Published in final edited form as:

J Pharmacol Exp Ther. 2008 August ; 326(2): 563–576. doi:10.1124/jpet.108.136630.

***N,N'*-Alkane-diyl-*bis*-3-picoliniums as Nicotinic Receptor Antagonists: Inhibition of Nicotine-induced Dopamine Release and Hyperactivity**

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Abstract

The current study evaluated a new series of *N,N'*-alkane-diyl-*bis*-3-picolinium (bAPi) analogs with C₆–C₁₂ methylene linkers as nicotinic receptor (nAChR) antagonists, for nicotine-evoked [³H]dopamine (DA) overflow, for blood-brain barrier choline transporter affinity and for attenuation of discriminative stimulus and locomotor stimulant effects of nicotine. bAPi analogs exhibited little affinity for $\alpha 4\beta 2^*$ and $\alpha 7^*$ high affinity ligand binding sites, nor for nAChRs modulating DA transporter function. With the exception of C₆, all analogs inhibited nicotine-evoked [³H]DA overflow (IC₅₀=2 nM–6 μ M; I_{max}=54–64%), with *N,N'*-dodecane-1,12-diyl-*bis*-3-picolinium dibromide (C₁₂, bPiDDB) being most potent. bPiDDB did not inhibit electrically-evoked [³H]DA overflow, suggesting specific nAChR inhibitory effects and a lack of toxicity to DA neurons. Schild analysis suggested that bPiDDB interacts in an orthosteric manner at nAChRs mediating nicotine-evoked [³H]DA overflow. To determine if bPiDDB interacts with α -conotoxin MII-sensitive $\alpha 6\beta 2$ -containing nAChRs, slices were exposed concomitantly to maximally-effective concentrations of bPiDDB (10 nM) and α -conotoxin MII (1 nM). Inhibition of nicotine-evoked [³H]DA overflow was not different with the combination compared with either antagonist alone, suggesting that bPiDDB interacts with $\alpha 6\beta 2$ -containing nAChRs. C₇, C₈, C₁₀ and C₁₂ analogs exhibited high affinity for the blood-brain barrier choline transporter in vivo, suggesting brain bioavailability. Although, none of the analogs altered the discriminative stimulus effect of nicotine, C₈, C₉, C₁₀ and C₁₂ analogs decreased nicotine-induced hyperactivity in nicotine-sensitized rats, without reducing spontaneous activity. Further development of nAChR antagonists that inhibit nicotine-evoked DA release and penetrate brain to antagonize DA-

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The University of Kentucky holds patents on the *N,N'*-alkane-diyl-*bis*-3-picolinium analogs described herein. A potential royalty stream to L.P.D., P.A.C. and J.T.A. may occur consistent with the University of Kentucky policy.

mediated locomotor stimulant effects of nicotine as novel treatments for nicotine addiction is warranted.

INTRODUCTION

Nicotine, the principal tobacco alkaloid, is an agonist at neuronal nicotinic receptor (nAChR) subtypes modulating dopamine (DA) release. Habitual tobacco smoking is maintained via rapid nicotine delivery to brain (Le Foll and Goldberg, 2005) and results from nicotine's intrinsic rewarding properties, believed due to increased DA release. Classical nAChR antagonists, mecamylamine and dihydro- β -erythroidine (DH β E), inhibit nicotine-evoked DA release and decrease the locomotor stimulant and reinforcing effects of nicotine in rats (Corrigall et al., 1994; Watkins et al., 1999; Rahman et al., 2004), suggesting a role for nAChR-mediated DA release in these abuse-related behavioral effects of nicotine. However, evidence supporting a primary role for DA in nicotine discrimination is lacking (Corrigall and Coen, 1994).

Substantia nigra DA neurons express mRNA for $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\beta 2$, $\beta 3$ and $\beta 4$ subunits (Klink et al., 2001; Azam et al., 2002). While $\beta 2$ -containing nAChRs play a critical role in mediating nicotine-evoked striatal DA release (Picciotto et al., 1998; Salminen et al., 2004; Scholze et al., 2007), a role for $\alpha 6$ - and $\beta 3$ -containing subtypes also has been implicated (Cui et al., 2003). A comprehensive molecular genetics study, in which an individual gene ($\alpha 4$, $\alpha 5$, $\alpha 7$, $\beta 2$, $\beta 3$ or $\beta 4$) was deleted, suggested that six different subtypes mediate nicotine-evoked DA release from mouse striatum, including α -conotoxin MII (α -CtxMII)-sensitive ($\alpha 6\beta 2\beta 3^*$, $\alpha 4\alpha 6\beta 2\beta 3^*$, $\alpha 6\beta 2^*$ and $\alpha 4\alpha 6\beta 2^*$) and α -CtxMII-resistant ($\alpha 4\beta 2^*$ and $\alpha 4\alpha 5\beta 2^*$) nAChRs, whereas deletion of $\beta 4$ and $\alpha 7$ had no effect (Salminen et al., 2004; Gotti et al., 2005). More recently, striatal synaptosomes from $\alpha 4$ and $\alpha 4/\beta 3$ knockout mice were used to isolate $\alpha 6$ -containing nAChRs to determine their involvement in nicotine-evoked DA release (Salminen et al., 2007). These results showed an increased EC₅₀ value for nicotine to evoke DA release with $\alpha 4$ deletion, and combined deletion of $\alpha 4$ and $\beta 3$ subunits resulted in a further 4–7-fold increase in EC₅₀ value. Results suggest that the $\alpha 4\alpha 6\beta 2\beta 3^*$ subtype constitutes ~50% of $\alpha 6$ -containing nAChRs on striatal DA terminals of wild-type mice, and that this subtype is most sensitive to nicotine activation and is a major subtype mediating nicotine-evoked DA release and behavior.

One approach to developing effective tobacco cessation pharmacotherapies is to discover molecules that selectively inhibit nicotine-evoked DA release. Although mecamylamine may have clinical efficacy as a cessation agent, adverse side effects due to inhibition of peripheral nAChRs limit its use (Rose et al., 1994). Development of antagonists selective for DA release-regulating nAChRs should retain therapeutic efficacy without producing peripheral side effects; unfortunately, no clinically-available compounds have this profile. In this regard, *N-n*-alkylnicotinium analogs with methylene linkers of C₇–C₁₂ potently inhibit nicotine-evoked [³H]DA overflow from rat striatal slices in an orthosteric manner and inhibit high affinity [³H]nicotine binding to rat brain membranes (Wilkins et al., 2002, 2003). Structurally-related *N-n*-pyridinium analogs with C₁₀–C₂₀ were also potent inhibitors of nicotine-evoked [³H]DA overflow, and longer chain analogs (C₁₅ and C₂₀) showed incomplete inhibition (Imax=50%), supporting the involvement of more than one nAChR subtype (Grinevich et al., 2003). These pyridinium analogs had little affinity for the [³H]nicotine binding site (Grinevich et al., 2003), indicating enhanced selectivity for nAChRs mediating nicotine-evoked DA release.

The *bis*-quaternary ammonium compounds, hexamethonium and decamethonium, which are considered to be simplified analogs of *d*-tubocurarine, have been used to differentiate

muscle and ganglionic nAChRs (Koelle, 1975). The current study exploited the *bis*-quaternary ammonium structural framework to enhance nAChR subtype selectivity and afford a new class of *N,N'*-alkane-diyl-*bis*-3-picolinium (bAPi) analogs (Fig. 1; Dwoskin et al., 2004). In microdialysis studies using rats, the lead analog, *N,N'*-dodecane-1,12-diyl-*bis*-3-picolinium dibromide (C₁₂, bPiDDB), decreased extracellular DA levels in nucleus accumbens following systemic nicotine (Rahman et al., 2007) and decreased intravenous nicotine self-administration (Neugebauer et al., 2006). Herein, we evaluated the bAPi series for their *in vitro* effects on nicotine-evoked DA release at striatal nAChRs and for their ability to inhibit the discriminative stimulus and/or locomotor stimulant centrally-mediated effects of nicotine. These polar and charged analogs are predicted to have poor brain bioavailability following systemic administration. However, previous work with structurally-related polar *mono*-nicotinium analogs revealed good affinity for the blood-brain barrier (BBB) choline transporter and active transport into brain (Allen et al., 2003). Therefore, an additional goal was to characterize these analogs for their affinity for the BBB choline transporter.

METHODS

Chemicals

[³H]Dopamine ([³H]DA; 3,4-ethyl-2-[*N*-³H] dihydroxyphenylethylamine; specific activity 28.0 Ci/mmol) and *S*(-)-[³H]nicotine (*S*(-)-[*N*-methyl-³H]; specific activity 81.0 Ci/mmol) were purchased from PerkinElmer Life Sciences Inc. (Boston, MA). L-Ascorbic acid, 1-[2-(*bis*[4-fluorophenyl]methoxy)ethyl]-4-[3-phenylpropyl]piperazine dihydrochloride (GBR 12909), bovine serum albumin (BSA), catechol, cytosine, decamethonium bromide (DEC), α -D-glucose, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES), hexamethonium chloride (HEX), methyllycaconitine citrate (MLA), *S*(-)-nicotine ditartrate, nomifensine maleate, nudicauline, pargyline hydrochloride and sucrose were obtained from Sigma Chemical Co. (St. Louis, MO). [³H] Methyllycaconitine ([³H]MLA) ([1 α ,4(*S*),6 β ,14 α ,16 β]-20-ethyl-1,6,14,16-tetramethoxy-4-([2-(³H)-methyl-2,5-dioxo-1-pyrrolidinyl]benzoyl)oxy)methyl)aconitane-7,8-diol; specific activity 25.8 Ci/mmol) was purchased from Tocris Cookson Ltd. (Bristol, UK). TS-2 Tissue solublizer and scintillation cocktail were purchased from Research Products International Corp. (Mount Prospect, IL). *d*-Tubocurarine (TBC) was purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals used in the *in vitro* assay buffers were purchased from Fisher Scientific (Pittsburgh, PA). α -CtxMII was synthesized as described previously (Cartier et al., 1996). bAPi salts (Fig. 1, structures 1–7) were synthesized by reacting 3-picoline with a variety of diiodo or dibromoalkanes (Dwoskin et al., 2004). All compounds were characterized by ¹H- and ¹³C-NMR spectroscopy, mass spectroscopy and elemental analysis. Chemical purity of all products was > 98%. bAPi analogs, *S*(-)-nicotine ditartrate, mecamlamine HCl and DH β E hydrobromide (Sigma, St. Louis, MO) were prepared in 0.9% NaCl (saline) for the behavioral experiments. Dilute NaOH was added to the nicotine solution to obtain a pH of ~7.4. All injections were administered *sc* in a volume of 1 ml/kg body weight. Doses of nicotine and the bAPi analogs are expressed as free base weights, and doses of mecamlamine and DH β E are expressed as salt weights.

Neurochemical Experiments

Subjects—Male Sprague-Dawley rats (200–225g) were obtained from Harlan (Indianapolis, IN) and were housed two per cage with free access to food and water in the Division of Laboratory Animal Resources, University of Kentucky. Experimental protocols involving the rats were in accordance with the NIH 1996 *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 Binding Assay—Striata from two rats were dissected, pooled and homogenized in 10 vol of ice-cold modified Krebs-HEPES buffer (in mM: 20 HEPES, 118 NaCl, 4.8 KCl, 2.5 CaCl₂, 1.2 MgSO₄, pH 7.5). Homogenates were incubated at 37°C for 5 min, and centrifuged (27,000g for 20 min at 4°C). Pellets were resuspended in 10 vol of ice-cold MilliQ water and incubated for 5 min at 37°C, followed by centrifugation (27,000g for 20 min at 4°C). Second and third pellets were resuspended in 10 vol of fresh, ice-cold 10% Krebs-HEPES buffer, incubated for 5 min at 37°C, and centrifuged. Final pellets were stored in 10% Krebs-HEPES buffer at –70 °C until assay. Upon assay, final pellets were resuspended in 20 vol of ice-cold MilliQ water, which afforded ~200 µg membrane protein/100 µl, using BSA as the protein standard. Binding assays were performed in duplicate in a final vol of 200 µl Krebs-HEPES buffer containing 200 mM Tris (pH 7.5 at 4°C). Assays were initiated by addition of 100µl membrane suspension to tubes containing final concentrations of 3 nM [³H]nicotine (50 µl) and 1 of 9 analog concentrations (50 µl; 10 nM–1 mM). Cytisine was used as a positive control for these experiments. Nonspecific binding was defined using 10 µM nicotine or cytisine (50 µM). After a 90 min incubation at 4 °C, reactions were terminated by dilution with 3 ml of ice-cold Krebs-HEPES buffer, followed by filtration through Schleicher and Schuell #32 glass fiber filters (presoaked in 0.5% PEI) using a BRANDEL cell harvester (Biomedical Research and Development Laboratories, Inc., Gaithersburg, MD). Filters were rinsed 3 times with 3 ml of ice-cold Krebs-HEPES buffer and transferred to scintillation vials. Scintillation cocktail (3 ml) was added and radioactivity was determined by liquid β-scintillation spectrometry (Packard model B1600 TR Scintillation Counter, Packard Instrument Company, Inc., Downer's Grove, IL).

[³H]MLA Binding Assay—Rat brain (minus cortex and cerebellum) was homogenized in 20 vol of ice-cold hypotonic buffer (in mM: 2 HEPES, 14.4 NaCl, 0.15 KCl, 0.2 CaCl₂ and 0.1 MgSO₄, pH 7.5). Homogenates were incubated at 37 °C for 10 min and centrifuged (29,000g for 17 min at 4°C). Pellets were washed 3 times by resuspension in 20 vol of the same buffer, followed by centrifugation. Final pellets were resuspended in the incubation buffer to yield ~150 µg membrane protein/100 µl. Assays were performed in duplicate, in a final vol of 250 µl of incubation buffer (in mM: 20 HEPES, 144 NaCl, 1.5 KCl, 2 CaCl₂, 1 MgSO₄ and 0.05% BSA, pH 7.5) initiated by addition of 100 µl membrane suspension to 150 µl of sample containing 2.5 nM [³H]MLA and one of nine analog concentrations (10 nM – 1000µM), and incubated for 2 h at room temperature. Nudicauline was used as a positive control for these experiments. Nonspecific binding was defined in the presence of 1 mM nicotine. Assays were terminated by dilution of samples with 3 ml of ice-cold incubation buffer followed by filtration through Schleicher and Schuell #32 glass fiber filters (presoaked in 0.5% PEI) using the cell harvester. Filters were rinsed and processed as previously described.

[³H]DA Overflow Assay—Coronal slices of rat striata (500 µm, 6–8 mg) were incubated for 30 min at 34 °C in Krebs' buffer (in mM: 118 NaCl, 4.7 KCl, 1.2 MgCl₂, 1.0 NaH₂PO₄, 1.3 CaCl₂, 11.1 α-D-glucose, 25 NaHCO₃, 0.11 L-ascorbic acid and 0.004 EDTA, pH 7.4, saturated with 95% O₂/5% CO₂). Slices were incubated for 30 min in buffer containing 0.1 µM [³H]DA and then transferred to glass chambers and superfused (1 ml/min) with buffer containing nomifensine (10 µM), a DA uptake blocker, and pargyline (10 µM), a monoamine oxidase inhibitor, to ensure that [³H]overflow primarily represented [³H]DA, rather than [³H]metabolites. Following superfusion for 60 min, three samples were collected to determine basal [³H]outflow. Slices were then superfused for 60 min in the absence (0 nM; control) or presence of analog (1 nM–10 µM) to determine the ability of the analog to evoke [³H]overflow using a within-subject design. Each slice was exposed to only one analog concentration, which remained in the buffer until the end of the experiment; all concentrations (including the 0 concentration) were exposed to striatal slices from each rat

(i.e., within-subject design). Following 60 min of superfusion, nicotine (10 μ M) was added to the buffer and samples collected for 60 min to determine the ability of the analog to inhibit nicotine-evoked [3 H]DA overflow. At least one striatal slice in each experiment was superfused for 60 min in the absence of analog, followed by superfusion with 10 μ M nicotine to determine nicotine-evoked [3 H]DA overflow in the absence of analog (nicotine control).

To determine if the analog inhibitory effects were specific for nicotine, the ability of bPiOI and bPiDDB (1 nM–10 μ M) to inhibit electrically-evoked [3 H]DA overflow was determined. Each slice was exposed to only one analog concentration and samples collected for 60 min. Electrical stimulation (1 Hz, 2 ms duration for 2 min; total 120 unipolar, rectangular pulses; SD9 stimulator; Grass Instruments, Quincy, MA) was applied and samples collected for 30 min. Stimulation parameters were chosen from a previous parametric analysis to provide [3 H]DA overflow similar to that evoked by 10 μ M nicotine, and resulting [3 H]DA overflow was shown to be calcium-dependent in previous studies (Dwoskin and Zahniser, 1986).

A Schild analysis was performed to determine if the interaction of the representative analog bPiDDB with nAChRs mediating nicotine-evoked DA release was orthosteric or allosteric. Using a repeated measures design, concentration-response curves for nicotine were determined in the absence and presence of one of three bPiDDB concentrations (1, 3 or 10 nM), based on IC₅₀ value for bPiDDB-induced inhibition of nicotine-evoked [3 H]DA overflow. Slices from a single rat were superfused for 60 min in both the absence and presence of one of the bPiDDB concentrations, followed by a 60-min period of superfusion in which one of six nicotine concentrations (0.1–100 μ M) was added to the buffer.

Experiments were conducted to determine if bPiDDB interacts with α -CtxMII-sensitive nAChRs. The concentration response for α -CtxMII was determined. Slices were superfused for 60 min followed by collection of two 4-min samples (2.4 ml/sample) to determine basal [3 H]outflow. Then, slices were superfused for 36 min in the absence (0 nM; control) or presence of α -CtxMII (0.1 nM – 30 nM). Following superfusion in the absence or presence of α -CtxMII, nicotine (10 μ M) was added to the buffer and samples collected for 36 min to determine the ability of the α -CtxMII to inhibit nicotine-evoked [3 H]DA overflow. In subsequent experiments, striatal slices were superfused for 36 min either with buffer only, a maximal concentration of bPiDDB (10 nM), a maximal concentration of α -CtxMII (1 nM), or to concomitant bPiDDB and α -CtxMII at their maximal inhibitory concentrations. Nicotine (10 μ M) was added to the buffer in the absence and presence of bPiDDB and/or α -CtxMII and superfusion continued for 36 min.

[3 H]DA Uptake Assay—Since nAChRs modulate DA transporter (DAT) function (Zhu et al., 2007), the ability of the analogs to interact with these receptors to alter DAT function was assessed. Also, since nomifensine was included in the superfusion buffer in [3 H]DA overflow assays, potential interactions with DAT would not be detected in the DA release assays. Striatum from an individual rat was homogenized in 20 ml of ice-cold 0.32 M sucrose containing 5 mM NaHCO₃ (pH 7.4) with 16 up and down strokes of a Teflon pestle homogenizer (clearance ~0.003 inches). Homogenates were centrifuged at 2000g for 10 min at 4°C and resulting supernatants centrifuged at 20,000g for 17 min at 4°C. Resulting pellets were resuspended in 2.4 ml of ice-cold assay buffer (in mM: 125 NaCl, 5 KCl, 1.5 MgSO₄, 1.25 CaCl₂, 1.5 KH₂PO₄, 10 α -D-glucose, 25 HEPES, 0.1 EDTA, 0.1 pargyline and 0.1 ascorbic acid, saturated with 95% O₂/5% CO₂, pH 7.4). Final protein concentration was ~400 μ g/ml, using BSA as a standard. Assays were performed in duplicate in a total volume of 500 μ l. Synaptosomes (50 μ l containing 20 μ g of protein) were added to tubes containing 350 μ l of buffer and 50 μ l of one of nine concentrations of analog (final concentration, 0.001

nM–100 μ M). GBR 12909 was used as a positive control for these experiments. Samples were incubated at 34°C for 5 min and 50 μ l of 0.1 μ M [3 H]DA was added. Incubations were continued at 34°C for 10 min. Reactions were terminated by addition of 3 ml of ice-cold assay buffer. Samples were filtered through Schleicher and Schuell #32 glass fiber filters (presoaked with 1 mM catechol buffer). Filters were washed 3 times with ice-cold buffer containing 1 mM catechol, transferred to vials, 10 ml of cocktail added, and radioactivity determined by liquid scintillation spectrometry.

Choline Transporter Experiments

Male Fischer 344 rats (250–300 g; Charles River Laboratories, Kingston, NY) were used to allow comparison with previous results obtained with these methods (Lockman et al., 2004). Unpublished results from our laboratory confirmed that results obtained using these assays do not differ significantly between Fischer-344 and Sprague Dawley rats. The *in situ* rat brain perfusion technique used to evaluate the effect of each bAPI analog on BBB transporter uptake of [3 H]choline has been described previously (Lockman et al., 2004). Briefly, a PE-60 catheter filled with heparinized saline (100 units/ml) was placed into the left common carotid artery after ligation of the left external carotid, occipital, and common carotid arteries (common carotid artery ligation was accomplished caudally to the catheter implantation site). The pterygopalatine artery was left open. Rat body temperature was monitored and maintained at 37 °C by a heating pad and feedback device (YSI Indicating Controller, Yellow Springs, Ohio). Buffered physiologic perfusion fluid was titrated to pH 7.4 (~290 mOsm) containing (in mM): 128 NaCl, 2.4 NaPO₃, 29.0 NaHCO₃, 4.2 KCl, 1.5 CaCl, 0.9 MgCl and 9.0 d-glucose. [3 H-Methyl]-choline chloride (80 Ci/mmol; final concentration, 14 nM) and [14 C]-sucrose (4.75 mCi/mmol; final concentration, 650 nM) were obtained from Dupont-New England Nuclear (Boston, MA) and added to the perfusion buffer.

Immediately prior to perfusion, the perfusion fluid containing drug was filtered, warmed to 37 °C and gassed with 95% air and 5% CO₂. Perfusion fluid was infused into the left carotid artery via infusion pump for 60 sec at 10 ml/min (Harvard Apparatus, South Natick, MA). This level of flow maintained carotid artery pressure at ~120 mm Hg. At time T, rats were decapitated, brain rapidly removed from the skull and perfused hemisphere dissected on ice after removal of the arachnoid membrane and meningeal vessels. Brain regions and perfusion fluid samples were digested overnight at 50 °C in 1 ml of 1.0 M piperidine. Scintillation fluid (10 ml, Scintisafe, Fisher Scientific, Pittsburgh, PA) was added to each vial and dual labeled scintillation counting of brain and perfusate samples accomplished with correction for quench, background and efficiency (LS 6500; Beckman Coulter, Fullerton, CA). Experiments were approved by the Texas Tech Animal Care and Use Committee and were conducted in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (1996).

Behavioral Experiments

Behavioral Apparatus—Nicotine discrimination experiments were conducted using six standard operant conditioning chambers (ENV-008, MED Associates, St. Albans, VT). Each chamber was housed in a sound-attenuating enclosure (ENV-018M, Med Associates) and operated by a personal computer interface (SG-502, MED Associates). A 5 × 4.2 cm opening that allowed access to a recessed food tray was located on the front panel of each operant conditioning chamber. Two metal response levers were located on either side of the food tray 7.3 cm above a metal-grid floor. A 28 V, 3-cm diameter, white cue light was centered 6 cm above each response lever.

Locomotor activity experiments were conducted using an automated Digiscan animal activity monitoring system (AccuScan Instruments, Columbus, OH). The system consisted of 12 clear chambers ($42 \times 42 \times 30$ cm) made opaque by attaching sheets of white plastic to their outer surface. Each chamber incorporated a horizontal 16×16 grid of photo beam sensors interfaced to a personal computer operating Digipro System software (v. 1.40, AccuScan Instruments); each beam was spaced 2.5 cm apart and 7.0 cm above the chamber floor. Horizontal activity was measured as photo beam interruptions and expressed as total distance traveled (cm).

Drug Discrimination—Adult male Sprague-Dawley rats (250–275 g at the beginning of the experiment; Harlan Industries), food-restricted to 85–90% of normal weight, were trained initially to respond for sucrose pellets (45 mg Noyes Precision Pellets, Research Diets, Inc., New Brunswick, NJ) under a fixed-ratio (FR) 1 schedule of reinforcement. Sprague-Dawley rats were used for these behavioral experiments to permit comparison to our previous work (Neugebauer et al., 2006). Only one lever was presented each daily session, and the lever presented (i.e., left or right) alternated each day. Over subsequent sessions, the FR value required for reinforcement was incremented to a terminal FR 25. Following 2 sessions of responding under the FR 25 schedule, nicotine discrimination training began during daily, 15-min sessions, occurring 6 days per week (Mon–Sat). From this point on, both levers were present in the chamber each session. Rats were trained to discriminate between injections of nicotine (0.2 mg/kg, sc) and saline by reinforcing responding on one lever (i.e., the nicotine-appropriate lever) following nicotine injections, and by reinforcing responding on the other lever (i.e., the saline-appropriate lever) following saline injections. Responding on the incorrect lever was recorded, but had no programmed consequence. Nicotine or saline was administered 5 min prior to each experimental session. After the rats were placed in the chamber, the cue lights were illuminated to signal the onset of the session. For half of the rats, the left lever was designated as the nicotine-appropriate lever and the right lever was designated the saline-appropriate lever; the reverse was true for the other half of the rats. Daily injections of nicotine and saline were administered according to a double-alternation sequence (i.e., NNSSNN or SSNNSS) that was counterbalanced across rats. Training sessions continued until rats met the following acquisition criteria on 8 of 10 consecutive sessions: (1) the first ratio of the session was completed on the injection-appropriate lever with ≤ 3 responses emitted on the incorrect lever; and (2) $\geq 85\%$ of the total responses emitted on the injection-appropriate lever.

Once acquisition criteria were met, test sessions were conducted twice per week (Tuesday and Friday). Baseline training sessions with the nicotine training dose or saline (random order) were conducted on intervening days. Stimulus conditions during test sessions were identical to training sessions, except that each test session was only 2 min in duration and no reinforcement was available. Prior to testing any novel pretreatments, rats were required to correctly complete 4 test sessions, 2 with nicotine and 2 with saline (counterbalanced order across rats). For all rats ($n=6$), a dose-response curve for nicotine (0.025, 0.05, 0.1, 0.2 or 0.4 mg/kg, sc) was first determined, followed by antagonism tests that determined the dose-response curves for the noncompetitive nAChR antagonist mecamylamine (0.1, 0.3, 0.56, 1.0 or 3.0 mg/kg, sc), the competitive nAChR antagonist DH β E (0.1, 0.3, 0.56, 1.0 or 3.0 mg/kg, sc) and each of the bAPi analogs (0.19–5.8 μ moles/kg, sc). Each antagonist was administered as a pretreatment 15 min prior to the 0.2 mg/kg training dose of nicotine using a Latin Square design to control for potential dose order effects. The 15-min pretreatment interval and dose range for the bAPi pretreatments was based on prior work from our laboratory (Neugebauer et al., 2006). Finally, substitution tests were conducted in which the 5.8 μ moles/kg dose of each *N,N'*-alkane-diyl-*bis*-3-picolinium analog was administered prior to saline to assess whether any of these analogs produced nicotine-like discriminative stimulus effects. Two dependent measures were collected during each test session: (1)

percentage of total responses occurring on the nicotine-appropriate lever (calculated as the number of responses on the nicotine-appropriate lever divided by the total number of responses on both levers $\times 100$); and (2) rate of responding in sec (calculated as the total number of responses on both levers divided by 120 sec). Lever selection data were excluded for rats that failed to complete one full ratio (FR 25) during a test session; however, all data were included for analysis of response rates.

Locomotor Activity—Adult male Sprague-Dawley rats (225–250 g at the beginning of the experiment; Harlan Industries) were sensitized initially to the locomotor stimulant effect of nicotine by administering nicotine (0.4 mg/kg, sc) on 21 consecutive days; locomotor activity was monitored for 60 min immediately following each nicotine injection. After completion of this repeated nicotine treatment phase, a test phase commenced on the following day (Day 22). For this phase of the experiment, rats ($n=6$ per group) were assigned randomly to receive pretreatment with mecamylamine (0.3–3 mg/kg, sc), DH β E (0.3–3 mg/kg, sc) or one of the bAPi analogs (0.58–5.8 μ moles/kg, sc). All doses were administered according to a within-subject Latin Square design to control for potential dose order effects. On the first day of the test phase (Day 22), the first pretreatment dose for a given rat was administered 15 min prior to nicotine (0.4 mg/kg), and locomotor activity was assessed for 60 min after nicotine administration. Over the next two days, rats were injected with nicotine (0.4 mg/kg) only; locomotor activity was assessed only during the second of these two nicotine maintenance days. On the following day (Day 25), the next pretreatment dose was administered; this 3-day cycle was repeated until testing of each analog dose was completed. Each rat was tested with only mecamylamine, DH β E or one of the bAPi analogs.

In a separate group of rats, the effect of each bAPi analog ($n=6$ per group) given alone following nicotine sensitization (21 days) was also assessed. The procedure was identical to that described above, with the exception that the bAPi analogs were administered prior to saline (no nicotine) on the test days. The dependent measure for locomotor activity experiments was the total horizontal distance traveled (cm). Protocols for all behavioral experiments were in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (1996) and were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

Data Analysis

For analog-induced inhibition of [3 H]nicotine and [3 H]MLA binding, data are expressed as fmol/mg protein and shown as a percent of control, i.e., specific [3 H]nicotine or [3 H]MLA binding in the absence of analog. Data were fit by a one-site, variable slope model, using nonlinear least-squares regression, such that $Y = B_t + ((T_p - B_t)/(1 + 10^{(\log IC_{50} - X) \cdot n}))$, where Y = specific [3 H]nicotine or [3 H]MLA binding, X = logarithm of analog concentration, B_t and T_p = minimum and maximum specific [3 H]nicotine or [3 H]MLA binding respectively, $\log IC_{50}$ = $\log$ [analog] which decreased [3 H]nicotine or [3 H]MLA binding by 50%, and n = the pseudo-Hill coefficient. Analog affinity constants (K_i values) were calculated from IC_{50} values using the Cheng-Prusoff equation.

In [3 H]DA overflow assays, fractional release in each sample was expressed as a percentage of total tissue tritium. Total [3 H]overflow was calculated by summing the increases of [3 H] above the basal [3 H]outflow resulting from exposure to the analog, α -CtxMII, nicotine, or the combination, and subtracting the basal [3 H]outflow for an equivalent period of analog exposure. Data were expressed as percent of nicotine control and were fitted by nonlinear least-squares regression using a variable slope, sigmoidal function. IC_{50} values for analog- or α -CtxMII-induced inhibition of nicotine-evoked [3 H]DA overflow were determined using PRISM version 4.0 (GraphPad Software, Inc., San Diego, CA). The mechanism by which

bPiDDB inhibited nicotine-evoked [^3H]DA overflow was determined using Schild analysis. Nicotine concentration response curves in the absence and presence of bPiDDB were generated by nonlinear fit of the data to a sigmoidal dose-response equation (variable slope): $\text{response} = B_t + (T_p - B_t) / [1 + 10^{(\log \text{EC}_{50} - X) \cdot n}]$, where X is the logarithm of the nicotine concentration and n is the Hill slope. Linear regression was used to generate the slopes and x-intercepts of the lines, which were compared statistically for parallelism. For each experiment, the dose ratio (dr) for each concentration of bPiDDB was calculated as that producing an equivalent response in the absence and presence of bPiDDB. The log of $\text{dr} - 1$ was plotted as a function of log of bPiDDB concentration to provide the Schild regression. Data were fit by linear regression, the slope determined, and linearity assessed. The effect of analog on [^3H]DA overflow and its ability to inhibit nicotine-evoked [^3H]DA overflow were analyzed by one-way repeated-measures analysis of variance (ANOVA), where analog or α -CtxMII concentration was a within-subjects factor (SPSS version 12.0, Chicago, IL). A two-way repeated measures ANOVA was performed to analyze the time course of analog or α -CtxMII inhibition of nicotine-evoked increase in the fractional release. If the two-way ANOVA revealed a significant concentration $\times$ time interaction, then one-way repeated measures ANOVAs were performed to determine the specific time points at which a concentration-dependent effect occurred. The ability of analogs to inhibit electrical field stimulation-evoked [^3H]DA overflow was analyzed *via* one-way repeated-measures ANOVA. The inhibitory effect of the combination of bPiDDB and α -CtxMII were compared to the inhibition produced by analog or α -CtxMII alone using a one-way ANOVA. For all analyses, differences were considered significant when $p < 0.05$.

For [^3H]DA uptake assays, data are expressed as pmol/min/mg protein as a percent of control ([^3H]DA uptake in the absence of analog) and were fit using a one-site, variable slope model, and a non-linear, non-weighted least-squares regression. The $\log \text{IC}_{50} = \log[\text{analog}]$ required to inhibit [^3H]DA uptake into striatal synaptosomes by 50%. Analog affinity constants (K_i values) were calculated from IC_{50} values using the Cheng-Prusoff equation.

In the choline transporter experiments, concentrations of [^3H]choline in brain and perfusion fluid were expressed as dpm/g brain or dpm/ml perfusion fluid, respectively. [^3H]Choline uptake into brain was unidirectional for at least 60 sec; K_{in} was quantified in single time-point experiments using the equation: $K_{in} = [Q^* - V_o C^*] / C^* T$, where Q^* is the quantity of [^3H]tracer in brain (dpm/g) at the end of perfusion, C^* is the perfusion fluid concentration of [^3H]choline (dpm/ml), T is the perfusion time (sec) and V_o is the extrapolated intercept ($T = 0$ sec; "vascular volume" in ml/g). In addition, [^{14}C]sucrose was incorporated into the perfusion fluid as a small molecular weight impermeant vascular marker to verify integrity of the BBB in each experiment. Vascular bound [^3H]choline was removed by a brief intravascular wash (15 sec) with tracer-free perfusion fluid. K_{in} values were converted to an apparent cerebrovascular permeability-surface area product (PS) using the Crone-Renkin equation: $PS = -F \ln (1 - K_{in}/F)$, where F is the cerebral perfusion fluid flow. In all instances, PA differed by $< 2\%$ from K_{in} , because F exceeded K_{in} by > 40 -fold. An apparent inhibition constant (K_i) for the analogs was determined from the equation: $[(PS_o - K_D) / (PS_i - K_D)] = 1 + C_i / K_i$ assuming competitive kinetics where PS_o is the [^3H]choline PS in the absence of competitor, PS_i is the [^3H]choline PS in the presence of inhibitor, and C_i is the perfusate concentration of inhibitor (250 μM). Apparent K_i was defined as the analog concentration that reduced saturable brain [^3H]choline influx by 50% at tracer choline concentration ($C_{pf} \ll K_m$).

For behavioral experiments, analyses of variance (ANOVA) for repeated measures, with dose as the within-subjects factor, were conducted on raw data. When the dose factor attained significance, a Dunnett's post-hoc test was used to compare the effect of each drug

dose to the saline control. For the drug discrimination experiment, ED₅₀ and AD₅₀ values ($\pm$ 95% confidence interval, CI) were determined, where appropriate, using nonlinear regression and were expressed as the dose estimated to produce 50% nicotine-appropriate responding (ED₅₀) or the antagonist dose estimated to reduce levels of nicotine-appropriate responding by the training dose to 50% (AD₅₀). Data were analyzed with SPSS v.12 (SPSS Inc., Chicago, IL, USA) and Prism v.4 (GraphPad Software, Inc., San Diego, CA, USA). Statistical significance was declared at $p < 0.05$.

RESULTS

Neurochemical Experiments

Inhibition of [³H]Nicotine and [³H]MLA Binding—Analogues were evaluated for their ability to inhibit [³H]nicotine and [³H]MLA binding to rat striatal membranes to determine affinity for the ligand binding site on $\alpha 4\beta 2^*$ and $\alpha 7^*$ nAChRs, respectively (Fig. 2). The bAPi analogs demonstrated low affinity for both $\alpha 4\beta 2^*$ and $\alpha 7^*$ nAChRs ($K_i = 49 \mu\text{M}$ to greater than $100 \mu\text{M}$). Cytisine and nudicauline were used as positive controls for the [³H]nicotine and [³H]MLA binding assays, respectively, and were found to have K_i values of $0.43 \pm 0.06 \text{ nM}$ and $1.58 \pm 0.30 \text{ nM}$, respectively. The classical quaternary ammonium compounds, DEC, TBC and HEX also showed low affinity at the [³H]nicotine binding site ($K_i = 5.7, 9.7$ and $22 \mu\text{M}$, respectively) and at the [³H]MLA binding site (K_i of $>100, >100$ and $4.8 \mu\text{M}$, respectively); results not shown.

Inhibition of Nicotine-Evoked [³H]DA Overflow—The ability of the analogs to evoke [³H]DA overflow from superfused rat striatal slices preloaded with [³H]DA was determined across a range of analog concentrations (1 nM – $10 \mu\text{M}$). None of the analogs evoked [³H]DA overflow across this concentration range (Table 1). Concentration response curves for the bAPi analogs (C_{6–12}) to inhibit nicotine-evoked [³H]DA overflow from rat striatal slices are illustrated in Fig. 3 (top panel). With the exception of bPiHxI (C₆), which showed no inhibition, one-way ANOVAs revealed significant concentration-dependent inhibition for each analog [bPiHpB (C₇), $F_{7,33} = 15.534$, $P < 0.001$; bPiOI (C₈), $F_{5,19} = 4.441$, $P = 0.007$; bPiNB (C₉), $F_{5,19} = 4.053$, $P = 0.011$; bPiDI (C₁₀), $F_{5,23} = 7.346$, $P < 0.001$; bPiUB (C₁₁), $F_{5,28} = 11.750$, $P < 0.001$; bPiDDB (C₁₂), $F_{5,23} = 13.282$, $P < 0.001$]. Dunnett's t -test revealed that bPiHpB (0.001 – $10 \mu\text{M}$), bPiOI (1 and $10 \mu\text{M}$), bPiNB ($10 \mu\text{M}$), bPiDI (1 and $10 \mu\text{M}$), bPiUB (1 and $10 \mu\text{M}$) and bPiDDB (0.001 – $10 \mu\text{M}$) significantly inhibited nicotine-evoked [³H]DA overflow when compared to the within-subject control (i.e., nicotine-evoked [³H]DA overflow in the absence of analog). A wide range of IC₅₀ values (2 nM to greater than $100 \mu\text{M}$) was observed among the bAPi analogs (Table 2). The most potent analog, bPiDDB (C₁₂), inhibited nicotine-evoked [³H]DA overflow with an IC₅₀ of 2 nM . bPiHpB (C₇) potently inhibited nicotine-evoked DA release with an IC₅₀ value of 60 nM . bPiDI (C₁₀) and bPiOI (C₈) exhibited intermediate IC₅₀ values of 180 and 300 nM , respectively; whereas bPiUB (C₁₁) and bPiNB (C₉) displayed lower potencies (IC₅₀= 1.12 and $5.81 \mu\text{M}$, respectively). Thus, the overall rank order of potency for the seven bAPi analogs to inhibit nicotine-evoked [³H]DA overflow was bPiDDB>bPiHpB>>bPiDI=bPiOI>>bPiUB>bPiNB >>bPiHxI, revealing a lack of correlation with the length of the methylene groups in the n -alkane linker ($r^2 = 0.3782$, $p = 0.1416$). Of note, these analogs did not inhibit nicotine-evoked [³H]DA overflow completely, and demonstrated maximal inhibition (Imax) in the range 54 – 64% .

The time course of the bPiDDB-induced inhibition of nicotine-evoked [³H]DA overflow is illustrated in Fig. 3 (bottom panel). Fractional release evoked by $10 \mu\text{M}$ nicotine reached a maximum 10 min after the addition of nicotine to the buffer and then gradually decreased, despite the presence of nicotine in the buffer throughout the remainder of the experiment.

Repeated measures two-way ANOVA revealed significant main effects of bPiDDB concentration ($F_{5, 25} = 17.605$, $p < 0.001$) and time ($F_{11, 53} = 67.742$, $p < 0.001$), and the concentration $\times$ time interaction was also significant ($F_{55, 249} = 1.868$, $p = 0.001$). Post hoc analysis revealed that 0.1, 1 and 10 μ M bPiDDB significantly inhibited nicotine-evoked fractional DA release across the time period from 15–60 min. Lower bPiDDB concentrations resulted in significant inhibition during a portion of the time course (at 25 min and 30 min with 1 nM; at 15–35 min and 50–55 min with 10 nM).

Field Stimulation-Evoked [3 H]DA Overflow—The ability of two representative *N,N'*-alkane-diyl-*bis*-3-picolinium analogs, bPiOI (C_8) and bPiDDB (C_{12}), to inhibit electrical field stimulation-evoked [3 H]DA overflow was determined to assess specificity of the inhibition of nicotine to evoke DA release. Consistent with the previous data, neither bPiOI or bPiDDB evoked [3 H]DA overflow prior to electrical stimulation (results not shown). At a single concentration (0.1 μ M), bPiOI inhibited electrically-evoked [3 H]DA overflow; however, inhibition was not concentration-dependent (Table 3). Importantly, bPiDDB did not inhibit field stimulation-evoked [3 H]DA overflow at any concentration examined.

Mechanism of bPiDDB Inhibition of Nicotine-evoked [3 H]DA Overflow—The mechanism of bPiDDB inhibition was assessed, as this analog was the most potent in the bAPi series for inhibition of nicotine-evoked [3 H]DA overflow. A Schild analysis was performed, such that for each experiment, striatal slices from a single rat were superfused with each of six concentrations of nicotine (0.1–100 μ M) in the absence and presence of one of three concentrations of bPiDDB (1 nM, 3 nM or 10 nM). Rightward shifts in the nicotine concentration-response curve were evident with increasing concentrations of bPiDDB, and inhibition was surmounted by increasing concentrations of nicotine (Fig. 4). The slopes of the linear portions of the curves were not different ($F_{3,196} = 2.150$, $p = 0.0953$), whereas the x-intercepts were different ($F_{3,199} = 11.119$, $p < 0.0001$), indicating that the curves are parallel. A linear fit ($r^2 = 0.92$) to the Schild-transformed data revealed a slope (1.2 ± 0.36) not different from unity (Fig. 4, inset). Further analysis revealed that the dose ratios were independent of response ($t_{42} = 18.8$, $p < 0.0001$). These results are consistent with bPiDDB acting as an orthosteric inhibitor of nAChRs mediating nicotine-evoked DA release.

The bAPi analogs produced incomplete inhibition of nicotine-evoked [3 H]DA overflow ($I_{max} = 54$ –64%; Table 2). In order to test the hypothesis that the representative analog, bPiDDB, interacts with nAChR subtypes sensitive to α -CtxMII, experiments were conducted to compare responses to concentrations of bPiDDB and α -CtxMII, which maximally inhibited nicotine-evoked [3 H]DA overflow, when each antagonist was presented alone and in combination. Initially, the concentration at which α -CtxMII produced maximal inhibition of nicotine-evoked [3 H]DA overflow was determined. Under the current experimental conditions, α -CtxMII inhibited nicotine-evoked [3 H]DA overflow in a concentration-dependent manner ($F_{5,15} = 5.65$, $p < 0.005$; $IC_{50} = 0.20 \pm 0.12$ nM, $I_{max} = 62 \pm 7.1\%$; Fig. 5). Post hoc tests revealed that 0.1 – 30 nM α -CtxMII significantly inhibited nicotine-evoked [3 H]DA overflow compared to the within-subject control (i.e., nicotine-evoked [3 H]DA overflow in the absence of the toxin). Concomitant exposure of rat striatal slices to maximally effective concentrations of bPiDDB (10 nM) and α -CtxMII (1 nM) resulted in significant inhibition of nicotine-evoked [3 H]DA overflow compared to the within-subject control ($F_{3,30} = 5.27$, $p < 0.01$; Fig. 6). Importantly, inhibition of nicotine-evoked [3 H]DA overflow resulting from concomitant exposure was not different ($p > 0.05$) from that produced by either antagonist alone. The time course of the response to nicotine shows clearly that the inhibition produced by the antagonists presented concomitantly was not different from that following either bPiDDB or α -CtxMII alone (Fig. 6 inset).

[³H]DA Uptake into Rat Striatal Synaptosomes—The ability of the bAPi analogs to inhibit [³H]DA uptake via DAT was determined using striatal synaptosomes (Fig. 7). None of bAPi analogs examined inhibited [³H]DA uptake across the concentration range 0.001 μM–100 μM, although inhibition of [³H]DA uptake was observed with the positive control, GBR 12909 ($K_i = 0.98 \pm 0.10$ nM). These results indicate that the ability of bAPi analogs to inhibit nicotine-evoked [³H]DA overflow did not result from inhibition of DAT function.

BBB Choline Transporter Experiments

Fig. 8 shows that the addition of 250 μM of each of the bAPi analogs in separate experiments significantly reduced the permeability of [³H]choline ($12.6 \pm 0.38 \times 10^{-4}$ mL/s/g). While all of the analogs produced greater inhibition than the natural substrate choline, there was no direct correlation between carbon chain linker length and ability to inhibit [³H]choline permeability. The [³H]choline PS reduction was lowest for bPiHxI (C_6 ; $7.7 \pm 0.14 \times 10^{-4}$ mL/s/g), bPiNB (C_9 ; $8.7 \pm 0.1 \times 10^{-4}$ mL/s/g), and bPiUB (C_{11} ; $7.9 \pm 0.4 \times 10^{-4}$ mL/s/g), whereas it was greatest for the C_{12} chain linker bPiDDB ($1.37 \pm 0.19 \times 10^{-4}$ mL/s/g). Intermediate inhibition of [³H]choline permeability was obtained with bPiHpB (C_7), bPiOI (C_8) and bPiDI (C_{10}). Apparent inhibition constants presented in Table 4 were calculated from the reduction of [³H]choline BBB PS shown in Fig. 8, according to Equation 3, estimating the relative affinity of each analog for the choline transporter. The vascular volume of [¹⁴C]-sucrose with a 60-sec perfusion was 0.01 ml/g or less in each experiment.

Behavioral Experiments

Drug Discrimination—Figs. 9a and 9b illustrate the dose effect of nicotine for percent nicotine-appropriate responding and rate of responding. As expected, nicotine elicited dose-dependent substitution for the nicotine training dose ($F_{5,35} = 61.79$, $p < 0.001$), with an $ED_{50} = 0.08$ (0.06–0.09) mg/kg. Nicotine at doses of 0.1, 0.2 and 0.4 mg/kg produced significantly greater levels of nicotine-appropriate responding relative to saline ($p < 0.01$). Only the highest nicotine dose (0.4 mg/kg) significantly reduced the rate of responding ($p < 0.05$).

Figs. 9c and 9d illustrate the dose effect of mecamylamine and DHβE administered 15 min prior to nicotine (0.2 mg/kg) for percent nicotine-appropriate responding and rate of responding. Mecamylamine dose-dependently and completely inhibited nicotine discrimination ($F_{5,35} = 30.23$, $p < 0.001$), with an $AD_{50} = 0.57$ (95% CI, 0.49–0.64) mg/kg. Similarly, DHβE dose-dependently and completely inhibited nicotine discrimination ($F_{5,35} = 29.17$, $p < 0.001$), with an $AD_{50} = 0.66$ (0.49–0.89) mg/kg. Complete inhibition of the nicotine cue was obtained with both mecamylamine and DHβE at doses that had no significant effect on the rate of responding.

In contrast to mecamylamine and DHβE, Figs. 9e and 9f illustrate that none of the bAPi analogs significantly altered nicotine discrimination, even when doses were administered that disrupted responding. Further, in substitution tests, none of the bAPi analogs at the 5.8 μmoles/kg dose elicited > 8% nicotine-appropriate responding (results not shown), indicating that these analogs lack nicotine-like discriminative stimulus effects.

Locomotor Activity—Across the initial 21-day repeated administration period, nicotine-induced hyperactivity increased progressively before reaching asymptotic levels on Day 16 (results not shown). Fig. 10 shows the dose effect of pretreatment with mecamylamine or DHβE on locomotor activity following nicotine administration in rats previously sensitized to the locomotor stimulant effect of nicotine. Nicotine-induced hyperactivity was evident by

elevated activity levels in nicotine-treated rats given nicotine alone on the test day (i.e., no mecamlamine or DH β E pretreatment), compared to nicotine-treated rats given saline alone on the test day (results not shown). For mecamlamine, ANOVA revealed a significant main effect of dose ($F_{3,15}=11.50$, $p < 0.01$), with 1 and 3 mg/kg of mecamlamine significantly reducing activity relative to saline pretreatment. For DH β E, ANOVA also revealed a significant main effect of dose ($F_{3,15}=5.20$, $p < 0.05$), with 1 and 3 mg/kg of DH β E significantly reducing activity relative to saline pretreatment. Thus, acute administration of both of these classical nAChR antagonists inhibited the locomotor stimulant effect of nicotine following repeated nicotine treatment.

Fig. 11 shows the dose effect of pretreatment with each of the bAPi analogs on activity following nicotine or saline in rats sensitized previously to the locomotor stimulant effect of nicotine. Nicotine-induced hyperactivity was evident as a significant increase in activity in rats given nicotine alone (no analog pretreatment) compared to rats given saline on the test day. Separate ANOVAs of the data from each analog in the presence of nicotine revealed significant main effects of dose for bPiOI pretreatment ($F_{3,15}=53.79$, $p < 0.001$), bPiNB pretreatment ($F_{3,15}=14.93$, $p < 0.001$), bPiDI pretreatment ($F_{3,15}=119.14$, $p < 0.001$), and bPiDDB pretreatment ($F_{3,15}=6.86$, $p < 0.01$); there was no significant effect of dose for bPiHxI, bPiHpB or bPiUB. Dunnett's tests revealed further that pretreatment with 5.8 μ moles/kg of bPiOI, 5.8 μ moles/kg of bPiNB, 1.9 and 5.8 μ moles/kg of bPiDI, and 1.9 and 5.8 μ moles/kg of bPiDDB significantly reduced activity relative to saline ($p < 0.05$). Within the dose range tested, inhibition of nicotine-induced hyperactivity was greatest with bPiOI and bPiDI. For both of these analogs, 5.8 μ moles/kg significantly decreased activity to a level below that observed in nicotine-sensitized rats given saline on the test day. In contrast to the effect of the analogs tested in the presence of nicotine, separate ANOVAs indicated no significant effects of any of the analogs tested following saline (Fig. 11, open circles).

DISCUSSION

The current study describes the development of bAPi analogs with interconnecting alkane C₆-C₁₂ linkers as antagonists at nAChRs mediating nicotine-evoked DA release and their effects on nicotine-stimulated behaviors in rats. In contrast to the structurally-related C₁₀ and C₁₂ *mono*-quaternary nicotinium analogs, which exhibited high affinity for $\alpha 4\beta 2^*$ nAChRs (Wilkins et al., 2003), the bAPi analogs displayed a lack of interaction at high affinity agonist binding sites on $\alpha 4\beta 2^*$ or $\alpha 7^*$ nAChRs. Thus, incorporation of a second cationic head group into the *mono*-quaternary structure eliminates interaction with high affinity agonist binding sites at $\alpha 4\beta 2^*$ and $\alpha 7^*$ nAChRs.

The bAPi analogs potently inhibited nicotine-evoked [³H]DA overflow, displaying a wide range of IC₅₀ values and incomplete maximal inhibition. Rank order of inhibitory potency was bPiDDB > bPiHpB >> bPiDI = bPiOI >> bPiUB > bPiNB >>> bPiHxI. bPiDDB (C₁₂) displayed the highest potency and incomplete maximal inhibition and was regarded as a lead compound for mechanistic evaluation. With the exception of bPiHxI, bAPi analogs were more potent than *bis*-quaternary ammonium antagonists, HEX and DEC, and the related compound, TBC (Grady et al., 1992). Schild analysis of bPiDDB inhibition revealed rightward shifts in the nicotine concentration-response curves and that increasing nicotine concentrations surmounted the inhibitory effect of bPiDDB. Slopes for the linear portions of the curves did not differ; however, x-intercepts were significantly different, indicating that parallelism was obtained. A linear fit to the Schild-transformed data revealed a slope not different from unity. At high nicotine concentrations (5 mM), mecamlamine inhibits DA release by ~50% (Grady et al., 1992), indicating that part of this effect is mediated by non-nAChR mechanisms; whereas nicotine concentrations of 500 μ M were completely inhibited by mecamlamine. Thus, some caution must be exercised with respect to the interpretation

of the current results across high nicotine concentrations. Despite this potential caveat, the results are consistent with the hypothesis that bPiDDB inhibits nAChRs mediating nicotine-evoked [^3H]DA overflow in an orthosteric manner.

bAPi analogs exhibited incomplete maximal inhibition of nicotine-evoked DA release from rat striatal slices, similar to results reported with α -CtxMII using rat striatal synaptosomes (Kulak et al., 1997), suggesting that at least two subtypes of nAChRs mediate nicotine-evoked DA release. Previous studies using knockout mice have also shown the critical involvement of β 2-containing subunits in subtypes mediating nicotine-evoked DA release (Picciotto et al., 1998). Six different β 2-containing nAChR subtypes have been suggested to mediate nicotine-evoked DA release in mouse striatum: α 4 β 2* and α 4 α 5 β 2* (not α -CtxMII-sensitive), and α 6 β 2*, α 6 β 2 β 3*, α 4 α 6 β 2* and α 4 α 6 β 2 β 3* (α -CtxMII-sensitive) (Salminen et al., 2004). The predominant subtype constituting ~50% of α 6-containing nAChRs mediating nicotine-evoked DA release in mouse striatum was α 4 α 6 β 2 β 3* (Salminen et al., 2007). Some caution is needed in extrapolating these results from mice to rats, because the impact of nAChRs on catecholamine release in the terminal fields differs between these species (Scholze et al., 2007).

The current results support the interpretation that bPiDDB interacts with α -CtxMII-sensitive, α 6 β 2-containing nAChR subtypes. In the current study, α -CtxMII inhibited (IC_{50} =0.20 nM; Imax =62%) nicotine-evoked DA release from rat striatal slices and was ~5-fold more potent and produced greater inhibition than previously reported (Imax =34–49%, Kulak et al., 1997). Discrepancies between studies may be due to different striatal preparations (slice vs synaptosomes), duration of α -CtxMII exposure, and/or concentration and duration of nicotine exposure. Nevertheless, concomitant exposure of rat striatal slices to maximally effective bPiDDB and α -CtxMII concentrations resulted in inhibition of nicotine-evoked DA release no greater than that produced by either antagonist alone, suggesting that bPiDDB and α -CtxMII inhibit the same nAChR subtypes. The nonselective antagonist, mecamylamine was shown to nearly completely inhibit (Imax =91%) nicotine-evoked DA release (Teng et al., 1997). Thus, these results support the interpretation that similar to α -CtxMII, bPiDDB is a selective high potency antagonist at a subset of nAChR subtypes containing α 6 and β 2, and likely inhibits α 6 β 2*, α 6 β 2 β 3*, α 4 α 6 β 2* and/or α 4 α 6 β 2 β 3*.

Although α 4- and β 2-containing nAChRs have been implicated in nicotine-evoked striatal DA release (Picciotto et al., 1998; Marubio et al., 2003), bAPi analogs showed little affinity for high affinity nicotine binding sites on α 4 β 2* nAChRs. Based on the Schild analysis, bAPi analogs may interact in an orthosteric manner at an alternative, lower affinity nicotine binding sites, perhaps in the channel of α 4 β 2* nAChRs, which would not be detected by [^3H]nicotine binding assays. However, results from the interaction study with α -CtxMII suggest that the subtypes mediating nicotine-evoked DA release are α -CtxMII-sensitive, which does not include α 4 β 2* nAChRs.

bAPi analogs also do not appear to interact with α 7* nAChRs, as these nAChRs likely are not involved in nicotine-evoked striatal DA release, although an indirect mechanism has been described (Kaiser and Wonnacott, 2000). α 7* nAChRs, detected using [^{125}I] α -bungarotoxin binding and immunoprecipitation, comprise only a small percentage of nAChRs in rat striatum (Zoli et al., 2002). Moreover, α 7* gene deletion has no effect on nicotine-evoked [^3H]DA release from striatal synaptosomes (Salminen et al., 2004). Moreover, α 7* antagonists do not inhibit nicotine-evoked [^3H]DA release from rat striatal slices (Cao et al., 2005), indicating that α 7* nAChRs do not play a major role in mediating this response.

The specificity of bAPi analogs to inhibit nicotine-evoked DA release was assessed by determining their ability to inhibit field-stimulation evoked DA release. Field-stimulation parameters were chosen to provide calcium-dependent DA release in an amount similar to that evoked by nicotine (Dwoskin and Zahniser, 1986). bPiDDB did not inhibit field-stimulation evoked DA release, indicating that inhibition of nicotine-evoked DA release is likely specific for nAChRs. These results indicate that bPiDDB exposure did not result in toxicity at DA neurons, since subsequent field-stimulation evoked DA release was not different from control.

Nicotine has been shown to enhance DAT function (Zhu et al., 2007), and nAChR antagonists may alter DAT function. However, results from synaptosomal [³H]DA uptake assays revealed that bAPi analogs do not inhibit [³H]DA uptake, indicating that bAPi analogs do not alter DAT function.

The current results also show that bAPi analogs have affinity for the BBB choline transporter. Vascular volume following exposure to each analog was shown to be <0.01 ml/g, indicating no BBB disruption (Lockman et al., 2004). bPiDDB exhibited the highest affinity ($K_i=5\text{ }\mu\text{M}$) for the BBB choline transporter. bPiHpB, bPiOI and bPiDI were also potent ($K_i<125\text{ }\mu\text{M}$) and were within the range suggestive of transport into brain via the BBB choline transporter.

Although nonselective nicotinic antagonists have been shown to block nicotine discrimination (Zakharova et al., 2005), bAPi analogs had no effect. Nonselective antagonists block the full complement of $\beta 2$ -containing nAChRs mediating nicotine-evoked DA release, whereas bAPi analogs inhibit only a subset of $\beta 2$ -containing nAChRs, thus partially inhibiting nicotine-evoked DA release. Importantly, however, the discriminative stimulus effect of nicotine does not appear to depend on DA-mediated mechanisms. Pretreatment with DA receptor antagonists does not block the nicotine cue (Corrigall and Coen, 1994; Le Foll et al., 2005). Thus, the difference between nonselective nAChR antagonists and the bAPi analogs in altering the discriminative stimulus effects of nicotine is not likely explained by their differential ability to inhibit nicotine-evoked DA release.

In contrast to the discriminative stimulus effect of nicotine, previous work points to a critical role of DA systems in mediating the locomotor stimulant effect of nicotine. Nicotine-induced hyperactivity is inhibited by local infusion of the DA D_2 antagonist eticlopride into nucleus accumbens (Boye et al., 2001) and 6-hydroxydopamine into nucleus accumbens or ventral tegmental area (Louis and Clarke, 1998). Locomotor sensitization produced by repeated nicotine administration is also associated with enhanced nicotine-evoked DA release in NAcc (Benwell and Balfour, 1992). In the current report, both mecamylamine and DH β E reversed nicotine-induced hyperactivity in nicotine-sensitized rats. Similarly, within the dose range tested, bPiOI, bPiNB, bPiDI and bPiDDB decreased nicotine-induced hyperactivity. Since none of the analogs reduced locomotor activity when co-administered with saline in nicotine-sensitized rats, the decrease in nicotine-induced hyperactivity was not likely due to nonspecific motor impairment. Instead, the attenuation in nicotine-induced hyperactivity produced by the bAPi analogs likely reflects a specific blockade of nicotine-evoked DA release. However, this conclusion must be tempered because a drug naive (no nicotine exposure) control group was not included in the experimental design to rule out completely nonspecific motor impairment.

Among the bAPi analogs evaluated, bPiOI and bPiDI administered with nicotine decreased nicotine-induced hyperactivity in sensitized rats to a level below the saline control value. In contrast, bPiOI and bPiDI did not alter activity when administered with saline in sensitized rats. Since both nicotine and nicotine-associated environmental cues may serve as

conditioned stimuli (Murray and Bevins, 2007), the novel analogs may have had a greater effect in the presence of nicotine than in the presence of saline because they blocked the compound cue elicited by nicotine and the nicotine-associated locomotor context. This is interesting since both nicotine and nicotine-associated cues elicit nicotine-seeking behavior in rats (Liu et al., 2007). Future experiments should determine whether these novel bAPi analogs would be useful for preventing cue-dependent relapse to tobacco smoking in animal models and clinical populations.

Acknowledgments

The authors thank Agripina G. Deaciuc, Joshua Cutshall, David Eaves, Laura Fenton, Rajendar K. Mattapalli, Lisa Price and Carmen Enid Ruiz for their technical assistance. We also acknowledge the expert consultation of Terry P. Kenakin, Ph.D., William A. Corrigall, Ph.D and David D. Allen, Ph.D.

This research was supported by NIH grants K02 DA00399, T32 DA007304 and U19 DA017548.

Nonstandard Abbreviations

ANOVA	analysis of variance
bAPi	<i>N,N'</i> -alkane-diyl- <i>bis</i> -3-picolinium
BBB	blood brain barrier
bPiDDB	<i>N,N'</i> -dodecane-1,12-diyl- <i>bis</i> -3-picolinium dibromide
bPiDI	<i>N,N'</i> -decane-1,10-diyl- <i>bis</i> -3-picolinium diiodide
bPiHpB	<i>N,N'</i> -heptane-1,7-diyl- <i>bis</i> -3-picolinium dibromide
bPiHxI	<i>N,N'</i> -hexane-1,6-diyl- <i>bis</i> -3-picolinium diiodide
bPiNB	<i>N,N'</i> -nonane-1,9-diyl- <i>bis</i> -3-picolinium dibromide
bPiOI	<i>N,N'</i> -octane-1,8-diyl- <i>bis</i> -3-picolinium diiodide
bPiUB	<i>N,N'</i> -undecane-1,11-diyl- <i>bis</i> -3-picolinium dibromide
BSA	bovine serum albumin
CI	95% confidence intervals
α-CtxMII	α -Conotoxin MII
DA	dopamine
DAT	dopamine transporter
DEC	decamethonium bromide
DHβE	dihydro- β -erythroidine
HEPES	<i>N</i> -(2-hydroxyethyl)piperazine- <i>N'</i> -(2-ethanesulfonic acid)
HEX	hexamethonium chloride
MLA	methyllycaconitine
nAChR	neuronal nicotinic acetylcholine receptor
NDDNI	<i>N-n</i> -dodecylnicotinium iodide
NDNI	<i>N-n</i> -decylnicotinium iodide
NEcPB	<i>N-n</i> -eicosylpyridinium bromide
NONI	<i>N-n</i> -octylnicotinium iodide

NPDPB	<i>N-n</i> -pentadecylpyridinium bromide
PEI	polyethylenimine
S.E.M	standard error of the mean
TBC	<i>d</i> -tubocurarine
*	indicates putative nAChR subtype assignment

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- 1** N,N'-hexane-1,6-diyl-bis-picolinium diiodide; bPiHxI (C6)
- 2** N,N'-heptane-1,7-diyl-bis-picolinium dibromide; bPiHpB (C7)
- 3** N,N'-octane-1,8-diyl-bis-picolinium diiodide; bPiOI (C8)
- 4** N,N'-nonane-1,9-diyl-bis-picolinium dibromide; bPiNB (C9)
- 5** N,N'-decane-1,10-diyl-bis-picolinium diiodide; bPiDI (C10)
- 6** N,N'-undecane-1,11-diyl-bis-picolinium dibromide; bPiUB (C11)
- 7** N,N'-dodecane-1,12-diyl-bis-picolinium dibromide; bPiDDB (C12)

Fig. 1.
Chemical structures, full chemical names and abbreviations of *N,N'*-alkane-diyl-*bis*-3-picolinium (bAPi) salts.

Fig. 2. At high concentrations, *N,N'*-alkane-diyl-*bis*-3-picolinium (bAPI) analogs inhibit [^3H]nicotine binding (top panel) and [^3H]MLA binding to rat brain membranes (bottom panel). Analog abbreviations are provided in Fig. 1. Cytisine and nudicauline were used as a positive control for [^3H]nicotine and [^3H]MLA binding assays, respectively. Inhibition of the binding of 3 nM [^3H]nicotine ([^3H]NIC, top panel) or 2.5 nM [^3H]MLA (bottom panel) was determined in separate series of experiments. Nonspecific binding was determined in the presence of 10 μM nicotine or cytisine for [^3H]nicotine binding assays and 1 mM nicotine for [^3H]MLA binding assays. Data are fmol/mg protein expressed as % of control. Control represents [^3H]nicotine binding (85.2 ± 6.93 fmol/mg protein; mean $\pm$ S.E.M) and [^3H]MLA binding (69.6 ± 9.72 fmol/mg protein; mean $\pm$ S.E.M), respectively, in the absence of analog; $n = 3\text{--}5$ rats/analog/binding assay. Curves were generated by nonlinear regression.

Fig. 3. *N,N'*-Alkane-diyl-*bis*-3-picolinium (bPi) analogs inhibit nicotine-evoked [³H]DA overflow from rat striatal slices (Top panel)

Analog abbreviations are provided in Fig. 1. Assay buffer contained nomifensine (10 μM) and pargyline (10 μM) throughout superfusion. Slices were superfused in the absence (control) or presence of analog for 60 min prior to nicotine addition to the buffer; superfusion continued for 60 min with nicotine added to the buffer. Control represents [³H]DA overflow in response to 10 μM nicotine (3.12 ± 0.16 total [³H]DA overflow as a percent of tissue [³H]content, mean $\pm$ S.E.M.). Data are expressed as % of nicotine control; $n = 5$ –10 rats/analog. Curves for concentration response were generated by nonlinear regression. Time course of bPiDDB-induced inhibition of nicotine-evoked [³H]DA overflow is shown in the bottom panel. Time course data were used to generate [³H]DA overflow data for bPiDDB, shown in the top panel. Slices were superfused with a range of concentrations (1 nM–10 μM) of bPiDDB for 60 min. After collection of the third sample, nicotine (10 μM) was added to the superfusion buffer in the absence (control) and presence of bPiDDB and superfusion continued for 60 min. In each experiment, a control slice was superfused in the absence of bPiDDB and presence of nicotine to determine nicotine-evoked total [³H]DA overflow. The arrow indicates the time point at which nicotine was added to the superfusion buffer. Data are expressed as fractional release as a percent of basal (mean $\pm$ S.E.M.), $n = 6$ rats. Basal was 0.94 ± 0.04 fractional release as % tissue [³H]content.

Fig. 4. Schild analysis for bPiDDB inhibition of nicotine-evoked [^3H]DA overflow from superfused rat striatal slices

Assay buffer contained nomifensine (10 μM) and pargyline (10 μM) throughout superfusion. After collection of the third sample, slices were superfused with buffer in the absence and presence of bPiDDB (1, 3 or 10 nM) for 60 min prior to the addition of nicotine (0.1 nM–100 μM) to the buffer, and superfusion continued for an additional 60 min. For each nicotine concentration, the control response is that for nicotine in the absence bPiDDB. Data are presented as mean $\pm$ S.E.M. of total [^3H]overflow during the 60 min exposure to nicotine in the absence and presence of bPiDDB; $n = 5$ rats. Concentration response curves were generated by nonlinear regression. Fig. 4 inset shows the Schild regression in which the log of $dr - 1$ was plotted as a function of log of bPiDDB concentration. Data were fit by linear regression.

Fig. 5. α -Conotoxin MII inhibition of nicotine (10 μ M)-evoked [3 H]DA overflow from superfused rat striatal slices is concentration-dependent, but incomplete

The concentration was determined at which α -conotoxin (α -CtxMII) produced maximal inhibition of nicotine-evoked [3 H]DA release from rat striatal slices using the current experimental conditions. Assay buffer contained nomifensine (10 μ M) and pargyline (10 μ M) throughout superfusion. Slices were superfused in the absence (control) or presence of α -CtxMII (0.1 – 30 nM) for 36 min, and superfusion continued for 36 min following addition of nicotine to the buffer. Control represents [3 H]DA overflow in response to 10 μ M nicotine (1.54 ± 0.18 total [3 H]DA overflow as a percent of tissue [3 H]content, mean $\pm$ S.E.M.). Data are expressed as mean $\pm$ S.E.M. of % of nicotine control for $n=6$ rats and the concentration-response curve was generated using nonlinear regression.

Fig. 6. Concomitant exposure to concentrations of bPiDDB and α -CtxMII produces inhibition of nicotine-evoked [3 H]DA overflow not different from that following exposure to either antagonist alone

Superfusion buffer contained nomifensine (10 μ M) and pargyline (10 μ M). Maximal inhibitory concentrations of α -CtxMII (1 nM) and bPiDDB (10 nM) were selected from previous concentration response analysis. After collection of the second sample, slices were superfused in the absence or presence of α -CtxMII, bPiDDB or α -CtxMII + bPiDDB for 36 min. Superfusion continued for 36 minutes following addition of nicotine (10 μ M) to the buffer. Control represents nicotine-evoked [3 H]DA overflow in the absence of antagonist (1.99 ± 0.52 total [3 H]DA overflow as a percent of tissue [3 H]content, mean $\pm$ S.E.M.). Data are expressed as mean $\pm$ S.E.M. of % control. * indicates different from control ($p < 0.01$). $n = 8$ rats. Inset: Time course of nicotine-evoked [3 H]DA overflow in the absence and presence of α -CtxMII, bPiDDB or α -CtxMII + bPiDDB. Data are expressed as fractional release as a percent of basal outflow. Arrow indicates the time point at which nicotine was added to the superfusion buffer.

Fig. 7. *N,N'*-Alkane-diyl-*bis*-3-picolinium (bAPI) analogs do not inhibit [³H]DA uptake into rat striatal synaptosomes

Analog abbreviations are provided in Fig. 1. GBR 12909 was used as a positive control for these experiments. Data are pmol/min/mg of specific [³H]DA uptake expressed as % of control; *n* = 3–4 rats. Control represents [³H]DA uptake in the absence of analog (33.6 ± 1.75 pmol/min/mg protein; mean $\pm$ S.E.M.). Nonspecific [³H]DA uptake was determined in the presence of 10 μ M nomifensine. Curves were generated using nonlinear regression; analog abbreviations are provided in Fig. 1.

Fig. 8. Reduction of cortical BBB [^3H]choline permeability (PS) in the presence of N,N' -alkane-diyl-*bis*-3-picolinium (bAPi) analogs (250 μM) using the in-situ rat brain perfusion technique, suggesting interaction with the BBB choline transporter

Data represent the mean ($\pm$ SEM) for 3–6 independent observations (***) $p < 0.001$.

Fig. 9. In contrast to mecamylamine and DHβE, the bAPi analogs do not inhibit the discriminative stimulus effect of nicotine

Discriminative stimulus and response rate effects of nicotine in rats ($n=6$) trained to discriminate nicotine (0.2 mg/kg, sc) from saline (panels a and b), and in nicotine-trained rats pretreated with either mecamylamine, DHβE (panels c and d), or bAPi analog (panels e and f). Analog abbreviations are provided in Fig. 1. Data points represent the mean ($\pm$ SEM) percentage of total responses occurring on the nicotine-appropriate lever (upper panels) and rate of responding (lower panels) as a function of nicotine (panels a and b) or pretreatment (panels c-f) dose. Asterisks indicate a significant difference relative to saline (S) control (* $p < 0.05$, ** $p < 0.01$).

Fig. 10. Mecamylamine and DHβE inhibit the locomotor stimulant effect of nicotine Mecamylamine (MEC; 0.1–3.0 mg/kg, sc; left panel) or DHβE (0.1–3.0 mg/kg, sc; right panel) pretreatment was given prior to nicotine (0.4 mg/kg) administration in nicotine-sensitized rats. Points represent the mean ($\pm$ SEM) total distance traveled (cm) as a function of pretreatment dose. Asterisks indicate a significant difference from the corresponding saline (S) control (* p < 0.05, ** p < 0.01, *** p < 0.001).

Fig. 11. bAPi analogs differentially inhibit the locomotor stimulant effects of nicotine
 bAPi analogs (0.58–5.8 μ moles/kg, sc) were given prior to nicotine (0.4 mg/kg) or saline administration in nicotine-sensitized rats. Analog abbreviations are provided in Fig. 1. Points represent the mean ($\pm$ SEM) total distance traveled (cm) as a function of pretreatment dose. Asterisks indicate a significant difference from corresponding saline (S) control (* p < 0.05, ** p < 0.01, *** p < 0.001).

TABLE 1
***N,N'*-Alkane-diyl-*bis*-3-picolinium (bAPi) analogs do not evoke [³H]DA overflow from superfused rat striatal slices**

Rat striatal slices were preloaded with [³H]DA. Superfusion buffer contained pargyline (10 μM) and nomifensine (10 μM). Slices were superfused with various concentrations (1 nM–10 μM) of bAPi analog. Each slice was exposed to only one concentration of analog. Data represent [³H]DA overflow during the 60-min superfusion period in the absence (Control) and presence of analog.

bAPi Analog	Control	0.001 μM	0.01 μM	0.1 μM	1 μM	10 μM
bPiHxI	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0 ^a
bPiHpB	0.09 ± 0.08	0.19 ± 0.13	0.13 ± 0.06	0.14 ± 0.05	0.23 ± 0.14	0.07 ± 0.04
bPiOI	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.08 ± 0.06
bPiNB	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
bPiDI	0 ± 0	0 ± 0	0.08 ± 0.05	0 ± 0	0 ± 0	0.29 ± 0.12
bPiUB	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.07 ± 0.06
bPiDDB	0 ± 0	0.14 ± 0.13	0.09 ± 0.07	0.05 ± 0.02	0.13 ± 0.07	0.14 ± 0.06

^aData are mean ± S.E.M. total [³H]DA overflow expressed as a percentage of tissue tritium; *n* = 5–10 rats/analog.

Summary of inhibitory activities of *N,N'*-alkane-diyl-*bis*-3-picolinium analogs (bAPi) in [³H]nicotine and [³H]MLA binding assays, [³H]DA overflow assay and [³H]DA uptake assay.

TABLE 2

Compound	[³ H]Nicotine Binding Assay <i>K_i</i> (μM)	[³ H]MLA Binding Assay <i>K_i</i> (μM)	[³ H]DA Overflow Assay		[³ H]DA Uptake Assay <i>K_i</i> (μM)
			IC ₅₀ (μM)	Imax (%)	
bPiHxI (C ₆)	>100 ^a	>100	>100	-	>100
bPiHpB (C ₇)	>100	>100	0.06 ± 0.03	54 ± 5	
bPiOI (C ₈)	>100	>100	0.30 ± 0.18	57 ± 9	>100
bPiNB (C ₉)	79.9 ± 16.6	>100	5.81 ± 5.07	61 ± 11	>100
bPiDI (C ₁₀)	>100	>100	0.18 ± 0.11	60 ± 9	>100
bPiUB (C ₁₁)	69.2 ± 28.9	>100	1.12 ± 0.65	62 ± 6	>100
bPiDDb (C ₁₂)	48.6 ± 17.2	>100	0.002 ± 0.001	64 ± 4	>100

^aData are mean ± S.E.M.; *n* = 5–10 rats/analog for [³H]DA overflow assay; *n* = 3–5 rats/analog for binding and uptake assays.

Effect of C₈ and C₁₂ N,N'-alkane-diyl-bis-3-picolinium analogs (bAPI) on field stimulation-evoked [³H]DA overflow from rat striatal slices

Slices were superfused with a range of concentrations (0.001–10 μM) of analog for 60 min followed by field stimulation (2 min, 1 Hz, 120 pulses) and superfusion continued for 30 min. Superfusion buffer contained nomifensine (10 μM) and pargyline (10 μM). Each slice was exposed to only one concentration of analog. Control represents the response to field stimulation in the absence of analog.

bAPI Analog	Control	0.001 μM	0.01 μM	0.1 μM	1 μM	10 μM
bPIOI (C ₈)	2.48 ± 0.26 ^a	1.87 ± 0.34	2.36 ± 0.34	1.23 ± 0.18 *	2.45 ± 0.31	2.45 ± 0.48
bPiDDb (C ₁₂)	2.75 ± 0.24	2.02 ± 0.24	2.27 ± 0.36	1.72 ± 0.37	1.91 ± 0.37	2.12 ± 0.42

^aData are mean ± S.E.M. total [³H]DA overflow expressed as a percentage of tissue tritium. n = 11–12 rats/analog;

* p < 0.05 different from control, Dunnett's post hoc t-tests.

TABLE 4

BBB choline transporter inhibition constants for *N,N'*-alkane-diyl-*bis*-3-picolinium (bAPi) analogs.

Substrate	CHT-Ki (μM)
Choline (K_m)	40 ± 3
bPiHxI (C_6)	324 ± 15
bPiHpB (C_7)	60 ± 7
bPiOI (C_8)	123 ± 17
bPiNB (C_9)	430 ± 13
bPiDI (C_{10})	79 ± 13
bPiUB (C_{11})	164 ± 21
bPiDDB (C_{12})	5 ± 3

The apparent inhibition constants (K_i) for choline and the bAPi analogs at the BBB choline transporter (CHT). Data represent the mean ($\pm$ SEM) for 3–6 independent observations.