Expedited Articles

Synthesis and Evaluation of *N*-[^{11}C]Methylated Analogues of Epibatidine as Tracers for Positron Emission Tomographic Studies of Nicotinic Acetylcholine Receptors

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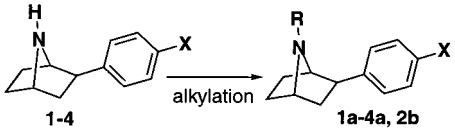
Four halogen-substituted analogues of *N*-methylepibatidine, a nicotinic acetylcholine receptor (nAChR) ligand, were synthesized. They were (±)-*exo*-*N*-methyl-2-(2-halogeno-5-pyridyl)-7-azabicyclo[2.2.1]heptanes, where halogeno = F (**1a**), Cl (**2a**), Br (**3a**), I (**4a**). (±)-*N*-Ethylepibatidine (**2b**) also was synthesized. The compounds **1a**, **2a**, **3a**, and **4a** and their corresponding normethyl analogues **1**, **2**, **3**, and **4** inhibited the in vitro binding of [^{3}H]epibatidine to nAChRs to a similar degree, with affinities in the 27−50 pM range. The binding affinity of *N*-ethylepibatidine (**2b**), however, was substantially lower. The *N*-[^{11}C]methyl derivatives of **1**, **2**, and **3** were synthesized from high-specific radioactivity [^{11}C]methyl iodide using a high-temperature/high-pressure technique. The corresponding radiolabeled compounds [^{11}C]**1a**, [^{11}C]-**2a**, and [^{11}C]**3a** were administrated to mice intravenously. The pattern of regional distribution of the three tracers in the mouse brain following intravenous administration matched those of [^{3}H]epibatidine, [^{3}H]norchloroepibatidine, and (±)-*exo*-2-(2-[^{18}F]fluoro-5-pyridyl)-7-azabicyclo-[2.2.1]heptane ([^{18}F]FPH), which are highly specific nAChR probes. The initial brain uptake of the ^{11}C analogues and the acute toxicity of the corresponding authentic nonlabeled compounds appeared to be related to their lipophilicity.

Introduction

As mediators of excitatory neurotransmission, central nicotinic acetylcholine receptors (nAChRs) have fundamental roles in brain function. They have been implicated in a variety of central processes, such as learning and memory,[1−4] and antinociception.[5] Postmortem studies of brains from patients with Alzheimer's disease[6,7] show deficits in neocortical and hippocampal concentrations of nAChRs, whereas brains from individuals who smoked cigarettes show elevated levels of nAChRs.[8]

Although in vivo studies of nAChRs in human brain would be useful in relating receptor parameters to brain function and to the progress of neurodegenerative diseases, until recently they have only been possible using the radiotracer [^{11}C]-(−)-nicotine.[9,10] Studies with this tracer have been carried out in nonhuman primates, healthy human volunteers, and patients with Alzheimer's disease. The high nonspecific binding and rapid clearance of [^{11}C]-(−)-nicotine from the brain, however, preclude its widespread use in the in vivo evaluation of nAChRs in human subjects.[9]

Scheme 1



FPH, **1** (X=F)
(+/-)-epibatidine, **2** (X=Cl)
BrPH, **3** (X=Br)
IPH, **4** (X=I)

1a (X=F; R=CH_3)
2a (X=Cl; R=CH_3)
2b (X=Cl; R=C_2H_5)
3a (X=Br; R=CH_3)
4a (X=I; R=CH_3)

Recent findings have indicated that (−)-epibatidine[13−16] (Scheme 1), an alkaloid from skin of the Ecuadorian frog *Epipedobates tricolor*,[11] is a promising parent structure on which to base future radiotracer development for in vivo labeling of nAChRs.[12] Our studies demonstrated high uptake of [^{3}H]epibatidine in the mouse brain, a regional distribution consistent with that of nAChRs, and slow clearance from the brain.[12] The in vivo biodistribution of the norchloro analogue of [^{3}H]epibatidine was similar to that of the parent compound.[17] Recently, [^{18}F]FPH ([^{18}F]**1**), the radiofluorinated analogue of epibatidine, has been synthesized,[18] and it was shown to label nAChR specifically in vivo in the mouse brain.[19−21] Because of its high uptake into the brain and its preferential localization to nAChR-rich areas rather than nAChR-poor regions, this radioligand appears to be well-suited for positron emission tomo-

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graphic (PET) imaging of nAChRs in the mammalian brain.[20] PET studies with [^{18}F]FPH in baboon brain demonstrated the feasibility of its use for imaging nAChRs in vivo.[21−23] Similar results were obtained in in vivo studies with [^{125}I]- and [^{123}I]IPH, radioiodinated analogues of epibatidine.[24] These radioiodinated tracers promise to be excellent ligands for single-photon emission tomographic (SPECT) imaging of nAChRs and autoradiographic studies, respectively.

Whereas the conventional dose (about 0.1 nmol/kg) of [^{18}F]FPH for a PET study in a baboon did not produce any noticeable pharmacological effects,[21,23] higher doses of epibatidine analogues induce powerful activation of nAChRs,[25] as well as prolonged catecholamine release,[26] and consequent cardiovascular effects. Because of a report that the enantiomers of *N*-methylepibatidine had one-half to one-fourth the affinities of the corresponding enantiomers of epibatidine in competition assays against [^{3}H]nicotine binding,[15] we reasoned that introduction of various alkyl groups into the epibatidine molecule might moderately decrease the binding affinity for nAChRs and toxicity as well while retaining high specific incorporation into brain regions enriched with nAChRs. We also assumed that modification of the lipophilicity without a substantial change in electronic influence of the substituents in the pyridine ring might optimize tracer uptake into the brain.

The objectives of the present study were to synthesize racemic *N*-alkyl derivatives of epibatidine and their halogen analogues,[27] to label them with the positron-emitting nuclide ^{11}C, to assess their ability to label central nAChRs in vivo, and to determine their acute toxicity in mice.

Results

In Vivo Kinetics and Regional Distribution. The results of the in vivo assays showed that [^{11}C]**1a**, [^{11}C]-**2a**, and [^{11}C]**3a** label nAChRs in the mouse brain with high affinity and pharmacology comparable to those of [^{18}F]FPH, which was previously developed for use in PET imaging.[19,21,23] Following intravenous administration of [^{11}C]**1a**, [^{11}C]**2a**, and [^{11}C]**3a** at doses ranging between 1.2 and 1.4 nmol/kg (25−35 μCi) in mice, all three compounds were avidly taken up into whole brain (Figure 1). At 2 min after injection the whole brain level of radioactivity was similar for [^{11}C]**1a** and [^{11}C]**2a** (4.8% and 5.1% of the injected dose (% I.D.), respectively) but lower for [^{11}C]**3a** (3.6% I.D.). Of the three compounds, [^{11}C]**2a** was most rapidly cleared from the brain (see 5-min value). Compound **2b** was not tested in vivo because of its relatively low affinity toward nAChRs in in vitro binding assay.

The kinetics of [^{11}C]**1a**, [^{11}C]**2a**, and [^{11}C]**3a** in the thalamus and cerebellum, areas with a high and a low density of nAChRs of the mouse brain, respectively,[30,31] were followed over a period of 60 min (Figure 2). All three tracers were rapidly taken up in thalamus and cerebellum followed by gradual decline from thalamus and rapid clearance from cerebellum. The rapid clearance of all three tracers from the cerebellum indicated that specific binding in this region of the mouse brain was relatively low.

The regional distribution of [^{11}C]**1a**, [^{11}C]**2a**, and [^{11}C]-**3a** in mouse brain at 45 min after intravenous injection showed highest concentrations of radioactivity in thalamus and superior colliculus, intermediate concentrations in striatum and cortex, and lower concentrations in hippocampus and cerebellum (Figure 3). The correlation coefficients between the distribution of labeled [^{11}C]**1a,** [^{11}C]**2a,** and [^{11}C]**3a** with that of [^{3}H]epibatidine[12] were 0.96, 0.95, and 0.97, respectively, and with that of [^{18}F]FPH,[19] they were 0.95, 0.92, and 0.88, respectively.

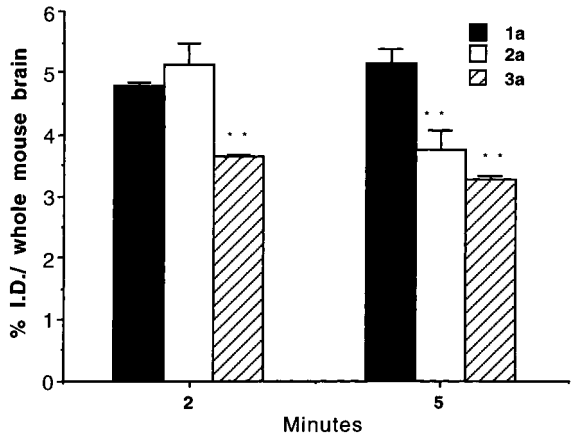


Figure 1. Uptake of [^{11}C]**1a**, [^{11}C]**2a**, and [^{11}C]**3a** into whole mouse brain at 2 and 5 min after intravenous injection (data are means ± SD; $n = 3$). Carrier dose values were 1.2−1.4 nmol/kg; $^{**}p < 0.01$ (2-way ANOVA with post hoc Dunnett's test) at 2 min, **2a** vs **3a** and **1a** vs **3a**; at 5 min, **1a** vs **2a** and **1a** vs **3a**.

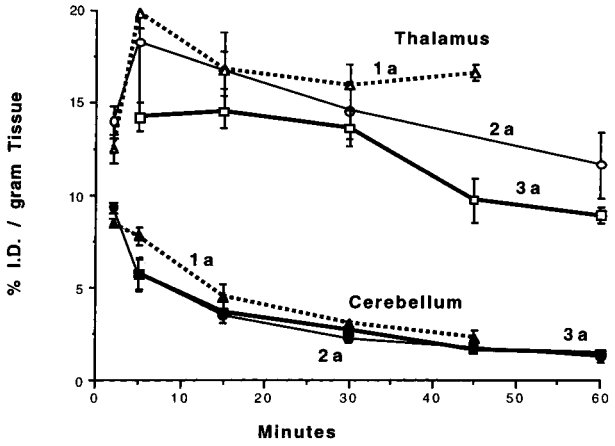


Figure 2. Kinetics of [^{11}C]**1a**, [^{11}C]**2a**, and [^{11}C]**3a** in thalamus and cerebellum after intravenous injection into mice (means ± SD; $n = 3$).

The uptake of [^{11}C]**1a** was high, particularly in thalamus and superior colliculus. It exceeded 15% of the injected dose per gram of tissue (I.D./g of tissue) at the carrier dose level of 1.4 nmol/kg. When the carrier dose was increased to 10 nmol/kg (200−250 μCi/mouse), the percentages of radioactivity measured in the different brain regions were reduced, demonstrating that nAChRs were partially saturated at this higher dose (Figure 4). The approximate percentage of receptor occupancy in thalamus at 45 min after administration of 1.4 and 10 nmol/kg [^{11}C]**1a** was calculated to be 15% and 55%, respectively.

Studies of Acute Toxicity in Mice. All three methylated compounds, [^{11}C]**1a**, [^{11}C]**2a**, and [^{11}C]**3a**, exhibited toxic effects at relatively low doses when

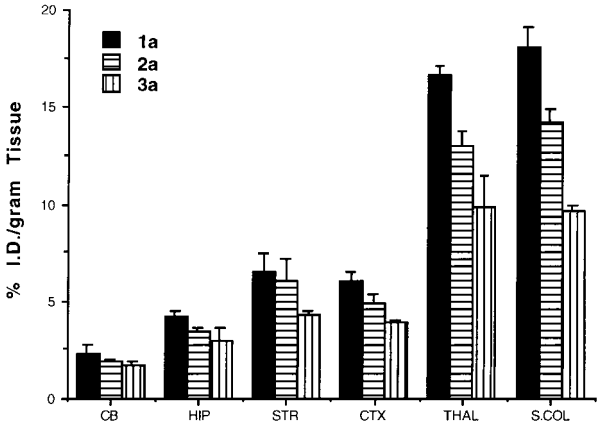


Figure 3. Regional distributions of radioactivity in mouse brain at 45 min after injection of [^{11}C]**1a**, [^{11}C]**2a**, and [^{11}C]-**3a**. Dose was 1.2−1.4 nmol/kg. Data are means ± SD; $n = 3$. CB, cerebellum; HIP, hippocampus; STR, striatum; CTX, parietal cortex; THAL, thalamus; SCOL, superior colliculus.

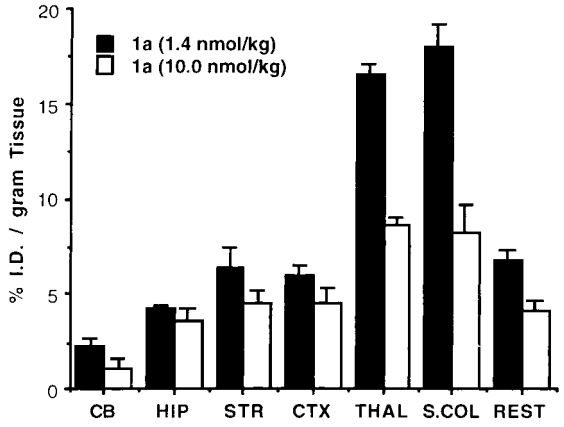


Figure 4. Regional distribution of radioactivity in mouse brain at 45 min after injection of 1.4 and 10.0 nmol/kg [^{11}C]-**1a**. Data are means ± SD ($n = 3$) and are expressed as percent of injected dose per gram of tissue. See Figure 3 for definition of abbreviations. Rest, remaining part of brain after dissection of all other areas presented.

injected intravenously into mice. However, the pharmacological effects of the three tracers varied slightly. At the 1.2−1.4 nmol/kg doses of [^{11}C]**1a** and [^{11}C]**3a**, no overt pharmacological signs were noted, whereas with [^{11}C]**2a** the animals had seizures and displayed Straub tail, and 1 of 19 mice died immediately. At a dose of 10 nmol/kg, however, animals given [^{11}C]**1a** or [^{11}C]**3a** also showed symptoms similar to those observed in mice that received [^{11}C]**2a** at the lower dose.

After intravenous injection of compounds **1**, **2**, **3**, and **4** into mice, approximate LD_{50} values of 40.6, 16.0, 13.6, and 66.7 nmol/kg were determined. For compounds **1a**, **2a**, **3a**, and **4a** the intravenous LD_{50} values were 24.3, 6.7, 11.2, and 79.6 nmol/kg, respectively.

For the correlation between the degree of lipophilicity of the different compounds in the two series with (1) their toxicity (expressed as $\log(1/LD_{50})$) and (2) their penetration into the mouse brain, the respective hydrophobic substituent constants (π values) were applied. Figure 5 shows a parabolic relationship between the lipophilicity of the respective compounds and (1) their toxicity and (2) their level of radioactivity in the whole brain at 2 min. These results are in concert with the Hansch equation,[32,33] which states that the biological responses of related drugs, including their toxicity, are a function of their lipophilicity and can be fitted to a parabolic curve with a slope symmetric around an "optimum".

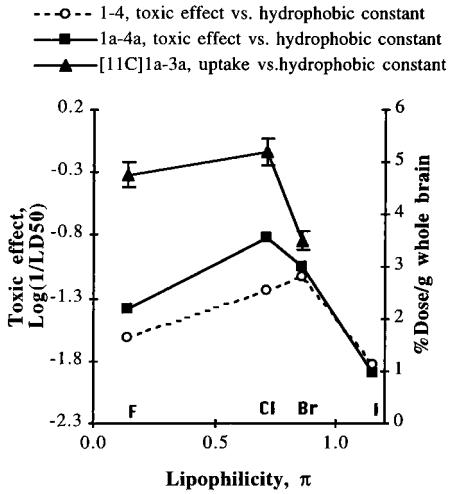


Figure 5. Mouse lethality after administration of epibatidine analogues **1**, **2**, **3**, **4** and **1a**, **2a**, **3a**, **4a** vs their lipophilicity and also radioactivity uptake into the whole mouse brain (2 min after administration) of epibatidine analogues [^{11}C]**1a**, [^{11}C]**2a**, [^{11}C]**3a** vs their lipophilicity.

Discussion

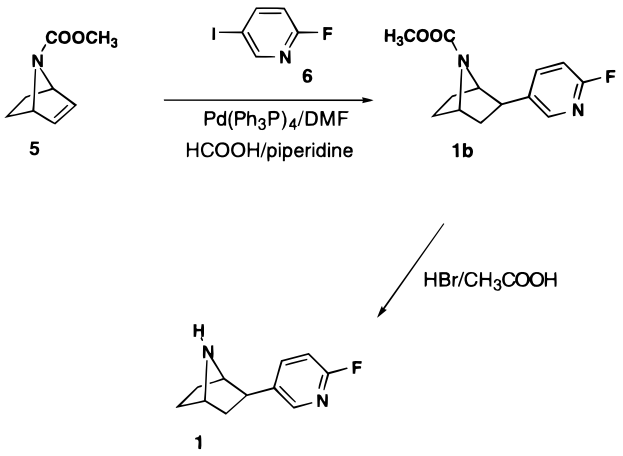
Organic Chemistry. Several procedures for the synthesis of epibatidine have been reported.[34,35] Microscale *N*-methylation of the enantiomers of epibatidine with formaldehyde has been described, however, without analytical details presented.[15]

Scheme 1 outlines the preparation of the *N*-alkylepibatidine analogues developed in this study. Our attempts to alkylate epibatidine (**2**) and its Br analogue (**3**) directly with methyl iodide in solutions of anhydrous solvents (CH_3CN, DMF, DMSO, acetone, ethanol) in the presence of a variety of basic catalysts gave very low methylation yields. Even under drastic conditions (150−180 °C, 24 h, high pressure), the highest yield was less than 10% (data not shown). Methylation of FPH (**1**) wih methyl iodide yielded only decomposition products. In contrast, a reductive methylation of racemic epibatidine (**2**),[15] BrPH (**3**), and IPH (**4**) produced the corresponding *N*-methyl derivatives (**2a**, **3a**, and **4a**) in high yield.

Attempts to *N*-methylate FPH (**1**) by reductive methylation failed due to instability of this compound under the reaction conditions. *N*-Methyl-FPH (**1a)** was easily synthesized, however, by treatment of **1** with methyl triflate at room temperature. Racemic *N*-ethylepibatidine (**2b**) was prepared by treatment of racemic epibatidine with ethyl triflate in a similar manner.

Previously, we described the microscale synthesis of the fluoro analogue of epibatidine, FPH (**1**), by a halogen-exchange substitution reaction.[18] For the preparation of milligram amounts of FPH (**1**) and its *N*-methyl analogue, a more effective method of FPH production was necessary. A synthetic scheme based on a Heck-type palladium-assisted coupling reaction[36] of 7-(methoxycarbonyl)-7-azanorbornene (**5**)[36] with 2-fluoro-5-iodopyridine (**6**)[27,28] followed by deprotection, as described

Scheme 2



previously,[18] produced hundreds of milligrams of FPH (**1**) with a 35% yield[27] (Scheme 2).

Radiosynthesis. *N*-[^{11}C]Methylepibatidine ([^{11}C]**2a)** and its analogues ([^{11}C]**1a** and [^{11}C]**3a**) were synthesized by reacting the corresponding normethyl derivatives (**1**, **2**, or **3**) with high-specific radioactivity [^{11}C]methyl iodide in a solution of acetonitrile at 185 ± 0.5 °C for 10 min followed by purification using reverse-phase HPLC.[27] The overall non-decay-corrected radiochemical yield was 4−15% for all three labeled compounds ([^{11}C]-**1a**, [^{11}C]**2a**, or [^{11}C]**3a**). Specific radioactivities were in the range of 1500−1800 mCi/μmol as calculated at end of synthesis (EOS). The average time of radiosynthesis, including HPLC purification, for the three compounds was 35 min. The labeled compounds ([^{11}C]**1a**, [^{11}C]**2a**, or [^{11}C]**3a**) were characterized by co-injection with authentic samples of the unlabeled compounds (**1a**, **2a**, or **3a**) on reverse-phase HPLC. The corresponding unreacted normethyl precursors (**1**, **2**, or **3**) were not detected by conventional HPLC (UV detector) in the final solutions of radiolabeled compounds.

Optimization of the [^{11}C]methylation of epibatidine analogues was performed with BrPH, **3**. When the previously reported procedure[37] for [^{11}C]methylation of epibatidine with [^{11}C]methyl iodide (CH_3CN, diisopropylethylamine, 100 °C, 5 min) was applied to BrPH, the yield of the final *N*-[^{11}C]methyl-BrPH ([^{11}C]**3a**) was about 1%. Solvent, catalyst, and temperature conditions were varied in attempts to increase the yield. Poor to no radiochemical yield of [^{11}C]**3a** was observed when DMF or toluene was used. Acetonitrile was found to be the solvent of choice for the radiomethylation of epibatidine analogues. The use of a variety of basic catalysts produced only low radiochemical yields; therefore none was used. The optimal radiomethylation temperature was 180−185 °C. At lower temperatures, the reaction mixture contained a substantial amount of unreacted [^{11}C]CH_3I. At temperatures higher than 200 °C, the radiochemical yield dropped and the reaction mixture contained unidentified labeled and unlabeled byproducts. Under the optimal conditions (180−185 °C, 10 min, CH_3CN, high-pressure vessel), the decay-corrected radiochemical yield of the labeled compound [^{11}C]**3a** was 4.5% after preparative HPLC separation and formulation.

The same high-temperature requirements were also needed for *N*-[^{11}C]methylation of epibatidine (**2**) and FPH (**1**). *N*-[^{11}C]Methylation of compounds **1** and **2** under the same reaction conditions provided 14.9% and 13.4% radiochemical yield (EOS) of *N*-[^{11}C]methyl compounds **1a** and **2a**, respectively.

Table 1. Inhibition of the in Vitro Binding of [^{3}H]Epibatidine to Central nAChRs in Rat Brain by Epibatidine Derivatives and Nicotine

compd	mean K_d(drug) ± SEM, nM	no. of assays
1a	0.028 ± 0.0026	5
2a	0.027 ± 0.0016	4
3a	0.036 ± 0.0042	3
1	0.037 ± 0.0045	3
2	0.027 ± 0.0006	4
3	0.045 ± 0.0025	4
2b	13.6 ± 0.21	4
(−)-nicotine	5.0 ± 0.29	8

In Vitro Binding Assay. Epibatidine is a highly potent agonist at central, ganglionic, and neuromuscular nAChRs.[12,13,15,38,39] It has exceptionally high affinity for the $\alpha_4\beta_2$ nAChR subtype (15 pM) and manifests very low nonspecific binding in the rat brain.[15] Such properties allow its use in binding assays performed at 4 °C, a condition that is recommended for other available tritiated nAChR ligands and especially for [^{3}H]-(−)-nicotine, which have much lower affinity and substantial nonspecific binding. Compounds with affinities in the low-picomolar range manifest very slow kinetics of association and dissociation, requiring long incubations (hours at room temperature and tens of hours at 4 °C) to reach equilibrium in binding assays. [^{3}H]-(±)-Epibatidine has therefore become a ligand of choice, especially for competition studies with nAChR ligands that have affinities in the picomolar range. The potential of [^{3}H]-(±)-epibatidine as a ligand for competition assays was demonstrated previously.[15] We used similar conditions, but we performed our assays at pH 6.8 instead of pH 7.4 as reported[15] to avoid a potential decomposition of fluoro analogues of epibatidine.

The affinities of (±)-epibatidine and (−)-nicotine measured in our assays were comparable to those published before.[15] Compared with results from competition assays with [^{3}H]-(−)-nicotine,[15] the K_i of (±)-epibatidine in our assays was slightly lower than those obtained with epibatidine stereoisomers, and the K_i of (−)-nicotine was about 5 times as high.[15] Since the previous studies with [^{3}H]-(−)-nicotine[15] were performed at 4 °C, this discrepancy may reflect an effect of the temperature.

Our results demonstrated that all three compounds (**1**, **2**, and **3**) have very high affinity, with K_d values ranging from 27 to 45 pM, and that *N*-methylation of these compounds, including (±)-epibatidine, does not produce any marked changes in their affinities (Table 1). In contrast, incorporation of an ethyl group into (±)-epibatidine decreased its binding affinity to 0.2% of that of the parent compound.

It was previously reported[15] that *N*-methylation of epibatidine stereoisomers significantly increases the K_i value of the (−)-isomer (260 ± 90 pM vs 45 ± 4 pM) with much less of an increase for the (+)-isomer (110 ± 30 pM vs 58 ± 7 pM). These results (mean ± SEM with $n = 3$) were obtained from competition assays with [^{3}H]-(−)-nicotine and rat cerebral cortical membrane preparations, incubated for 120 min at 4 °C at pH 7.4. Based

on the K_i values of the stereoisomers, it can be predicted that the affinity of the *N*-methyl-(±)-epibatidine under these conditions should be about 3 times as high as that of (±)-epibatidine (150 pM vs 50 pM). Taking into consideration the dispersion (SEM) of these measurements, the ratio might vary, roughly from 1.5- to 6-fold. The lack of any effect of *N*-methylation on the affinity of (±)-epibatidine in our study may reflect an effect of incubation temperature or pH that contributed to discrepancy in the results from these two studies. Alternatively, the products of *N*-methylation of the epibatidine stereoisomers may have much slower binding kinetics than those of the parent compounds. In that case, underestimation of the affinity of these methylated compounds could result in insufficient time of incubation to reach equilibrium in the assays with [^{3}H]-(−)-nicotine. Finally, the discrepancy may result from differences in the relative concentrations of nAChR subtypes, specifically $\alpha_4\beta_2$ and $\alpha_3\beta_4$, in the tissue preparations used and differences in the selectivity of (−)-nicotine and (±)-epibatidine for these receptor subtypes.

In the rat brain, most of the high-affinity nAChR sites that bind [^{3}H]-(−)-nicotine[41] and [^{3}H]-(±)-epibatidine[15] are of the $\alpha_4\beta_2$ subtype. Nonetheless, a minor component of the [^{3}H]-(±)-epibatidine binding in the rat forebrain at the ligand concentration used (30−40 pM) may be related to an interaction with the $\alpha_3\beta_4$ receptor subtype.[15] In some brain regions this subtype is seen in reasonable concentrations.[42] The observed K_i values of the ligands studied may therefore reflect interactions with the $\alpha_3\beta_4$ receptor subtype to some extent. In this regard, it is notable that in contrast to results of competition studies with [^{3}H]-(−)-nicotine or [^{3}H]-(±)-epibatidine, *N*-methyl-(−)-epibatidine was 8 times as potent as *N*-methyl-(+)-epibatidine and 3 times as potent as (−)-epibatidine in activation of the $\alpha_3\beta_4$ subtype of nAChRs in PC12 cells.[16] Additional studies with stereoisomers of *N*-methylepibatidine and nAChR subtype-selective ligands may be warranted.

In Vivo Regional Brain Biodistribution and Toxicity. The *N*-[^{11}C]methyl derivatives ([^{11}C]**1a**, [^{11}C]-**2a**, and [^{11}C]**3a)** showed rapid accumulation into the whole mouse brain with the peak occurring at 2 min after intravenous administration (4.8%, 5.1%, and 3.6% I.D. for [^{11}C]**1a**, [^{11}C]**2a**, and [^{11}C]**3a**, respectively) (data is not shown). Thereafter, the radioactivity declined gradually over the next 45−60 min. This pattern of kinetics in the whole mouse brain is similar to that of the ^{18}F-labeled fluoroanalogue of epibatidine ([^{18}F]-FPH)[19] but differs from those of [^{3}H]epibatidine and [^{3}H]-norchloroepibatidine, tracers which show gradual uptake with a peak at 30 min after injection.[12,17]

The three *N*-[^{11}C]methyl derivatives ([^{11}C]**1a**, [^{11}C]**2a**, and [^{11}C]**3a**) displayed similar regional distributions in the mouse brain (Figure 2), matching those of [^{18}F]-FPH[19] and [^{3}H]epibatidine.[12] Although total brain radioactivity declined over the 45-min observation period, specific binding, estimated as ratios of thalamus-to-cerebellum, climbed to about 7.0 (range 6.0−7.3) for all three *N*-[^{11}C]methyl analogues.

Epibatidine and its derivatives are known to be highly potent in producing biological effects. Intravenous administration of epibatidine, even at very low doses (2.5−9.6 nmol/kg), transiently increases arterial pressure and discharge of superior cervical or splanchnic sympathetic nerve.[25] Moreover, at doses of 200−400 nmol/kg sc in mice, epibatidine produces convulsions and death.[14] The approximate LD_{50} values of epibatidine, its analogues (**1**, **2**, **3**, and **4**), and corresponding *N*-methyl derivatives (**1a**, **2a**, **3a**, and **4a**) determined after intravenous injection into mice ranged between 7 and 68 nmol/kg. Within each series, toxicity depended on the lipophilicity of the particular analogue, and a parabolic relationship between the toxic effect expressed as $\log(1/LD_{50})$ and the π-hydrophobic constant of the respective halogen atom in the molecule was observed (Figure 5). A similar parabolic relationship was observed between the radioactivity due to ^{11}C-labeled tracers (**1a**, **2a**, and **3a**) in thalamus at 2 min postinjection and the π-hydrophobic constant of the respective halogen atom in the molecule.

The high degree of toxicity observed in mice dictates that utmost caution must be exercised in the preparation of these radiotracers for use in nonhuman primates and humans. The specific radioactivity must be maximized (>2000 mCi/μmol), and contamination with unlabeled product must be avoided.

Experimental Section

Chemistry. All reagents used were ACS or HPLC grade. A high-pressure tube (Ace Glass) was used for the radiomethylation. ^{1}H NMR spectra were recorded on a Bruker AM 300 (300 MHz) instrument; chemical shifts (δ) were recorded in parts per million (ppm) downfield from TMS. High-resolution electronic ionization mass spectra were recorded on an AEI MS-30 spectrometer (University of Minnesota Mass Spectra Laboratory). HPLC analysis and purification were performed with two Waters 590EF HPLC pumps, an in-line fixed wavelength (254 nm) detector, and a single, 5-cm NaI crystal radioactivity detector. HPLC chromatograms were recorded by a Rainin Dynamax dual channel control/interface module connected to a Macintosh computer with appropriate program software (Dynamax, version 1.3). A dose calibrator (Capintec CRC-12R) was used for all radioactivity measurements. Elemental analyses were performed at Atlantic Microlab, Atlanta, GA. All newly synthesized final compounds gave satisfactory elemental analyses (C, H, N, Cl, Br, ±0.4%).

(±)-*exo*-2-(2-Fluoro-5-pyridyl)-7-(methoxycarbonyl)-7-azabicyclo[2.2.1]heptane (1b). The Heck-type coupling of 7-(methoxycarbonyl)-7-azabicyclo[2.2.1]heptane (**5**)[36] (0.65 g, 4.52 mmol) and 2-fluoro-5-iodopyridine (**6**)[27,28] (1.43 g, 6.8 mmol) was performed using the general coupling conditions described elsewhere.[36] After the completion of the coupling the reaction mixture was diluted with water and extracted with CH_2Cl_2. The extract was washed with water (3 × 25 mL) and dried over Na_2SO_4. The solvent was evaporated, and CH_3CN (3 mL) was added to redissolve the residue. The solution was purified by preparative HPLC (Waters PrepPak μBondapak C18 HPLC cartridge, 25 mm × 100 mm) (mobile phase, CH_3CN-H_2O, 40:60; flow rate 12 mL/min). The peak containing the final product (t_R 10 min) was collected, and the solvent was partially evaporated on a rotary evaporator. The residual solution was saturated with Na_2SO_4 and extracted with CH_2Cl_2 (3 × 50 mL). The organic phase was dried over anhydrous Na_2SO_4, filtered, and evaporated to yield the final product as a transparent oil (0.4 g, 35%). NMR, high-resolution mass spectra, and HPLC characteristics were identical to those of compound **1b** synthesized as described previously.[18]

***N*-Methyl-(±)-*exo*-2-(2-fluoro-5-pyridyl)-7-azabicyclo-[2.2.1]heptane (N-Me-FPH) (1a).** Methyl triflate (30.6 μL, 0.27 mmol) was added to a vial containing 52 mg (0.27 mmol) of FPH[18] free base (**1**) in 1 mL of methylene chloride (anhydrous) at room temperature. The reaction mixture was stirred

overnight, the solvent was removed under vacuum, and the residue was redissolved in 5 mL of methanol. The solution was evaporated to dryness, and the oily residue was redissolved in 1.5 mL of HPLC mobile phase and injected into the HPLC column (Waters RCM Porapak (8 × 100 mm); mobile phase hexane−2-propanol−triethylamine, 100:5:0.02; flow rate 5 mL/min). The final product **1a** (t_R 2.5 min) was obtained as a white solid with a yield of 12 mg (22%) after evaporation of the solvent under vacuum: mp 44−45 °C (hexane); ^{1}H NMR ($CDCl_3$; multiplicity, *J*(Hz)) δ 8.09 (1H, d, 2.22, H$^{6'}$), 8.01 (1H, ddd, 8.25, 8.25, 2.43, H$^{4'}$), 6.82 (1H, dd, 8.49, 2.91, H$^{3'}$), 3.32 (1H, t, 4.02), 3.13 (1H, d, 3.75), 2.25 (3H, s, N−CH_3), 1.95−1.41 (6H, m, overlapping); MS *m*/*z* (rel intensity) [M^+] 206.1219 (7%); calculated for $C_{12}H_{15}FN_2$, M 206.1225.

(±)-*N*-Methylepibatidine (2a). A mixture of 200 mg of (±)-epibatidine free base (**2**),[36] 1 g of paraformaldehyde, and 7 mL of formic acid was stirred at 100−120 °C in a sealed vial for 5 h. The reaction mixture was diluted with 70 mL of H_2O and chilled, and pH was adjusted to 9−10 with 6 N sodium hydroxide solution. The resulting mixture was saturated with sodium chloride and extracted with methylene chloride (5 × 100 mL). Combined extracts were partially evaporated under vacuum, and the product (**2a**) was separated by semipreparative HPLC (t_R 4.5 min; Hamilton PRP-1 C18 column, 10 × 250 mm; mobile phase CH_3OH−CH_3CN−buffer (0.017 M NaH_2PO_4, 0.008 M Na_2HPO_4), 2:2:1; flow rate 7 mL/min). After collection of the product peak, the solution was partially evaporated, the pH was adjusted to 9−10 with 6 N NaOH, and the mixture was extracted with CH_2Cl_2 (80 mL × 3). The final product (**2a**) was obtained as a white solid with a yield of 189 mg (88.7%): mp 84−85 °C (hexane); ^{1}H NMR ($CDCl_3$; multiplicity, *J*(Hz)) δ 8.29 (1H, d, 2.43, H$^{6'}$), 7.88 (1H, dd, 8.31, 2.49, H$^{4'}$), 7.21 (1H, d, 8.22, H$^{3'}$), 3.34 (1H, s), 3.15 (1H, s), 2.66 (1H, dd, 9.21, 5.11, H^2), 2.26 (3H, s, N−CH_3), 1.94−1.41 (6H, overlapping, H^{3,3a,5,5a,6a,6}); MS *m*/*z* (rel intensity) [M^+] 222.0919 (13%); calculated for $C_{12}H_{15}ClN_2$, M 222.0924.

(±)-*N*-Ethylepibatidine (2b). Ethyl triflate (28.5 μL, 0.22 mmol; Aldrich) was added at −70 °C to a vial containing 47 mg (0.22 mmol) of (±)-epibatidine free base (**2**)[36] in 0.2 mL of methylene chloride (anhydrous). The cooling bath was removed, and the reaction mixture was stirred for 30 min. The reaction mixture was mixed with 50 mL of ether, 0.2 mL of triethylamine, and 0.2 mL of water, and the solvents were evaporated down. The residue was separated by semipreparative HPLC (t_R 3.5 min; Hamilton PRP-1 C18 column, 10 × 250 mm; mobile phase CH_3OH−CH_3CN−buffer (0.1 M ammonium formate, 0.2% triethylamine), 2:2:1; flow rate 10 mL/min). After collection of the product peak, the solution was partially evaporated, the pH was adjusted to 9−10 with 6 N NaOH, and the mixture was extracted with ether (100 mL × 3) yielding **2b** as a transparent oil (36.5%): ^{1}H NMR ($CDCl_3$; multiplicity, *J*(Hz)) δ 8.34 (1H, d, 2.4), 7.93 (IH, dd, 8.4, 2.43), 7.20 (1H, d, 8.4), 3.42 (1H, t, 4.05), 3.24 (1H, d, 3.3), 2.62 (1H, dd, 9.12, 4.89), 2.39 (2H, multiplet), 1.85−1.38 (6H, overlapping), 1.08 (3H, t, 7.17); MS *m*/*z* (rel intensity) [M^+] 236.1086 (5%); calculated for $C_{13}H_{17}ClN_2$, M 236.7441.

***N*-Methyl-(±)-*exo*-2-(2-bromo-5-pyridyl)-7-azabicyclo-[2.2.1]heptane (N-Me-BrPH) (3a).** Compound (**3a**) was prepared by reductive methylation of normethyl analogue **3**[18] according to the procedure for N-Me-epibatidine (**2a**) with 53% yield: mp 88−89 °C (hexane). The conditions for the semipreparative HPLC were Hamilton PRP-1 C18 column (10 × 250 mm); mobile phase CH_3OH−CH_3CN−buffer (0.1 M ammonium formate, 0.1% triethylamine, pH 7.0), 2:2:1; flow rate 7 mL/min; t_R 5 min. **3a**: ^{1}H NMR ($CDCl_3$; multiplicity, *J*(Hz)) δ 8.27 (1H, d, 2.31, H$^{6'}$), 7.79 (1H, dd, 8.28, 2.49, H$^{4'}$), 7.36 (1H, d, 8.28, H$^{3'}$), 3.32 (1H, t, 3.8), 3.11 (1H, d, 3.66), 2.62 (1H, dd, 9.12, 4.95, H^2), 2.24 (^{3}H, s, N−CH_3), 1.97−1.40 (6H, overlapping, H^{3,3a,5,5a,6a,6}); MS *m*/*z* (rel intensity) [M^+] 266.0422/268.0380 (9%/9%); calculated for $C_{12}H_{15}N_2{}^{79}Br$/$C_{12}H_{15}N_2{}^{81}Br$, M 266.0419/268.0399.

***N*-Methyl-(±)-*exo*-2-(2-iodo-5-pyridyl)-7-azabicyclo[2.2.1]-heptane (N-Me-IPH) (4a).** To 31 mg (0.1 mmol) of IPH[24] dissolved in 1.5 mL of dry acetonitrile were added 70 μL of 37% formaldehyde in water (0.93 mmol, 9.3 equiv) and 18 mg of sodium cyanoborohydride (0.29 mmol, 2.9 equiv). The reaction was stirred for 6 h at room temperature. The acetonitrile was evaporated under a gentle stream of argon, and the residue was partitioned between water (5 mL) and methylene chloride (5 mL). The organic layer was separated and the aqueous layer extracted two more times with methylene chloride (2 × 5 mL). The extracts were combined, dried over anhydrous sodium sulfate, and evaporated. The residual oil was dissolved in 1 mL of 90/10 hexane/2-propanol and purified via chiral HPLC (1 cm × 25 cm, Chiracel OD column; Daicel Chemical Industries Inc., Fort Lee, NJ). The mobile phase consisted of 90/10 hexane/2-propanol at 3.5 mL/min, and 13 mg of each enantiomer of N-Me-IPH (t_R 6.9 and 7.8 min) was collected to the same flask (82% yield): ^{1}H NMR ($CDCl_3$; multiplicity, *J*(Hz)) δ 8.28 (1H, br s), 7.58 (2H, m), 3.33 (1H, m), 3.12 (1H, d, 3.7), 2.60 (1H, dd, 9.1, 5.0), 2.24 (3H, s), 1.40−1.95 (6H, m); MS *m*/*z* (rel intensity) [M^+] 314.0280 (17%); calculated for $C_{12}H_{15}N_2I$, M 314.0279.

***N*-[^{11}CH$_3$]Methylepibatidine Analogues [^{11}C]1a, [^{11}C]2a, and [^{11}C]3a.** The following radiochemical synthesis of (±)-*N*-[^{11}C]methyl-*exo*-2-(2-bromo-5-pyridyl)-7-azabicyclo[2.2.1]-heptane ([^{11}C]**3a**) is given as an example of the general method used for preparation of [^{11}C]**1a**, [^{11}C]**2a**, and [^{11}C]**3a**.

No-carrier-added ^{11}C-labeled methyl iodide was swept by nitrogen flow into the pressure vessel containing a solution of 1−2 mg of normethyl precursor (**3**) in 300 μL of anhydrous acetonitrile. The reaction vessel was sealed and heated for 10 min at 185−195 °C. The reaction mixture was cooled, diluted with 300 μL of the preparative HPLC mobile phase, injected into a Hamilton PRP-1 column (10 × 250 mm), and eluted with CH_3CN−CH_3OH−0.025 M sodium phosphate buffer (pH 6.5), 2:2:1, at a flow rate of 7 mL/min. The radioactive peak of the methylated product corresponding to authentic **3a** with retention time 6.8 min was collected into a flask containing 100 μL of acetic acid, and the solvent was removed on a rotary evaporator. The product was redissolved in 5 mL of sterile water.

An aliquot of the final solution of known volume and radioactivity was applied to an analytical reverse-phase HPLC column (Hamilton PRP-1 column, 250 mm × 4.6 mm; mobile phase CH_3CN−CH_3OH−0.025 M sodium phosphate buffer (pH 6.5), 2:2:1; flow rate of 2.5 mL/min). The desired product was eluted with a retention time of 4.0 min. The area of the UV absorbance peak at 254 nm corresponding to product was measured and compared to a standard curve relating mass to UV absorbance. The average specific radioactivity of the final product ([^{11}C]**3a**) was 1600 mCi/mmol. The radiochemical product was also coeluted with a sample of authentic **3a**.

In Vitro Assays of Binding to nAChRs. 1. Tissue Preparation: Brains were obtained from adult male rats. The cerebellum, pons, and medulla were removed, and the brains were frozen for up to 3 months before assay. On the day of the assay, the tissue was thawed and homogenized in 10 volumes of cold 55.6 mM Tris-HCl buffer (pH 6.8). The homogenate was centrifuged at 37200*g* for 15 min. Supernatant fluid was discarded, and the pellet was washed twice in fresh buffer and then suspended in buffer for use in the assay.

2. Competition Assays: Binding assays were performed as described by Houghtling et al.[16] but at lower pH (6.8 vs 7.4) to protect the chemically unstable compounds **1** and **1a**. The assays were performed using a single concentration of [^{3}H]-epibatidine (NEN; specific radioactivity 53.2 Ci/mmol) that varied slightly but was between 30 and 40 pM and 12 concentrations of the competing ligand. For compounds **1**, **2**, **3**, **1a**, **2a**, and **3a**, the competing drug concentrations ranged between 3×10^{-8} and 1×10^{-12} M; for compound **2b** the concentrations ranged from 3×10^{-6} to 1×10^{-10} M. Nonspecific binding was defined using (−)-nicotine hydrogen tartrate (3×10^{-4} M). Incubations were run at 25 °C for 3 h. The total assay volume was 5 mL, and each tube contained 10 mg of tissue, nicotine or other competing ligand, and radiotracer in 50 mM Tris HCl buffer, pH 6.8. Nicotine was dissolved in water, and other competing ligands were initially

dissolved and stored in ethanol at concentrations between 3 and 6 mM. Water was used to make dilutions. Incubations were terminated by rapid vacuum filtration through Whatman GF/C filters that had been presoaked in 0.5% poly(ethylenimine) to reduce binding to the filter. The filters were washed three times with 4-mL aliquots of ice-cold buffer and placed in liquid scintillation vials. Formula 989 (Packard Instrument Co., Meriden, CT) liquid scintillation cocktail was added to the vials. After allowing bound radioactivity to dissociate from the filter overnight, the radioactivity in the vials was determined by liquid scintillation counting (Beckman model #3801). Assays using concentration of each competing ligand were run in triplicate, and each ligand was assayed at least 3 times on 3 separate days.

3. Data Analysis: K_d(drug) values were calculated using nonlinear regression analyses (Competition Program in the KELL software package v. 4, Biosoft), using total binding and a one-site model assuming the ligands were only competing against the high-affinity binding site for epibatidine. The published value of 15 pM for the high-affinity binding site was used to calculate K_d(drug).[16]

In Vivo Kinetic and Distribution Studies. Biodistribution studies in mice (male, CD-1 mice weighing 25−35 g; Charles River Laboratories, Wilmington, MA) were performed as previously described.[19] Briefly, the animals were sacrificed by cervical dislocation at selected times after tracer injection into a lateral tail vein. The brains were quickly removed from the skull and dissected on ice as previously described.[40] Samples were weighed and placed into small plastic tubes, and their radioactivity was determined, along with that of aliquots of the injected dose, in an automatic gamma counter (1282 Compugamma CS; LKB Wallace, Gaithersburg, MD).

Determination of Approximate LD_{50} Values. Approximate LD_{50} values were determined by administering nonradioactive drugs**,** dissolved in a volume of 0.2 mL of saline, intravenously to male CD-1 mice (n = 3−4 mice/dose level) and observing the animals for 1 h after injection. Doses close to the LD_{50} value were varied in 4−5 increments of 1−2.5 μg/kg. The dose at which 2 of 4 animals tested died was assumed to be the LD_{50}. Doses near the LD_{50} caused severe pharmacological effects, such as increased respiratory rate and heart rate, severe seizures, hind leg paralysis, and Straub tail. At lethal doses, death typically occurred within the first 10 s after injection.

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