NDDPI concentration \( (F_{3,15} = 36.616, \ p < 0.001) \) and time \( (F_{11,55} = 80.497, \ p < 0.001) \), and a significant concentration x time interaction \( (F_{33,165} = 4.215, \ p < 0.001) \). Fractional release evoked by nicotine peaked 10 min after its addition to the buffer and subsequently decreased toward basal levels, despite the presence of nicotine in the buffer throughout the remainder of the experiment. The time course illustrates the concentration-dependent inhibition, with low concentrations of NDDPI (0.01- 1.0 µM) inhibiting the response to nicotine across the time course of exposure.

Mechanism of N-n-Alkylpyridinium Inhibition of Nicotine-evoked [3H]DA Overflow. The competitive versus noncompetitive nature of inhibition of nicotine-evoked [3H]DA overflow was determined by Schild analysis for the representative analog, NDDPI. The concentration-response for nicotine was determined in the absence and presence of three NDDPI concentrations (0.1, 0.3 and 0.6 µM; Fig. 4). Inclusion of 0.3 and 0.6 µM NDDPI in the buffer produced rightward shifts in the concentration-response curves for nicotine relative to the response curves obtained under the control condition (in the absence of NDDPI). A linear fit \( (r^2 = 0.9889) \) to the Schild-transformed data (Fig. 4, inset) revealed a slope not different from unity, and the regression did not deviate significantly from linearity, as determined by runs test. The log \( K_B \) value of \( -6.774 \) for NDDPI was derived from the \( x \)-intercept. The anti-log transform provided a \( K_B \) value of 0.17 µM, which was in good agreement with the \( IC_{50} \) value of 0.26 µM obtained by determining NDDPI-induced inhibition of 10 µM nicotine.
Furthermore, NDDPI did not augment $[^3]$H][DA overflow evoked by low concentrations (1 nM – 1 µM) nicotine. Thus, the results from the Schild analysis indicate that NDDPI interacts in a competitive manner with nAChRs mediating nicotine-evoked $[^3]$H][DA overflow.

**NDDPI Does Not Inhibit Field Stimulation-Evoked $[^3]$H][DA Overflow.** $[^3]$H][DA-preloaded striatal slices were superfused for 60 min in the absence or presence of NDDPI (1 nM – 1 µM), and were subsequently field stimulated with 120 electrical pulses (1 Hz stimulation for 2 min). Table 4 provides the results demonstrating that electrical field stimulation-evoked $[^3]$H][overflow was not inhibited by NDDPI. Electrical field stimulation resulted in total $[^3]$H][overflow of 2.24 ± 0.35% of $[^3]$H][ tissue content. ANOVA revealed that the main effect of NDDPI concentration was not significant ($F_{4,20} = 2.309$; $p > 0.05$). Thus, NDDPI did not inhibit electrical stimulation-evoked $[^3]$H][overflow.
DISCUSSION

Structural simplification of a series of $N$-$n$-alkylnicotinium antagonists was achieved by removal of the $N$-methylpyrrolidino moiety, which afforded $N$-$n$-alkylpyridinium analogs with carbon chain lengths ranging from $C_1$ to $C_{20}$. These $N$-$n$-alkylpyridinium analogs had generally greater water solubility than the respective $N$-$n$-alkylnicotinium analogs, which allowed for evaluation of longer chain compounds ($C_{15}$ and $C_{20}$) in the current study. $N$-$n$-Alkylpyridinium analogs had low affinity for $[^3H]$nicotine binding sites, i.e., 1-3 orders of magnitude lower than those of the respective $N$-$n$-alkylnicotinium analogs (Wilkins et al., 2003a; present results). Similar to the $N$-$n$-alkylnicotinium antagonists, the $N$-$n$-alkylpyridinium analogs exhibited no inhibition of $[^3H]$MLA binding. These results indicate that the $N$-methylpyrrolidino moiety in the $N$-$n$-alkylnicotinium series is a structural requirement for potent inhibition of $\alpha_4\beta_2^*$ nAChRs probed by high affinity $[^3H]$nicotine binding.

This series of $N$-$n$-alkylpyridinium compounds was initially evaluated for intrinsic activity in the DA release assay (i.e., analog-induced $[^3H]$DA overflow). As in the $N$-$n$-alkylnicotinium series, relatively high concentrations of analogs with $n$-alkyl chain lengths greater than or equal to $C_{10}$ exhibited intrinsic activity in the DA release assay; however, in contrast to the $N$-$n$-alkylnicotinium analogs, intrinsic activity produced by the current series of analogs was not related to chain length (Wilkins et al., 2002; present results). Mecamylamine, which inhibits all known nAChR subtypes including the $\alpha_4\beta_2^*$ subtype probed by $[^3H]$nicotine binding, was used to evaluate if the $N$-$n$-alkylpyridinium analogs produced intrinsic activity via stimulation of nAChRs. However, mecamylamine did not inhibit intrinsic activity induced by the representative analog, NDDP. These results indicate that $N$-$n$-alkylpyridinium analog-induced intrinsic activity is not the result of stimulation of nAChRs (i.e., these analogs do not act as nAChR agonists). Thus, intrinsic activity induced by these compounds is likely the result of stimulation of non-nicotinic receptors.
The most significant finding in the current study is that \(N-n\)-alkylpyridinium analogs, with alkyl chains ranging from \(C_{10}-C_{20}\), potently and selectively inhibited nicotine-evoked \([^{3}H]\)DA overflow (IC\(_{50}\) = 0.12-0.49 \(\mu\)M). The representative \(N-n\)-alkylpyridinium analog NDDPI inhibited nicotine-evoked \([^{3}H]\)DA overflow at low concentrations (0.1-1.0 \(\mu\)M), whereas NDDPI inhibited \([^{3}H]\)nicotine binding at high concentrations of 100 \(\mu\)M. These results support the suggestion that different nAChRs mediate nicotine-evoked DA release and \([^{3}H]\)nicotine binding (also see Introduction), and moreover, illustrate the greater than 2-orders of magnitude selectivity of NDDPI as an antagonist at nAChRs mediating nicotine-evoked DA release in striatum relative to \(\alpha_4\beta_2\) nAChRs. Furthermore, the IC\(_{50}\) value for NDDPI to inhibit nicotine-evoked \([^{3}H]\)DA overflow was 0.26 \(\mu\)M, whereas significant intrinsic activity for NDDPI was observed only at a 40-fold higher concentration of 10 \(\mu\)M. As such, NDDPI exhibits 40-fold selectivity as an antagonist at nicotinic receptors mediating nicotine-evoked DA release relative to its action to intrinsically evoke DA release likely via a non-nicotinic receptor-mediated mechanism. With the exception of NPDPB, which exhibits similar selectivity to NDDPI, the remaining analogs exhibit 330-fold selectivity for the nicotinic receptor mediating nicotine-evoked DA release (IC\(_{50}\) ~0.3 \(\mu\)M), whereas intrinsic activity (stimulation of DA release) was observed at relatively higher concentrations (100 \(\mu\)M). Thus, the 40-330 fold separation between analog-induced inhibition of nicotine-evoked dopamine release and analog-induced intrinsic activity supports the contention that these compounds are relatively selective as antagonists at nicotinic receptors mediating nicotine-evoked dopamine release.

The \(N-n\)-alkylpyridinium analogs completely inhibited the effect of nicotine in the DA release assay, with the exceptions of NEcPB (\(C_{20}\) analog) and NPDPB (\(C_{15}\) analog), both compounds exhibiting maximal inhibition of only ~50%. High concentrations (10-100 \(\mu\)M) of NPDPB produced significant amounts of intrinsic activity, which may have contributed to the observed incomplete inhibition of the effect of nicotine. However, only the high concentration (100 \(\mu\)M) of NEcPB elicited a small, but
significant, amount of intrinsic activity. At lower concentrations (10 µM), NEcPB induced maximal inhibition and no intrinsic activity was observed. Thus, maximal incomplete inhibition was observed at a concentration of NEcPB eliciting no intrinsic activity. The Conus snail neurotoxin, α-conotoxin-MII (Fig. 1, Structure 5), also has been shown to inhibit ~50% of the response to nicotine in the DA release assay (Kulak et al., 1997; Kaiser et al., 1998; Kaiser and Wonnacott, 2000). Whether the latter observations indicate that NEcPB and α-conotoxin-MII act at the same nAChR subtype remains to be determined. α-Conotoxin-MII inhibits acetylcholine electrophysiological responses in Xenopus oocytes expressing α3/α6 and α6/α4 nAChRs containing either β2 or β4 subunits (Luetje et al., 1990; Cartier et al., 1996; Kuryatov et al., 2000). Also, α-conotoxin-MII binds with high affinity to immunopurified α6β2* (Zoli et al., 2002), implicating nAChR subtypes containing these two subunits as mediating this response. Furthermore, [3H]-α-conotoxin-MII binding was eliminated in α6-knockout mice, but not in α3-knockout mice, suggesting an interaction with native α6-containing nAChRs (Champtiaux et al., 2002; Whiteaker et al., 2002). Additionally, studies using β2-knockout mice also implicate β2-containing nAChRs in nicotine-evoked DA release (Picciotto et al., 1998; Grady et al., 2002). The fact that α-conotoxin-MII and NEcPB inhibited only 50% of nicotine-evoked DA release indicates that more than one subtype of nAChR is involved (Kulak et al., 1997; Kaiser et al., 1998; present results). Since nigral neurons express α3-α7 and β2-β4 mRNAs (Wada et al., 1989; Deneris et al., 1989; Le Novere et al., 1996; Charpentier et al., 1998; Arroyo-Jimenez et al., 1999; Klink et al., 2001; Azam et al., 2002; Zoli et al., 2002), it is quite possible that a diversity of nAChR subunit combinations and subtypes are involved in this response. Evidence is emerging which suggests that different DA neurons in the nigra can be categorized based upon the expression of particular nAChR subtypes with varying compositions of nAChR subunits (Azam et al., 2002). Although speculative at the current time, it may be that the expression of nAChR subtypes by dopaminergic neurons is a dynamic process, which is regulated in part by the history of the organism, including environmental exposure and drug experience. With respect to drug discovery, NEcPB is a small molecule, but appears to produce the same functional effect (i.e., ~50%
maximal inhibition) as the relatively larger neuroactive peptide, α-conotoxin-MII. The discovery of NEcPB sets the precedence for the development of small, non-peptide molecules that are selective antagonists for a subset of nAChR subtypes mediating nicotine-evoked DA release.

In terms of structure-activity relationships, analogs in the current $N$-$n$-alkylpyridinium series required longer $n$-alkyl substituents (i.e., C$_{10}$-C$_{20}$) compared to those in the respective $N$-$n$-alkynicotinium series (C$_{8}$-C$_{12}$) to obtain a comparable affinity (IC$_{50}$ = 0.1-0.5 µM) for nAChRs mediating nicotine-evoked DA release from striatum. Furthermore, in the $N$-$n$-alkynicotinium series, $n$-alkyl chain length was linearly related to nAChR affinity; whereas in the $N$-$n$-alkylpyridinium series, no such linear relationship was observed. In this respect, C$_{10}$-C$_{20}$ analogs were equipotent in inhibiting nicotine-evoked DA release, whereas analogs with alkyl chain lengths of less than C$_{10}$ did not inhibit nicotine-evoked [$^{3}$H]DA overflow. Interestingly, the C$_{10}$ analog, NDPI, was a potent inhibitor (IC$_{50}$ = 0.13 µM) of nicotine-evoked DA release (present results). This is in marked contrast to the C$_{10}$ analog, NDNI, which had no inhibitory activity in the nicotine-evoked DA release assay (Wilkins et al., 2002). In the latter study, NDNI was proposed to exhibit a unique conformation to explain its unusual pharmacological profile; this unique conformation is obviously not maintained in the NDPI structure, after removal of the $N$-methylpyrrolidino moiety.

Although it appears that the nature of the cationic head group and the size of the $n$-alkyl substituent are both important in establishing affinity for nAChR subtypes mediating nicotine-evoked DA release, the lack of a linear relationship in the C$_{10}$-C$_{20}$ analogs of the $N$-$n$-alkylpyridinium series indicates that antagonist potency is insensitive to chain length within this 10-carbon range. Thus, the inhibitory effect of these C$_{10}$-C$_{20}$ $N$-$n$-alkylpyridinium analogs may be more generally related to a common physicochemical property of these molecules rather than to their differences in chemical structure.
The mechanism of inhibition of a representative analog in this structural series, NDDPI, was determined by Schild analysis. Nicotine concentration response curves were obtained in the absence and presence of 3 concentrations of NDDPI. NDDPI produced a rightward shift in the concentration response curve for nicotine, and a linear Schild regression with slope not different from unity, providing a $K_B$ value of 0.17 µM. These results are indicative of competitive and potent antagonism at nAChRs mediating nicotine-evoked DA release. The $K_B$ value from the Schild analysis was in good agreement with the IC$_{50}$ value obtained from the inhibition analysis with the single concentration of nicotine (10 µM). Furthermore, the observation that low concentrations of NDDPI (0.01-0.6 µM) did not augment DA release evoked by the very low concentrations (1 nM – 1 µM) of nicotine provides additional support for the contention that NDDPI is not acting as an agonist at nAChRs mediating nicotine-evoked DA release. Moreover, the findings from the Schild analysis indicate that NDDPI is a competitive antagonist at nAChRs mediating nicotine-evoked DA release. Thus, the simplified N-n-alkylpyridinium analogs are potent, selective and competitive antagonists of nAChRs mediating nicotine-evoked DA release, indicating that the N-methylpyrrolidino moiety is not a structural requirement for interaction with these nAChR subtypes. Competitive inhibition most likely results from the pyridinium moiety of NDDPI, which may interact competitively with the agonist-binding region on the nAChR protein that normally accommodates the protonated N-methylpyrrolidino moiety of the nicotine molecule. In this respect, it is important to note that NDDPI did not inhibit depolarization-induced release evoked by electrical field stimulation, which provides additional supporting evidence that these analogs selectively interact with the agonist-binding site on nAChRs mediating nicotine-evoked DA release.

Evidence indicates that high micromolar concentrations (30-100 µM) of nicotine activate $\alpha7^*$ homomeric nAChRs to evoke DA release through an indirect mechanism of action, i.e., nicotine-induced stimulation of $\alpha7^*$ nAChRs releases glutamate from glutamatergic terminals, which then indirectly releases DA from dopaminergic terminals (Kaiser and Wonnacott, 2000). Furthermore, glutamate
receptor antagonists have been reported to inhibit ~20-50% of nicotine-evoked DA release from striatal slices, but not from striatal synaptosomes (Wonnacott et al., 2000), indicating that local circuitry within the striatal slice is sufficient to detect this indirect effect of nicotine on DA release. The concentration of nicotine (10 μM) used in the present study to evoke DA release may not have been sufficient to activate this indirect glutamate-mediated mechanism of DA release. Moreover, the observation that N-n-alkylpyridinium analogs have no affinity for the [3H]MLA binding site excludes the involvement of homomeric α7* nAChRs as a possible target for these antagonists.

In summary, structural simplification of N-n-alkynicotinium antagonist molecules (e.g., NONI and NDNI), by removal of the N-methylpyrrolidino moiety, afforded N-n-alkylpyridinium analogs, which were found to be selective, potent and competitive antagonists at nAChRs mediating nicotine-evoked DA release from superfused rat striatal slices. Of note, one of the compounds in the series, NEcPB, sets the precedence for drug discovery and development of small, non-peptide molecules that are selective antagonists for a subset of nAChR subtypes mediating nicotine-evoked DA release. Furthermore, the current results indicate that the N-methylpyrrolidino moiety in the N-n-alkynicotinium series is a structural requirement for potent inhibition of α4β2* nAChRs, but is not necessary for inhibition of nAChRs mediating nicotine-evoked DA release. Results from the current studies are beginning to delineate the specific pharmacophore requirements for subtype-selective nAChR antagonism. Such subtype-selective nAChR antagonists will aide in the elucidation of the physiological function of specific nAChR subtypes. Furthermore, identification of specific nAChR antagonist pharmacophores will be of value in the drug discovery process, and in the further development of selective pharmacological agents, which may have therapeutic value in diseases such as schizophrenia, depression, neurodegenerative diseases and as treatments for drug abuse.
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distinguish between different neuronal nicotinic acetylcholine receptor subunit combinations. J 

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Acetylcholine receptors containing the β2 subunit are involved in the reinforcing properties of 


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FOOTNOTES

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FIGURE LEGENDS

Fig. 1  Chemical structures of nicotine (1), N-n-alkylnicotinium analogs NONI (2a) and NDNI (2b), N-n-alkylpyridinium analogs (3a-n), methyllycaconitine (4) and α-conotoxin-MII (5).  \( R = \text{alkyl substituent; } X = \text{the halide anion.} \)

Fig. 2  Inhibition of specific \(^3\text{H}\)nicotine binding to rat striatal membranes by N-n-alkylpyridinium analogs.  Analog-induced inhibition is illustrated in three panels for clarity of presentation.  Assays used a range of analog concentrations (0.1 \(\mu\)M – 1 mM) to inhibit binding of 3 nM \(^3\text{H}\)nicotine.  Data are fmol/mg protein expressed as a percent of control \(^3\text{H}\)nicotine binding and represent the mean ± S.E.M. of 52 independent experiments (\(n = 4\) independent experiments/analog).  Control \(^3\text{H}\)nicotine binding was \(37.2 \pm 0.54\) fmol/mg protein, range 34 - 45 fmol/mg protein, in the absence of analog.  Curves were generated by nonlinear regression.  Numbers inside the parentheses indicate the length of the \(n\)-alkyl chain; analog abbreviations are provided in the text.

Fig. 3  Concentration-dependent inhibition of nicotine-evoked \(^3\text{H}\)DA overflow by N-n-alkylpyridinium analogs.  Superfusion buffer contained nomifensine (10 \(\mu\)M) and pargyline (10 \(\mu\)M).  N-n-Alkylpyridinium analog was added to the buffer following 60 min of superfusion, and superfusion continued for 60 min with analog before addition of nicotine (10 \(\mu\)M) to the buffer.  Slices were superfused with analog plus nicotine for an additional 60 min.  In each experiment, one striatal slice was superfused with 10 \(\mu\)M nicotine in the absence of analog and served as the nicotine control.  Top panel illustrates the data for NDNI, NUPI, NDDPI, NPDPB and NEcPB as mean ± S.E.M. total \(^3\text{H}\)DA overflow expressed as a percentage of nicotine control.  The response to nicotine (10 \(\mu\)M) under control conditions was \(2.14 \pm 0.19\) total \(^3\text{H}\)DA overflow (mean ± S.E.M.).  Numbers in parentheses indicate the number of carbons in the \(n\)-alkyl chain for each analog.  Curves were generated by nonlinear regression.  Bottom panel illustrates the time course of the inhibition of nicotine-evoked fractional release by the
representative analog NDDPI. The vertical arrow indicates addition of nicotine to the buffer. Data are expressed as mean ± S.E.M. as a percentage of basal [³H]outflow as a function of time of superfusion (min). Basal [³H]outflow prior to nicotine exposure was 0.89 ± 0.04 fractional release. n = 5-6 rats/analag.

Fig. 4 NDDPI competitively inhibits nicotine-evoked [³H]DA overflow from superfused rat striatal slices. Superfusion buffer contained nomifensine (10 µM) and pargyline (10 µM) from the start of superfusion. After 60 min of superfusion, slices were superfused in the absence or presence of 0.1, 0.3 or 0.6 µM NDDPI for a 60-min period prior to the addition of nicotine (1.0 nM - 100 µM) to the buffer, and superfusion continued for an additional 60 min. In each experiment examining the effect of a single concentration of NDDPI, a set of slices was superfused with 1 nM to 100 µM nicotine in the absence of NDDPI, serving as the nicotine control condition. Since the results from the nicotine control condition (i.e., concentration response for nicotine in the absence of NDDPI) were not significantly different among the series of experiments (F2,10 = 2.92, p > 0.05), these data were pooled for graphical presentation. An additional slice in each experiment was superfused in the absence of either drug and served as the buffer control, and indicated by Control. Data are expressed as mean ± S.E.M. total [³H]DA overflow during the 60-min period of exposure to nicotine in the absence and presence of NDDPI as a function of log nicotine concentration. Curves were generated by nonlinear regression fit of the data to a sigmoid dose-response equation. Schild regression plot of the data are presented in the inset; slope determined by linear regression. n = 3-5 rats/NDDPI concentration.
**TABLE 1**

Kᵢ values and pseudo Hill slopes (nₕ) for N-n-alkylpyridinium analog inhibition of [³H]nicotine binding to rat striatal membranes.

IC₅₀ values and 95% confidence intervals were derived from a nonlinear regression fit of the data to a one-site model. Pseudo nₕ values were determined from a variable slope sigmoid fit.

<table>
<thead>
<tr>
<th>Analog</th>
<th>Chain Length</th>
<th>Pseudo nₕ</th>
<th>Kᵢ (95 % CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMPI</td>
<td>1</td>
<td>-0.95</td>
<td>20.1 (15.6 – 25.9)</td>
</tr>
<tr>
<td>NEPI</td>
<td>2</td>
<td>-0.91</td>
<td>28.7 (22.3 – 37.0)</td>
</tr>
<tr>
<td>NPPrPI</td>
<td>3</td>
<td>-1.00</td>
<td>42.3 (30.2 – 59.3)</td>
</tr>
<tr>
<td>NBuPI</td>
<td>4</td>
<td>-0.94</td>
<td>9.65 (7.57 – 12.3)</td>
</tr>
<tr>
<td>NPPI</td>
<td>5</td>
<td>-0.91</td>
<td>8.72 (5.68 – 13.4)</td>
</tr>
<tr>
<td>NHxPI</td>
<td>6</td>
<td>-0.98</td>
<td>9.07 (6.12 – 13.5)</td>
</tr>
<tr>
<td>NHPI</td>
<td>7</td>
<td>-0.90</td>
<td>15.0 (13.0 – 17.2)</td>
</tr>
<tr>
<td>NOPI</td>
<td>8</td>
<td>-1.01</td>
<td>20.1 (16.3 – 24.8)</td>
</tr>
<tr>
<td>NNPI</td>
<td>9</td>
<td>-1.06</td>
<td>19.0 (16.4 – 22.1)</td>
</tr>
<tr>
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<td>-1.01</td>
<td>16.7 (13.6 – 20.4)</td>
</tr>
<tr>
<td>NUPI</td>
<td>11</td>
<td>-1.07</td>
<td>17.0 (14.4 – 20.1)</td>
</tr>
<tr>
<td>NDDDPI</td>
<td>12</td>
<td>-1.17</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>NPDPB</td>
<td>15</td>
<td>-0.96</td>
<td>38.0 (28.2 – 51.3)</td>
</tr>
<tr>
<td>NEcPB</td>
<td>20</td>
<td>-1.12</td>
<td>&gt; 100</td>
</tr>
</tbody>
</table>

* CI, confidence interval. n = 4 independent experiments/analog.
TABLE 2

*N-n*-Alkylpyridinium analog-induced \([^{3}\text{H}]\text{DA}\) overflow from rat striatal slices.

Slices were superfused with various analog concentration added to the superfusion buffer. Data represent \([^{3}\text{H}]\text{DA}\) overflow during the 60-min period of superfusion in the absence and presence of analog.

<table>
<thead>
<tr>
<th>Analog</th>
<th>(n)</th>
<th>Control</th>
<th>0.01</th>
<th>0.1</th>
<th>1</th>
<th>10</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDPI (10)</td>
<td>6</td>
<td>0 ± 0(^a)</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>2.71 ± 0.92</td>
<td>29.5 ± 5.53*</td>
</tr>
<tr>
<td>NUPI (11)</td>
<td>6</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>8.07 ± 1.38</td>
<td>48.0 ± 6.41*</td>
</tr>
<tr>
<td>NDDPI (12)</td>
<td>6</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0.83 ± 0.32</td>
<td>13.8 ± 3.59*</td>
<td>58.7 ± 12.4*</td>
</tr>
<tr>
<td>NPDPB (15)</td>
<td>5</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0.34 ± 0.23</td>
<td>24.8 ± 4.23*</td>
<td>47.2 ± 6.91*</td>
</tr>
<tr>
<td>NEcPB (20)</td>
<td>5</td>
<td>0 ± 0</td>
<td>0.05 ± 0.03</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0.05 ± 0.04</td>
<td>0.91 ± 0.53*</td>
</tr>
</tbody>
</table>

\(^a\) Data are mean ± S.E.M. total \([^{3}\text{H}]\text{DA}\) overflow expressed as a percentage of tissue tritium.

\(* p < 0.05\), compared to control (absence of analog).

\(n\) indicates number of rats/analog.

Number in parenthesis indicates \(n\)-alkyl chain length for each analog.
TABLE 3

Concentration (IC$_{50}$) of N-$n$-alkylpyridinium analogs required to reduce nicotine-evoked [$^3$H]DA overflow from superfused rat striatal slices by 50%.

IC$_{50}$ values and 95% confidence intervals (CI) were derived from the sigmoid fits to the percent control transformed data shown in Fig. 2.

<table>
<thead>
<tr>
<th>Analog</th>
<th>IC$_{50}$ (95% CI)</th>
<th>µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDPI (10)</td>
<td>0.13 (0.02 – 0.87)</td>
<td>0.13 (0.02 – 0.87)</td>
</tr>
<tr>
<td>NUPI (11)</td>
<td>0.49 (0.01 – 16.9)</td>
<td>0.49 (0.01 – 16.9)</td>
</tr>
<tr>
<td>NDDPI (12)</td>
<td>0.26 (0.02 – 4.23)</td>
<td>0.26 (0.02 – 4.23)</td>
</tr>
<tr>
<td>NPDPB (15)</td>
<td>0.32 (0.11 – 0.87)</td>
<td>0.32 (0.11 – 0.87)</td>
</tr>
<tr>
<td>NEcPB (20)</td>
<td>0.12 (0.01 – 2.38)</td>
<td>0.12 (0.01 – 2.38)</td>
</tr>
</tbody>
</table>

Number in parenthesis indicates $n$-alkyl chain length for each analog.
TABLE 4

NDDPI does not inhibit electrical field stimulation-evoked [3H]overflow from [3H]DA preloaded striatal slices. Superfusion buffer contained pargyline (10 µM) and nomifensine (10 µM). Slices were superfused with a range of NDDPI concentrations. Each slice was exposed to only one concentration of NDDPI and subsequently was stimulated with 120 pulses (1 Hz) of electrical field stimulation. Data represent [3H]overflow during the 60 min period of superfusion following electrical field stimulation.

<table>
<thead>
<tr>
<th>NDDPI (µM)</th>
<th>120 Pulses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0)</td>
<td>2.24 ± 0.35a</td>
</tr>
<tr>
<td>0.001</td>
<td>1.47 ± 0.28</td>
</tr>
<tr>
<td>0.01</td>
<td>2.45 ± 0.39</td>
</tr>
<tr>
<td>0.1</td>
<td>2.23 ± 0.36</td>
</tr>
<tr>
<td>1.0</td>
<td>1.23 ± 0.38</td>
</tr>
</tbody>
</table>

aData are mean ± S.E.M. of total [3H]overflow expressed as a percentage of tissue tritium. n = 6 rats.
Nicotine

1

a) R = n-octyl NONI
b) R = n-decyl NDNI

c) R = methyl NMPI X = I
d) R = ethyl NEPI X = I
e) R = n-propyl NPrPI X = I
f) R = n-butyl NBuPI X = I
g) R = n-hexyl NHxPI X = I
h) R = n-heptyl NHPI X = I
i) R = n-octyl NOPI X = I
j) R = n-nonyl NNPI X = I
k) R = n-decyl NDPI X = I
l) R = n-undecyl NUPI X = I
m) R = n-pentadecyl NPDPB X = Br
n) R = n-eicosyl NEcPB X = Br

Methyllycaconitine (MLA)

4

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

α-Conotoxin MII

5
Total Tritium Overflow

log [Nicotine] (M)

log (dose ratio - 1)

0 µM

0.1 µM

0.3 µM

0.6 µM

Control

-9

-8

-7

-6

-5

-4

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