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Discovery of a novel nicotinic receptor antagonist for the treatment of nicotine addiction: 1-(3-Picolinium)-12-triethylammonium-dodecane dibromide (TMPD)

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ABSTRACT

Limitations in efficacy and high relapse rates of currently available smoking cessation agents reveal the need for more efficacious pharmacotherapies. One strategy is to develop subtype-selective nicotinic receptor (nAChR) antagonists that inhibit nicotine-evoked dopamine (DA) release, the primary neurotransmitter involved in nicotine reward. Simple alkylation of the pyridino N-atom converts nicotine from a potent agonist into a potent antagonist. The classical antagonists, hexamethonium and decamethonium, differentiate between peripheral nAChR subtypes. Using a similar approach, we interconnected varying quaternary ammonium moieties with a lipophilic linker to provide *N*,*N*′-bis-nicotinium analogs, affording a lead compound, *N*,*N*′-dodecyl-1,12-diyl-bis-3-picolinium dibromide (bPiDDB), which inhibited nicotine-evoked DA release and decreased nicotine self-administration. The current work describes a novel compound, 1-(3-picolinium)-12-triethylammonium-dodecane dibromide (TMPD), a hybrid of bPiDDB and decamethonium. TMPD completely inhibited (IC_{50} = 500 nM) nicotine-evoked DA release from superfused rat striatal slices, suggesting that TMPD acts as a nAChR antagonist at more than one subtype. TMPD (1 μM) inhibited the response to acetylcholine at α3β4, α4β4, α4β2, and α1β1εδ receptors expressed in *Xenopus* oocytes. TMPD had a 2-fold higher affinity than choline for the blood–brain barrier choline transporter, suggesting brain bioavailability. TMPD did not inhibit hyperactivity in nicotine sensitized rats, but significantly and specifically decreased nicotine self-administration. Together, the results suggest that TMPD may have the ability to reduce the rewarding effect of nicotine with minimal side effects, a pharmacological profile indicative of potential clinical utility for the treatment of tobacco dependence.

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1. Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) are CNS targets for the development of drugs to treat a variety of diseases including tobacco dependence, drug addiction, Parkinson's disease and depression, among others. Tobacco addiction is a major health problem in the US, accounting for more preventable illnesses and deaths than any other single factor [1]. Currently available tobacco use cessation agents (i.e., nicotine, bupropion and varenicline) have limited efficacy and relapse rates are reported to be high, revealing a continuing need for the development of alternative, more efficacious smoking cessation pharmacotherapies [1–3]. Dopamine (DA) release evoked by nicotine via activation of presynaptic nAChRs is thought to mediate nicotine-induced reward, leading to tobacco dependence [4–8]. Nicotine-evoked DA release is completely inhibited by the nonselective, noncompetitive nAChR antagonist, mecamylamine [9–11]. Mecamylamine has some efficacy as a tobacco cessation agent, but its therapeutic use is limited by peripherally-mediated side-effects [12]. One therapeutic strategy is to develop novel subtype-selective nAChR antagonists that inhibit nicotine-evoked DA release, which may prove efficacious as smoking cessation agents with fewer side-effects due to their nAChR subtype-selective action.

Nicotine, the major alkaloid in tobacco, activates with varying potency all known nAChRs [13], and these nAChRs modulate release of various neurotransmitters [14–16]. nAChRs are pentameric and have discrete expression patterns [17–19]. Genes for α2-α7 and β2-β4 subunits have been identified in mammalian brain [20,21]. Heteromeric nAChRs exist as combinations of α and β subunits, and variations in subunit compositions contribute to differences in nAChR function and pharmacology [22–24]. nAChRs are located on DA cell bodies and terminals, including substantia nigra and striatal terminal fields. Substantia nigra DA neurons express α3, α4, α5, α6, α7, β2, β3 and β4 mRNA [25–27]. Variations in subunit combinations may be involved in the formation of nAChR subtypes which modulate DA release at striatal presynaptic DA terminals. Native subtypes by convention have an asterisk designation to indicate putative composition [28]. Results show that α-conotoxin MII (α-CtxMII), the Conus snail peptide neurotoxin, as well as small quaternary ammonium molecules, *N*-*n*-pentadecylpyridinium bromide, *N*-eicosylpyridinium bromide and *N*,*N*′-dodecyl-1,12-diyl-bis-3-picolinium dibromide (bPiDDB), only partially inhibit nicotine-evoked striatal DA release, indicating involvement of more than one population of nAChR subtype [29–34]. Studies using β2 knockout mice have shown the involvement of β2-containing nAChRs in nicotine-evoked DA release [31,35–37]. α4β2*, α6β2*, and α4α6β2* subtypes are suggested to mediate the DA response to nicotine [38]. Results from a comprehensive molecular genetics study in which an individual subunit gene (i.e., α4, α5, α7, β2, β3, or β4) was deleted, suggested that at least six different subtypes mediate nicotine-evoked DA release from mouse striatal synaptosomes, including two classes of nAChRs: α-CtxMII-sensitive nAChRs (i.e., α6β2β3*, α4α6β2β3* and possibly a small amount of α6β2* or α4α6β2* subtypes) and α-CtxMII–resistant nAChRs (i.e., α4β2* and α4α5β2* subtypes), whereas deletion of β4 and α7 subunits had no effect [39]. Also, α6- and β3-containing nAChRs have been implicated in nicotine-evoked DA release [24,40,41]. Additionally, substantia nigra neurons express high levels of both α6 and β3 mRNA [26,40–43] consistent with their involvement in mediating nicotine-evoked DA release.

To further complicate the association of a particular response with a specific nAChR subtype, studies using recombinant receptors report that when the ratio of subunit pairs is varied, different subtype classes are formed and the function of these different subtype classes is dependent on subunit ratio [44]. Exposure to drug also influences nAChR subtype stoichiometry and function in recombinant receptor systems [44–46]. Different DA neurons can be categorized based upon the expression of the specific subtype composition [27]. CNS nAChRs and their modulation of neurotransmitter release have been reviewed recently [47,48]. Thus, the presence of specific mRNAs encoding a particular subunit, the relative ratio of transcribed subunits, specific expression of nAChR subtypes by different DA neurons, and pharmacological history are all important to neuronal function and potentially play a collective role in the response to nicotine and to drugs which block the effect of nicotine.

The observation that nAChR subtypes which mediate nicotine-evoked DA release are pharmacologically different suggests that subtype-selective antagonists can be developed. As part of our drug development efforts, the nicotine molecule has been modified to obtain subtype-selective nAChR antagonists. Simple alkylation of the pyridino N-atom converts nicotine from a nonselective agonist into a potent, subtype-selective, competitive antagonist [49–51], leading to the discovery of a new class of nAChR antagonists resulting from *N*-alkylation of the pyridine moiety of nicotine [49–56]. These novel analogs exhibit potent inhibition of nAChR subtypes mediating nicotine-evoked DA release from striatal nerve terminals. Quaternization of the pyridine N-atom of nicotine with a lipophillic *N*-alkyl substituent to afford *N*-alkylnicotinium analogs and/or interconnecting varying quaternary ammonium moieties with a lipophilic linker to afford *N*,*N*′-bis-quaternary ammonium analogs generates subtype-selective nAChR antagonists, which could have potential as novel smoking cessation agents.

bis-Trialkylammonium salts such as hexamethonium chloride and decamethonium bromide are regarded as simplified analogs of d-tubocurarine and have been utilized to differentiate between subtypes of peripheral nicotinic receptors. We adopted a similar approach and generated a sub-library of compounds based on bis-nicotinium analogs by incorporating a variety of head groups and linkers varying in length and unsaturation, which resulted in the identification of a lead compound, bPiDDB, that potently inhibited nicotine-evoked DA release in vitro and decreased nicotine self-administration in rats in vivo [57,58,33].

Our current research focuses on the development of novel, potent and selective antagonists at nAChRs mediating nicotine-evoked DA release through structural modification of bPiDDB. We describe the effects of a novel compound, 1-(3-picolinium)-12-triethylammonium-dodecane dibromide (TMPD), which is a structural hybrid of bPiDDB and the C_{10} neuromuscular blocking agent, decamethonium. One quaternary ammonium head group of TMPD is a 3-picolinium

moiety and the other head group is triethylammonium moiety; both head groups are separated by a C_{12} linker identical to that found in the bPiDDB molecule.

Decamethonium and hexamethonium do not cross the blood–brain barrier. The blood–brain barrier is a major limiting factor for brain distribution, with ~98% of small molecules being excluded from brain because they do not diffuse across the blood–brain barrier or they are subject to efflux. In addition, considering that hydrophilic or charged compounds do not readily permeate cell membranes, the blood-to-brain drug permeation of these molecules is typically less than 1% [59–61]. However, bPiDDB was able to access the brain by being transported via the blood–brain barrier choline transporter. The structural change from bPiDDB to TMPD is not expected to alter the transport properties of TMPD at the blood–brain barrier choline transporter. Thus, the current report describes the synthesis, neurochemical and behavioral properties of our lead compound, TMPD, and the ability of this compound to interact with the blood–brain barrier choline transporter.

2. Methods

2.1. Synthesis of TMPD

1-(3-Picolinium)-12-triethylammonium-dodecane dibromide (TMPD) was prepared by dissolving 3-picoline (1.63 g, 17.50 mmol) and 1,12-dibromododecane in 150 ml acetonitrile and heating the solution at 50 °C for 24 h (Fig. 1). After the mixture was cooled to room temperature, solvent was removed in vacuo using a rotary evaporator. The residue was suspended in diethyl ether (100 ml), and the precipitate was removed by filtration, washed with diethyl ether, and dissolved in water (80 ml). The resulting aqueous solution was extracted with diethyl ether (40 ml × 3) and then chloroform (50 ml × 3). The combined chloroform extracts were dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo. The resulting solid residue was triturated with diethyl ether, filtered and dried in vacuo, to afford 4.86 g of 1-(3-picolinium)-12-bromododecane bromide as a pale yellow powder; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.35 (s, 1H), 9.20 (d, J = 6.0 Hz, 1H), 8.23 (d, J = 7.8 Hz, 1H), 8.00 (dd, J = 7.8, 6.0 Hz, 1H), 4.84 (t, J = 7.5 Hz, 2H), 3.33 (dt, J = 6.9, 3.3 Hz, 2H), 2.59 (s, 3H), 2.56 (t, J = 7.5 Hz, 2H), 1.95 (m, 2H), 1.77 (m, 2H), 1.05–1.22 (m, 14H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 145.6, 144.4, 142.2, 139.6, 127.9, 61.9, 34.4, 32.9, 32.1, 29.52, 29.46, 29.43, 29.2, 28.8, 28.2, 26.2, 18.9 ppm. The above product (2.0 g) was mixed with triethylamine (10 ml) and the mixture stirred at 60 °C for 24 h. The residual triethylamine was removed in vacuo using a rotary evaporator, and the residue was dissolved in water (20 ml). The resulting aqueous solution was extracted with chloroform (20 ml × 3), the water layer was evaporated to dryness under vacuum and the resulting residue was dried in vacuo for 24 h to afford 2.27 g of TMPD as a viscous amber oil; ^{1}H NMR (300 MHz, D_2O) δ 8.70 (s, 1H), 8.64 (d, J = 5.7 Hz, 1H), 8.35 (d, J = 7.8 Hz, 1H), 7.92 (dd, J = 7.8, 5.7 Hz, 1H), 4.55 (t, J = 7.5 Hz, 2H), 3.10–3.33 (m, 8H) 2.54 (s, 3H), 2.00 (m, 2H), 1.66 (m, 2H), 1.10–1.43 (m, 25H) ppm; ^{13}C NMR (75 MHz, D_2O) δ 146.0, 143.8, 141.4, 140.0, 127.5, 61.9, 56.95, 56.92, 52.71, 52.68, 52.64, 47.0, 30.7, 28.9, 28.8, 28.7, 28.5, 28.3, 25.9, 25.5, 21.1, 18.0, 8.6, 7.0 ppm.

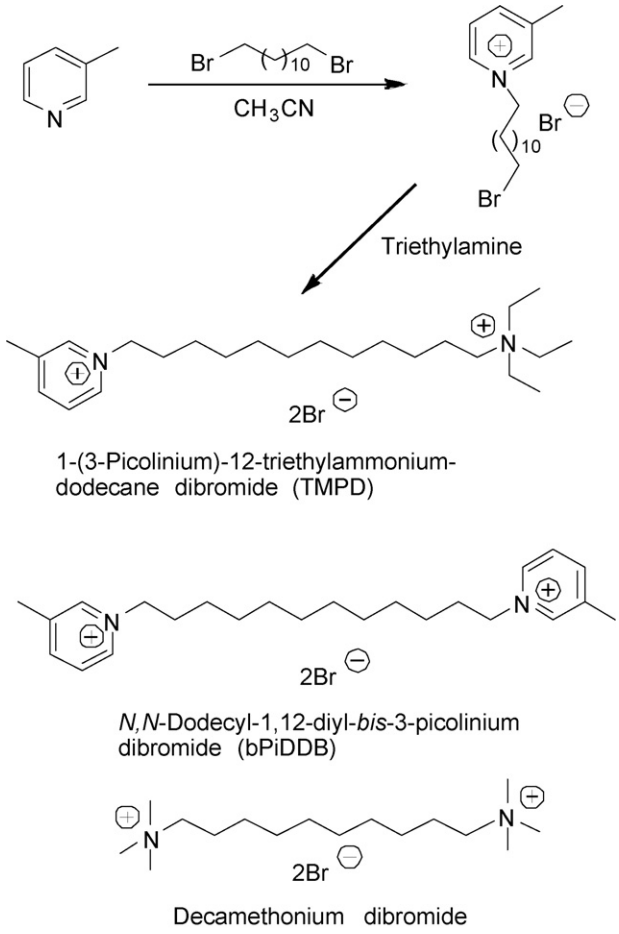

Fig. 1 – Preparation of 1-(3-picolinium)-12-triethylammonium-dodecane dibromide (TMPD) and its structural relationship to bPiDDB and decamethonium dibromide.

2.2. Animals

For DA release and behavioral assays, male Sprague-Dawley rats (200–225 g) from Harlan Industries (Indianapolis, IN) were used. For blood–brain barrier choline transporter assays, Fischer 344 rats (220–250 g) from Charles River Laboratories, (Kingston, NY, USA) were used. Rats had unlimited access to food and water in the home cage, except as noted. Rats were maintained on a 14:10 h light/dark cycle in which the lights came on at 06:00 h and went off at 20:00 h. All experiments were conducted during the light phase of the cycle. In the behavioral studies, rats were acclimated to the animal colony for at least 5 days and were handled briefly on 3–5 consecutive days prior to the start of the experiment. The Institutional Animal Care and Use Committee of the University of Kentucky and Texas Tech University Health Sciences Center approved the conduct of the experiments described herein. The experiments conformed to the guidelines established by the NIH Guide for the Care and Use of Laboratory Animals (1996 Edition).

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

Nicotine-evoked overflow of [^{3}H]DA (28 Ci/mmol, Perkin-Elmer, Boston, MA) from striatal slices preloaded with [^{3}H]DA was determined using a previously published method with minor modifications [56,62,63]. Briefly, striatal slices were prepared using a McIlwain tissue chopper (Mickle Laboratory Engineering Co Ltd., Surrey, England). Slices were incubated at 34 °C in Krebs' buffer containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 1.3 mM $CaCl_2$, 11.1 mM α-D-glucose, 25 mM $NaHCO_3$, 0.11 mM L-ascorbic acid, and 0.004 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4, saturated with 95%O_2/5%CO_2) in a metabolic shaker for 30 min. Slices were transferred to fresh buffer, 0.1 μM [^{3}H]DA added and incubation continued for 30 min. Subsequently, slices were rinsed with Krebs' buffer and transferred to superfusion chambers maintained at 34 °C (Brandel suprafusion system 2500, Gaithersburg, MD) and were superfused (flow rate = 0.6 ml/min) for 60 min with oxygenated Krebs' buffer containing both nomifensine (10 μM), a DA uptake inhibitor, and pargyline (10 μM), a monoamine oxidase inhibitor. Two samples (2.4 ml/sample, sample collection at 4 min intervals) were collected for determination of basal [^{3}H]outflow. Slices were superfused for 36 min in the absence (0 nM; control, which was superfused with Krebs' buffer only) or presence of mecamylamine or TMPD (1 nM to 10 μM; vehicle for both antagonists was Krebs' buffer) to determine the effect of the compound alone. Nicotine (10 μM) was added to the buffer; superfusion continued and samples were collected for 36 min to determine the ability of mecamylamine or TMPD to inhibit nicotine-evoked [^{3}H]DA overflow. At the end of the experiment, slices were removed from the chambers and were solubilized with 1 ml of TS-2 tissue solubilizer (Research Products International Corp, Prospect, IL). Scintillation cocktail (4 ml) was added to superfusate and solubilized tissue samples. Radioactivity was determined by liquid scintillation spectroscopy using a 1600 TR Tri Carb Liquid Scintillation Analyzer (Packard, Downer's Grove, IL). Fractional release was determined by dividing the [^{3}H] in each superfusate sample by the tissue-[^{3}H] at the time of collection and expressed as percent of total tissue tritium. Basal [^{3}H]outflow was determined as the mean of fractional release in the two basal samples collected prior to the introduction of mecamylamine or TMPD in the superfusion buffer. Total [^{3}H]overflow was the sum of fractional release above basal following addition of nicotine to the buffer. IC_{50} represents the antagonist concentration which decreased nicotine-evoked [^{3}H]DA overflow by 50% of the maximal effect and was calculated using an iterative nonlinear least squares curve-fitting program, PRISM version 4.0 (Graph PAD Software, Inc., San Diego, CA). A one-way ANOVA with Dunnett's post hoc analysis was used to determine if mecamylamine or TMPD inhibited nicotine-evoked total [^{3}H]DA overflow.

2.4. Preparation of RNA and expression in Xenopus oocytes

Rat neuronal nAChR clones and mouse muscle α1, β1 and δ nAChR cDNA clones were obtained from Dr. Jim Boulter (UCLA). The mouse muscle ε clone was provided by Dr. Paul Gardner (Univ. Massachusetts Med. School). A form of the α6/α3 chimera [64] was obtained from Michael McIntosh (University of Utah) and corrected for a point mutation as previously reported [65]. Mature (>9 cm) female *Xenopus laevis* African toads (Nasco, Ft. Atkinson, WI) were used as a source of oocytes. The Institutional Animal Care and Use Committee of the University of Florida approved the conduct of the experiments described. Experiments were conducted as previously described, [66] using OpusXpress 6000A (Molecular Devices, Union City CA). Each oocyte received initial control applications of acetylcholine (ACh), then co-applications of ACh and 1 μM TMPD, and then a follow-up control application of ACh. The control ACh concentrations utilized in α1β1εδ, α7, α4β4, α4β2 receptor experiments were 3 μM, 60 μM, 10 μM, and 10 μM, respectively, and 100 μM in α3β4, α4/6β4 and α6/3β2β3 receptor experiments. These ACh control values were chosen because they evoke robust and reproducible responses for each of the respective subtypes, minimizing the chance that rundown or desensitization would be misinterpreted as inhibition. Both the peak amplitude and net charge [67] of the responses were measured for each drug application and calculated relative to the preceding ACh control responses to normalize the data, compensating for the varying levels of channel expression among the oocytes. Net charge values were used to report inhibitory effects. Means and standard errors (S.E.M.) were calculated from the normalized responses of at least four oocytes for each experiment.

2.5. Blood–brain barrier [^{3}H]choline uptake assay

Uptake of [^{3}H]choline (79.2 Ci/mmol, Perkin-Elmer, Boston, MA) into brain was assessed using the in situ rat brain perfusion technique. Briefly, Fischer 344 rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). A PE-60 catheter filled with heparinized saline (100 U/ml) was placed into the left common carotid artery after ligation of the left external carotid, occipital and common carotid arteries. The pterygopalatine artery was left open during the experiments. Rectal temperature was maintained at 37 °C by a feedback device (YSI Indicating Controller, Yellow Springs, Ohio). The catheter was connected to a syringe containing a warmed (37 °C), gassed (95% O_2 and 5% CO_2), buffered physiologic perfusion fluid containing 128 mM NaCl, 2.4 mM $NaPO_3$, 29.0 mM $NaHCO_3$, 4.2 mM KCl, 1.5 mM $CaCl_2$, 0.9 mM $MgCl_2$ and 9 mM D-glucose (pH = 7.35 and 290 mOsm) with 1 μCi/ml [^{3}H]choline and 0.3 μCi/ml [^{14}C]sucrose (4.75 mCi/mmol, Perkin-Elmer, Boston, MA). The perfusion fluid was infused for 60 s at 10 ml/min (Harvard Apparatus, South Natick, MA. U.S.A.), maintaining a carotid artery pressure of ~120 mm Hg. After 60 s perfusion, the perfusion fluid was changed immediately to a tracer-free perfusion fluid for 15 s to wash out residual nonspecific [^{3}H]choline in the vasculature. Cerebral samples were obtained and digested at 50 °C in 1 ml of 1 M piperidine solution. Ten milliliter of scintillation cocktail (Beckman, Fullerton, CA, USA) was added and tracer contents assessed by dual-label liquid scintillation spectroscopy. Brain uptake of [^{3}H]choline was determined by calculation of a single time point blood-to-brain transfer coefficient (K_{in}), as previously described [68] from the relationship $K_{in} = [C_{tot} - V_v C_{pf}]/(C_{pf}/T)$, where $C_{tot} = C_{br} + C_{vas}$, the sum of

the amount of choline remaining in the perfusate in the blood–brain vessels and the amount of choline penetrating into brain. Cerebral vascular volume and cerebral perfusion flow rate were determined in separate experiments as previously described [69,70], where C_{pf} is the perfusion fluid concentration of tracer choline and T is the net perfusion time with the assumption that uptake is linear. An apparent cerebrovascular permeability surface-area product (PS) was then determined using the relationship, $PS = F \ln(1-k_{in}/F)$, where F is the cerebral blood flow determined for each region of the brain from the uptake of [^{14}C]diazepam (76.0 Ci/mmol, Perkin-Elmer, Boston, MA). Inhibition of [^{3}H]choline brain uptake was determined by the inclusion of unlabeled choline or TMPD (250 μM) in the perfusion fluid. An apparent inhibition constant (K_i) for choline and TMPD was determined from the equation $[(PA_o-K_D)/(PA_i-K_D)] = 1 + C_i/K_i$ assuming competitive kinetics where PA_o is the [^{3}H]choline PA in the absence of TMPD, PA_i is the [^{3}H]choline PA in the presence of TMPD, and C_i is the perfusate concentration of TMPD. Apparent K_i is defined as the TMPD concentration that reduces saturable brain [^{3}H]choline influx by 50% at tracer choline concentration ($C_{pf} \ll K_m$) and in the absence of other competing compounds. Data are from the frontal cerebral cortex, and are expressed as mean ± S.E.M. for $n = 3$–4 independent determinations for each compound evaluated. Data were analyzed by ANOVA with Bonferroni correction for multiple comparisons (GraphPad Prism 4, Graphpad Software, San Diego, CA).

2.6. Locomotor activity assays

Locomotor activity was recorded automatically using an animal activity monitoring system with Versamax System software (AccuScan Instruments Inc., Columbus, OH). Rats were placed into a monitoring chamber (42 × 42-cm square and 30 cm high) made opaque by attaching sheets of white plastic to each outer surface. The chamber incorporated a horizontal 16 × 16 grid of photo beam sensors, with each beam 2.5 cm apart and 7.0 cm above the chamber floor. Locomotor activity was recorded for either 30 or 60 min, depending on the experiment. Activity was measured by photo beam interruptions and expressed as total distance traveled (cm).

The first experiment assessed the locomotor effect of TMPD alone. Rats were assigned randomly to one of two different treatment groups that received either TMPD (0.58, 1.94 and 10.9 μmol/kg, s.c., 1 ml/kg body weight) or 0.9% NaCl (saline). TMPD was prepared in saline. Rats given TMPD were first injected with the middle dose (1.94 μmol/kg) on each of 5 consecutive days (Mon–Fri), followed by a two-day (Sat–Sun) rest period. Fifteen minutes after injection, rats were monitored for locomotor activity for 30 min. This procedure was repeated over the next 2 weeks, with the dose changed to 0.58 and 10.9 μmol/kg for weeks 2 and 3, respectively. The saline control group was treated similarly, except all injections were saline. To be consistent with the procedure used in the operant conditioning experiments (see below), rats were restricted throughout the experiment to 20 g food given at the end of each locomotor session (Mon–Fri) or during the afternoon (Sat–Sun). Food consumption and any signs of toxicity (lethargy, ataxia, failure to groom) were monitored daily. Since the highest dose of TMPD (10.9 μmol/kg) produced pronounced hypoactivity and lethargy in the first experiment, only the two lower doses (0.58 and 1.94 μmol/kg) were tested for their ability to block nicotine-induced hyperactivity in the second experiment.

In the second experiment, rats were first sensitized to the locomotor stimulant effects of nicotine (0.4 mg/kg, s.c., given once daily on Mon–Fri for 3 weeks); locomotor activity was monitored for 60 min beginning 15 min following each injection. Nicotine ditartrate (Sigma, St. Louis, MO) was prepared in saline, to which NaOH was added to obtain a pH of 7.4. Following the sensitization phase, rats were tested under each of the following six different treatment conditions in a random order: (1) saline + saline; (2) 0.58 μmol/kg TMPD + saline; (3) 1.94 μmol/kg TMPD + saline; (4) saline + nicotine; (5) 0.58 μmol/kg TMPD + nicotine; and (6) 1.94 μmol/kg TMPD + nicotine. On these treatment days, rats were first given an injection of TMPD (0.58 or 1.94 μmol/kg, s.c.) or saline, followed 15 min later with nicotine (0.4 mg/kg) or saline; locomotor activity was assessed for 60 min beginning 15 min after the second injection. All injections were s.c. in the middle region of the back. Intervening between each treatment, rats were given 1 day of maintenance on the initial sensitization regimen, i.e., nicotine (0.4 mg/kg) alone. All rats in this experiment were food restricted (20 g/day at the end of the locomotor session) for the duration of the experiment and only the data from the last 30 min of the session were used, as nicotine-induced hyperactivity is most pronounced during this period [71]. A correlated *t*-test was first used to compare the total distance traveled from 30 to 60 min post-injection in the saline + saline and saline + nicotine pretreatment conditions to determine if nicotine-induced locomotor sensitization was obtained. A one-way ANOVA was then conducted to determine the effect of TMPD in the presence and absence of nicotine, using TMPD dose as a within-subject factor.

2.7. Nicotine self-administration and sucrose-maintained responding

Experiments were conducted in operant conditioning chambers (ENV-001; Med Associates, St. Albans, VT, USA), housed in sound-attenuated outer chambers using a Med Associates Interface model SG-503 with MED-IV software. The end walls of the operant conditioning chamber were aluminum, front and back walls were made of clear Plexiglass, and the floor consisted of 18 stainless steel rods (4.8 mm in diameter and placed 1.6 cm apart). Located in the bottom center of one of the end walls was an opening (5 cm × 4.2 cm) for a recessed food tray, into which a food hopper could dispense sucrose pellets individually. Located on either side of the food tray was a response lever. A 28-V white cue light was located 6 cm above each response lever. An infusion pump (Med-Associates, St. Albans, VT) delivered drug reinforcement via a silastic tube attached to a swivel mounted on the outside of the back wall.

For nicotine self-administration, rats were initially given brief lever-press training for food presentations (45 mg Precision Pellets, Bio-Serv, Frenchtown, NJ). Rats were food deprived to 85% of their ad libitum weights by restricting their intake of rat chow to 8-10 g/day for 5 days. Rats were then briefly trained to press a lever in a two-lever operant chamber using a fixed ratio 1 (FR 1) schedule, which was incrementally

increased to a FR 5 across 7 sessions, during 15 min sessions. Rats were given 20 g/day of food following each lever-press training session. After training for food reinforcement, rats were allowed ad libitum access to food in the home cage for 7 days and were then implanted with an indwelling jugular catheter. Rats were anesthetized by injections of ketamine (80 mg/kg, i.p.) and diazepam (5 mg/kg, i.p.) and a silastic catheter was inserted into the jugular vein. The free end of the catheter exited through the skin and was secured to an acrylic head mount attached to the skull. Drug infusions were delivered via an infusion pump which was connected to the head mount via silastic tubing protected by a metal leash during the self-administration sessions. The nicotine self-administration procedure was similar to that described previously [72]. Following recovery from catheter surgery (7 days), rats were reintroduced to operant conditioning chambers for 60-min daily sessions; food restriction was maintained for the duration of the experiment (17–20 g/day, given in the home cage after the session). Responses made on one lever (active) were recorded and were followed by an infusion of nicotine (0.03 mg/kg/infusion, 100 μl delivered over 5.9 s), whereas responses made on the other lever (inactive) were recorded, but had no scheduled consequence. The unit dose of nicotine (0.03 mg/kg/infusion) was chosen based on previously published work [72]. Nicotine was administered i.v. and dose is expressed as the free base weight. This dose produces optimal responding on a FR schedule with limited access. Completion of the FR requirement resulted in simultaneous activation of the infusion pump and cue lights, which signaled a 20-s time-out period during which responding on either lever had no consequence. The FR 1 schedule was gradually increased across sessions to a terminal FR 5 schedule. Rats were trained on the FR 5 schedule until stable responding was achieved, defined by the following criteria: (1) minimum of 10 infusions per session; (2) less than 20% variability in responding for 3 consecutive sessions; and (3) minimum of 2:1 (active:inactive) response ratio. After responding for nicotine stabilized, the effect of acute pretreatment with TMPD on nicotine self-administration was determined. Each rat was administered TMPD (1.1 or 1.94 μmol/kg, s.c.) or saline 15 min prior to the session, with the order of pretreatments determined randomly.

For food-reinforced operant responding, a separate group of rats was trained to lever press for food reinforcement using a FR5 schedule of reinforcement during 60-min sessions. In order to reduce the food reinforcement rate to a level comparable to the rate observed in the nicotine self-administration experiment, a long signaled time-out (210-s, cue lights on) was used as described previously [73]. Criteria for pretreatment were the same as those used in the nicotine self-administration experiments. Subsequently, each rat was administered TMPD (1.1 or 1.94 μmol/kg) or saline 15 min prior to the session, with the order of pretreatments determined randomly. In the nicotine and food operant reinforcement experiments, data were first expressed as a percent change from the baseline (no pretreatment) control response rate within each experiment. A two-way ANOVA was then used to assess the effect of TMPD on operant responding, using reinforcer type (nicotine versus food) as a between-subject factor and dose (1.1 versus 1.94 μmol/kg) as a within-subject factor. In all cases, statistical significance was declared at $p < 0.05$.

3. Results

TMPD was prepared via the initial formation of 1-(3-picolinium)-12-bromododecane bromide followed by subsequent reaction with triethylamine (Fig. 1). Mecamylamine was evaluated for its ability to inhibit nicotine-evoked [^{3}H]DA overflow from superfused rat striatal slices (Fig. 2). The time course shows that the mecamylamine-induced inhibition of nicotine-evoked [^{3}H]DA release was concentration dependent across the concentration range of 10 nM to 10 μM (Fig. 2, top panel). Fractional release evoked by nicotine (10 μM) peaked 8 min after its addition to the buffer and subsequently decreased towards basal level despite the presence of nicotine in the buffer throughout the remainder of the superfusion experiment. Complete inhibition of the effect of nicotine-evoked fractional release was observed at 10 μM

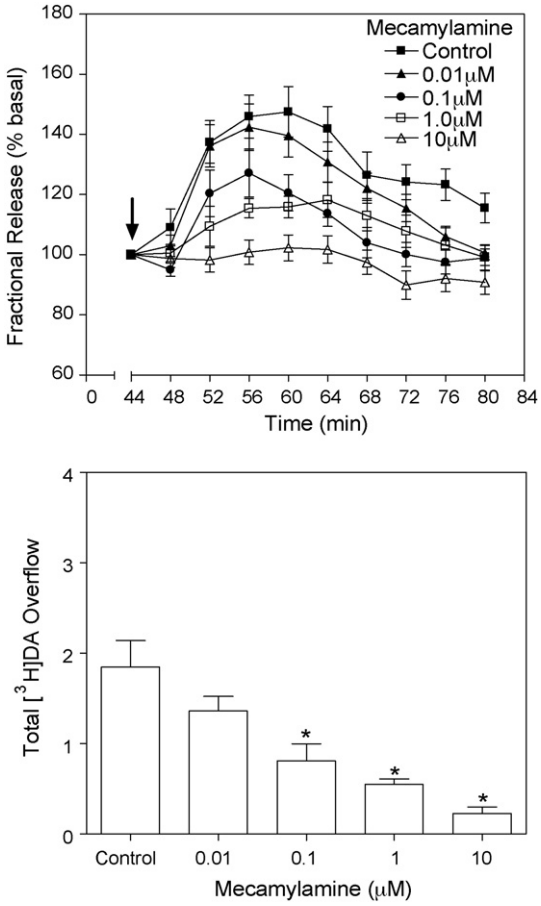

Fig. 2 – Time course of the concentration-dependent mecamylamine inhibition of nicotine-evoked fractional [^{3}H]DA release (top panel) and total [^{3}H]DA overflow (bottom panel). Slices were superfused in the absence (control) or presence of mecamylamine for 36 min prior to the addition of nicotine (10 μM) to the buffer (indicated by the arrow). Data are expressed as mean ± S.E.M. ($n = 7$ rats). *$p < 0.01$ for 0.1, 1, and 10 μM vs. control).

mecamylamine. Expression of the data as total [^{3}H]DA overflow also shows the concentration dependence of the mecamylamine-induced inhibition of the effect of nicotine (Fig. 2, bottom panel, $F(4,38) = 16.64$, $p < 0.01$). The IC_{50} for mecamylamine to inhibit the effect of nicotine was about 100 nM. Similar to mecamylamine, TMPD completely inhibited nicotine-evoked fractional release and nicotine-evoked total [^{3}H]overflow (Fig. 3, top and bottom panels, respectively; $F(4,19) = 10.05$, $p < 0.01$). The IC_{50} for TMPD was 500 nM and complete inhibition of the effect of nicotine was observed at 10 μM TMPD.

nAChR subunit combinations α3β4, α4β4, α4β2, α6/3β2β3, α7 and α1β1εδ were expressed in *Xenopus* oocytes. Control responses to ACh were obtained before and after washout, ACh was co-applied with TMPD at a concentration of 1 μM. Fig. 4 shows the net charge of the co-application responses normalized to the responses to ACh alone. Pairwise *t*-tests between the responses of single cells to ACh applied alone or co-applied with 1 μM TMPD indicated that there was significant inhibition of α3β4 ($p < 0.01$), α4β4 ($p < 0.01$), α4β2 ($p < 0.05$, $n = 4$), and muscle-type α1β1εδ receptors ($p < 0.01$).

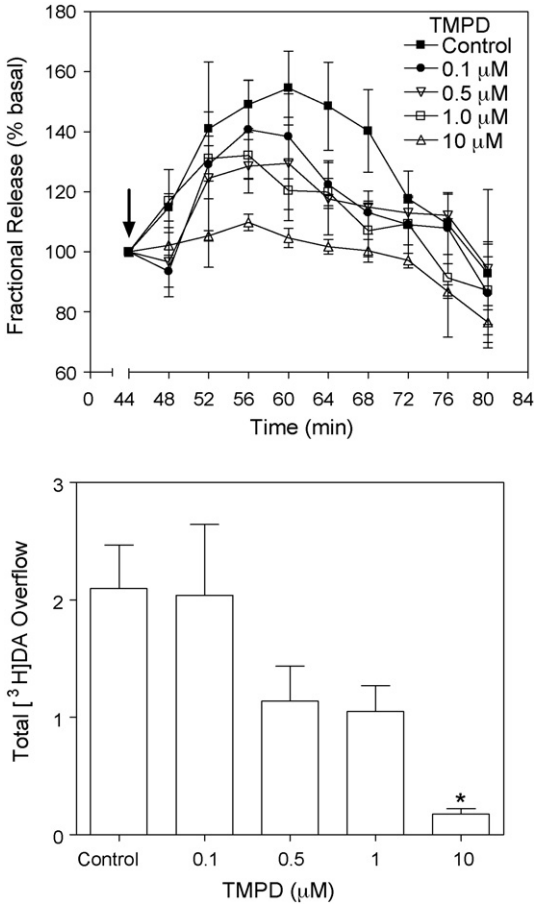

Fig. 3 – Time course of the concentration-dependent TMPD inhibition of nicotine-evoked fractional [^{3}H]DA release (top panel) and total [^{3}H]DA overflow (bottom panel). Slices were superfused in the absence (control) or presence of TMPD for 36 min prior to the addition of nicotine (10 μM) to the buffer (indicated by the arrow). Data are expressed as mean ± S.E.M. ($n = 4$ rats). *$p < 0.01$ for 10 μM vs. control).

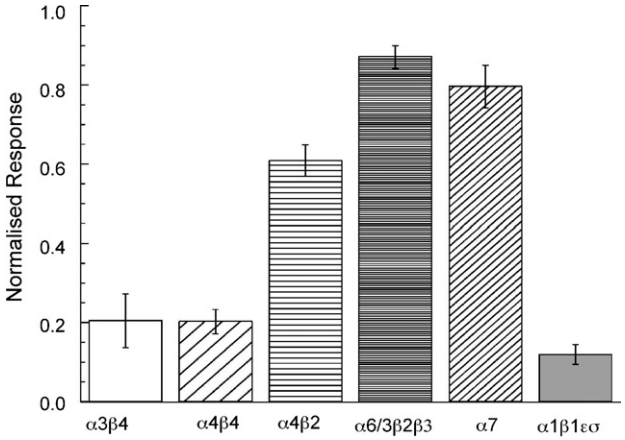

Fig. 4 – Inhibition of nAChR subtypes expressed in *Xenopus* oocytes. Plotted are the average responses (±S.E.M.) of four or more oocytes to the co-application of ACh at control concentrations (see Section 2) and 1 μM TMPD, normalized to the responses obtained from the same cells to ACh applied alone. Pairwise *t*-tests of the responses to ACh alone compared to ACh plus TMPD indicated that TMPD produced significant inhibition of the ACh-evoked responses of cells expressing rat α3β4 ($p < 0.01$), α4β4 ($p < 0.01$), α4β2 ($p < 0.05$) or mouse muscle-type (α1β1εδ) ($p < 0.01$) subunits. Based on these data, the likely IC_{50} values for TMPD are estimated to fall in the following ranges; 160–380 nM, 200–300 nM, 1.3–1.9 μM and 100–160 nM, for α3β4, α4β4, α4β2, and muscle-type, α1β1εδ receptors, respectively.

TMPD showed only 18–20% inhibition at α6/3β2β3 and α7 receptors. After a washout period, cells were retested with control applications of ACh alone. Only the responses of α3β4-expressing oocytes, which were 73 ± 5% of their original amplitude, remained significantly lower than the original ACh controls ($p < 0.05$; data not shown).

The blood–brain barrier permeability surface-area product for [^{3}H]choline in this study was 1.29 ± 0.07 μl/s/g, which approximates previously determined values [69]. Addition of 250 μM of TMPD to the perfusate fluid reduced [^{3}H]choline permeability surface-area product at the blood–brain barrier to 0.21 ± 0.02 μl/s/g (Fig. 5), affording an apparent K_i of 22.3 ± 2.5 μM. To calculate the K_i of TMPD competitive kinetics were assumed and the following relationship was used: $[(PA_o - K_D)/(PA_i - K_D)] = 1 + C_i/K_i$, where PA_o is the [^{3}H]choline PA in the absence of TMPD, PA_i is the [^{3}H]choline PA in the presence of TMPD, and Ci is the perfusate concentration of TMPD. As such, the apparent K_i was defined as the TMPD concentration that reduces saturable brain [^{3}H]choline influx by 50% at a tracer choline concentration ($C_{pf} \ll K_m$) and in the absence of other competing compounds. These data provide evidence of the degree of blood–brain barrier choline transport interaction. The decrease in [^{3}H]choline produced by TMPD is similar to that observed upon addition of 250 μM choline (0.29 ± 0.01 μl/s/g; Fig. 5), affording an apparent K_m of 44.3 ± 1.5 μM for choline [74,69]. In the latter studies, the K_m for choline was determined using multiple concentrations (250 nM to 20 mM) of unlabeled choline. Thus, TMPD has

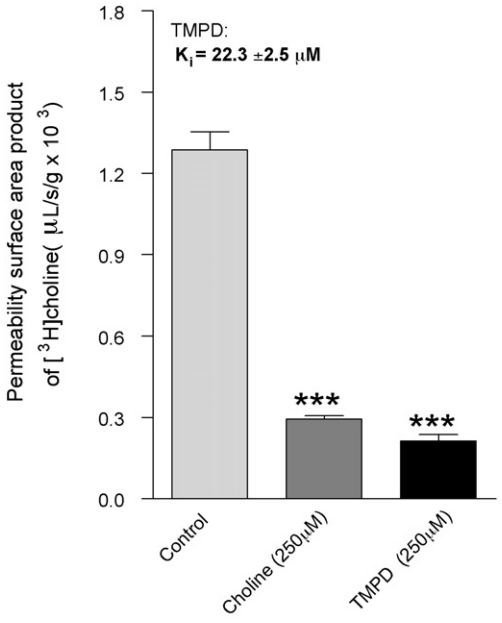

Fig. 5 – TMPD has a 2-fold higher affinity for the blood–brain barrier choline transporter than choline. A significant reduction (*; $p < 0.001$) of the cortical blood–brain barrier PS of [^{3}H]choline was observed by the addition of 250 μM of unlabeled choline or TMPD in the vascular perfusate. Similar significant reductions were noted in 8 cortical and sub-cortical brain regions (data not shown). The apparent calculated K_i is defined as the concentration that reduces saturable brain [^{3}H]-choline (tracer concentration) influx by 50%. Data are mean + S.E.M.; n = 3–4.**

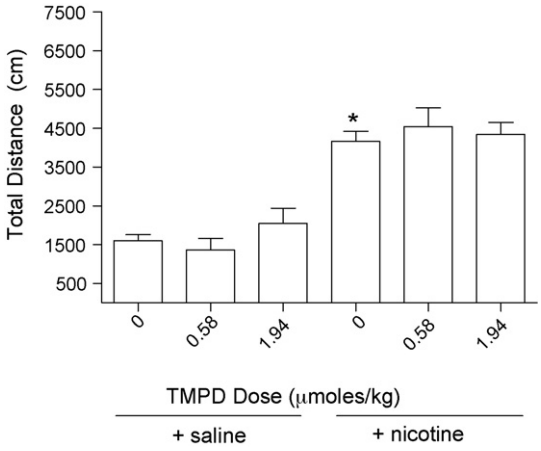

Fig. 6 – Mean (±S.E.M.) distance traveled during the last 30 min of the 60-min session in rats pretreated with saline or TMPD (0.58 or 1.94 μmol/kg), followed by saline (left 3 bars) or nicotine (right 3 bars). Note that rats were initially sensitized to repeated daily nicotine injections. Asterisk (*) represents a significant difference compared to saline alone, $p < 0.05$. n = 4 per bar.

2-fold higher affinity for the blood–brain barrier choline transporter compared with the endogenous substrate choline.

Fig. 6 illustrates locomotor activity exhibited by rats sensitized initially to nicotine and subsequently pretreated with saline or TMPD (0.58 or 1.94 μmol/kg) in the absence or presence of nicotine. As expected, rats tested under the influence of nicotine alone were significantly more active than rats tested with saline alone ($t = 8.43$; $df = 6$, $p < 0.05$). However, one-way ANOVA revealed no significant effect of TMPD dose, indicating that TMPD did not alter nicotine-induced activity within the dose range tested. In addition, in the experiment in which each dose of TMPD was administered alone across 5 repeated daily injections, no significant change in activity was observed following either acute or repeated treatment (results not shown).

In the self-administration experiment, under baseline control conditions, rats earned 14.2 ± 0.2 (mean ± S.E.M.) nicotine infusions or 17 ± 0 food reinforcers. Fig. 7 illustrates the number of nicotine and food reinforcers earned as a function of TMPD dose, expressed as a percent change relative to the baseline control condition (no pretreatment) within each experiment. A two-way ANOVA revealed significant main effects of dose ($F = 5.21$; $df = 1,7$; $p < 0.05$) and reinforcer type ($F = 5.45$; $df = 1,7$; $P < 0.05$); there was no significant interaction between dose and reinforcer type. Thus, regardless of the dose tested, TMPD produced a greater decrease in the number of nicotine reinforcers earned compared to the number of food reinforcers earned.

4. Discussion

The current study introduces for the first time a novel nicotinic receptor antagonist, TMPD, which is characterized as a bis-quaternary ammonium analog that is a hybrid of the classical neuromuscular channel blocker, decamethonium dibromide, and the neuronal nicotinic receptor antagonist, bPiDDB. In our search for novel nicotinic receptor antagonists, the nicotine-evoked DA release assay is used as an initial screen to identify lead compounds, since the ability of nicotine to release DA is believed to be associated with the rewarding properties of

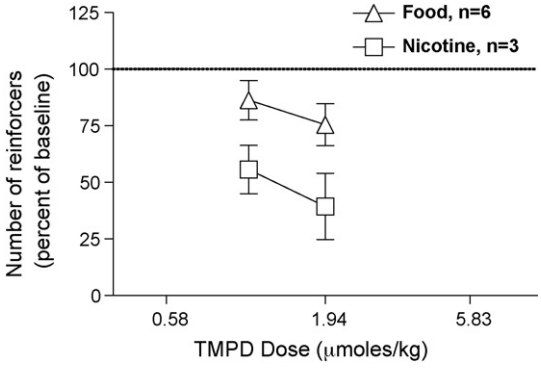

Fig. 7 – Mean (±S.E.M.) percent decrease in number of nicotine infusions or food pellets earned following pretreatment with TMPD (1.1 or 1.94 μmol/kg). The line at 100% represents baseline number of reinforcers earned without any pretreatment.

nicotine, and striatum is important for identifying and anticipating rewards and organizing goal directed behavior [75–78]. TMPD was identified as a hit in this screen because it produced greater than 40% inhibition of nicotine-evoked DA release at a single probe concentration of 100 nM. In the full concentration–response analysis, TMPD was observed to completely inhibit nicotine-evoked DA release from superfused rat striatal slices, with an IC_{50} value of 500 nM. TMPD was 5-fold less potent than mecamylamine in this regard. As such, TMPD graduated from a ''hit'' to a lead compound. Thus, replacing one of the two 3-picolinium head groups of bPiDDB with a triethylammonium moiety resulted in a compound with a 100-fold lower potency compared to bPiDDB, and that completely inhibited nicotine-evoked DA release from striatal slices. These results suggest that TMPD is likely acting as an antagonist at more than one nAChR subtype, since bPiDDB produces maximal but incomplete inhibition of nicotine-evoked DA release from rat striatal slices.

As part of our drug discovery process, TMPD was evaluated in oocyte expression experiments against the indicated panel of receptor subtypes at a probe concentration of 1 μM, along with many other candidate compounds. Data obtained at a single probe concentration can be quite informative, such that IC_{50} values can be estimated to fall within the following ranges; 160–380 nM, 200–300 nM, 1.3–1.9 μM and 100–160 nM, for α3β4, α4β4, α4β2, and α1β1εδ receptors, respectively. While higher concentrations of TMPD might have also produced significant inhibition of α6/3β2β3 and α7 receptors, the current data suggest that IC_{50} values for these latter subtypes would be greater than 5 μM and 3 μM, respectively. Note that in the oocyte expression experiments, TMPD produced a significant inhibition of muscle-type receptors, as well as several neuronal subtypes of nAChR, including α3β4 and α4β4, but not at α6/3β2β3 or α7 subtypes. α7 nAChRs are not strongly implicated in the addictive processes [79], and lack of inhibition by TMPD at this subtype suggests that TMPD could have a low side-effect liability. The effects of TMPD on muscle receptors were readily reversible and consistent with a use-dependent mechanism. Specifically, inhibition was observed to accumulate during the course of the response so that while there was a total inhibition of 88 ± 2% of the net charge, peak currents were inhibited by only 65 ± 5%. The time to peak current was also significantly advanced in the presence of TMPD, so that while in response to ACh alone, peak responses occurred in 5.6 ± 0.8 s (basically the same as the time constant for solution exchange), in the presence of TMPD peak responses occurred in 1.5 ± 0.3 s. These features are consistent with, and indicative of, use-dependent inhibition [80]. Since the effects of co-application were fully reversible after only a brief washout, a strict test of use dependence (i.e., application of the drug in the absence of agonist) would not have been informative. Therefore, it seems unlikely that effects on muscle receptors would, in fact, represent a serious side-effect liability due to the enormous receptor reserve at neuromuscular junctions.

Brain penetration of TMPD was expected to be severely limited given that it is a charged di-cationic molecule. However, in our recent studies, the blood–brain barrier choline transporter was shown to bind to a large number of structurally-related quaternary ammonium cations, and this transporter was shown to facilitate the brain bioavailability of at least two of these charged molecules, specifically, the monocationic quaternary ammonium compound, *N*-octylnicotinium iodide (NONI), and the bis-quaternary ammonium compound, bPiDDB [81]. Thus, while affinity does not equate to transport across the blood–brain barrier choline transporter, affinity coupled with demonstrated CNS activity (i.e., the current results from behavioral studies) suggest that TMPD utilizes the blood–brain barrier choline transporter as a vector to access the brain. Future kinetic analyses will be performed using radiolabeled-TMPD to directly determine the brain bioavailability of TMPD. In this respect, given that TMPD had a 2-fold higher affinity than choline for the blood–brain barrier choline transporter, and that choline plasma levels are only about 25% of the K_m of the blood–brain barrier choline transporter, the carrier has the capacity to transport TMPD without interrupting the supply of choline to the brain [81].

Since TMPD decreased nicotine-evoked DA release, we anticipated that if TMPD crosses the blood–brain barrier in sufficient concentrations following systemic administration, TMPD would inhibit the behavioral effects of nicotine. Surprisingly, TMPD did not inhibit the hyperactivity in nicotine sensitized rats, but did significantly decrease nicotine self-administration. The lack of effect on activity by TMPD in the dose range tested is important because it suggests that the decrease in nicotine self-administration is not due to a general sedative effect. Furthermore, the TMPD-induced decrease in nicotine self-administration was relatively specific, since at both doses of TMPD, a greater decrease in nicotine self-administration was observed compared to the decrease in responding for food reinforcement. Thus, these results suggest that TMPD may have the ability to reduce the rewarding effect of nicotine with minimal side effects, a pharmacological profile indicative of potential clinical utility for the treatment of tobacco dependence.

In summary, when one of the 3-picolinium head groups of bPiDDB is replaced by a triethylammonium moiety to afford TMPD, the outcome is a loss of potency and selectivity at native nAChRs mediating nicotine-evoked DA release. Interaction of TMPD with various nAChRs expressed in *Xenopus* oocytes was performed to begin to determine the nAChR subtype with which TMPD interacts. Nevertheless, similar to mecamylamine, TMPD is an efficacious inhibitor of nicotine self-administration in rats.

Acknowledgments

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