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John Daly's Compound, Epibatidine, Facilitates Identification of Nicotinic Receptor Subtypes

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Abstract

The diversity of nicotinic acetylcholine receptor (nAChR) subtypes was explored by measuring the effects of gene deletion and pharmacological diversity of epibatidine binding sites in mouse brain. All epibatidine binding sites require expression of either the $\alpha 7$, $\beta 2$, or $\beta 4$ subunit. In agreement with general belief, the $\alpha 4\beta 2^*$ -nAChR and $\alpha 7$ -nAChR subtypes are major components of the epibatidine binding sites. $\alpha 4\beta 2^*$ -nAChR sites account for approximately 70% of total high- and low-affinity epibatidine binding sites, while $\alpha 7$ -nAChR accounts for 16% of the total sites all of which have lower affinity for epibatidine. The other subtypes are structurally diverse. Although these minor subtypes account for only 14% of total binding in whole brain, they are expressed at relatively high concentrations in specific brain areas indicating unique functional roles.

Keywords

Neuronal nicotinic acetylcholine receptors; Epibatidine; Null mutant mice; α -Bungarotoxin; Cytisine; α -Conotoxin MII

Introduction

Ligand binding assays have been used for over 40 years to identify and characterize nicotinic cholinergic receptors (nAChRs) in brain. Indeed, the first studies that showed that [¹²⁵I]- α -bungarotoxin (Patrick and Stallcup 1977) and [³H]-nicotine (Romano and Goldstein 1980) bind with high affinity to membranes derived from rat brain provided some of the first evidence that suggested that nAChRs might be expressed in brain. Subsequent studies that compared the anatomical distributions and biochemical properties of these binding sites in rat (Clarke et al. 1985) and mouse (Marks and Collins 1982) brain yielded the first evidence that more than one nAChR subtype is expressed in brain. When the nine nAChR subunit genes that are expressed in mammalian brain ($\alpha 2$ – $\alpha 7$, $\beta 2$ – $\beta 4$) were cloned and sequenced nearly 20 years ago, the number of potential subtypes expanded dramatically. Much of the recent research in the nAChR field is attempting to identify the subunit compositions of those receptor subtypes that are actually expressed (i.e., native receptors) in brain as well as other tissues (reviewed in Millar and Gotti 2008).

Pharmacological approaches have been used for many years in attempts to identify different nAChR subtypes. For example, the findings that α -bungarotoxin and decamethonium are potent inhibitors of the nAChR expressed at the neuromuscular junction whereas mecamylamine and hexamethonium are potent inhibitors of nAChR(s) expressed at autonomic ganglia (reviewed in Collins et al. 2009) were cornerstones of the growing data set that indicated that the nAChRs expressed at these two peripheral sites are different. These successes prompted several groups to develop and characterize additional nicotinic ligands as potential new tools for studying nAChRs. Early studies that used [³H]-methylcarbamylcholine (Abood and Grassi 1986), [³H]-acetylcholine (Martino-Barrows and Kellar 1987), and [³H]-cytisine (Pabreza et al. 1991) demonstrated that these compounds bind with high affinity to rodent brain membranes, but subsequent studies showed that these ligands bind to the same sites that had been successfully measured using [³H]-nicotine (Anderson and Arneric 1994). These ligands had several advantages when compared with [³H]-nicotine but were quickly replaced by radiolabeled epibatidine shortly after Badio and Daly (1994) reported that (+/–)epibatidine binds with extraordinarily high affinity (pM Kd) to rat brain membranes. The first binding studies done with epibatidine (Badio and Daly 1994; Houghtling et al. 1994) suggested that it binds to a single high-affinity site, the same sites that are measured with [³H]-nicotine. However, saturation analyses that used a broader range of ligand concentrations demonstrated that both high- and low-affinity epibatidine binding sites can be detected in rat, human (Houghtling et al. 1995), and mouse (Marks et al. 1998, 1999) brain. These findings, coupled with the report that [³H]-epibatidine binding is significantly greater than [³H]-cytisine binding in many brain regions (Perry and Kellar 1995), that it is a potent activator of $\alpha 3\beta 2$, $\alpha 3\beta 4$, $\alpha 4\beta 2$, $\alpha 7$, and $\alpha 8$ nAChRs expressed in *Xenopus laevis* oocytes (Gerzanich et al. 1995), and that it binds to heteromeric receptors expressed in *Xenopus* (Parker et al. 1998), suggest that the ligand might be useful for measuring several, perhaps many, different nAChR subtypes.

The studies reported here review our use of pharmacological (competition binding) and genetic (null mutant or gene knockout) strategies to characterize epibatidine binding in mouse brain. The results of these experiments indicate that epibatidine can be used to measure a large number of different binding sites in brain.

Materials and Methods

Mice

All procedures involving mice were reviewed and approved by the Animal Care and Utilization Committee of the University of Colorado, Boulder. Mice were bred in the

Specific Pathogen-Free Colony at the Institute for Behavioral Genetics, University of Colorado, weaned at 25 days of age and housed with like-sexed littermates. Animals were maintained on a 12-h light/12-h dark cycle (lights on 7 a.m.–7 p.m.) and allowed free access to food and water. Mice differing in $\beta 2$ nAChR genotype were originally obtained from Marina Picciotto, Yale University (Picciotto et al. 1995), were derived by mating heterozygotes, and had been backcrossed to C57BL/6 J for at least ten generations. Tail clippings were obtained from mice about 40 days of age, and genotype was determined as described previously (Marks et al. 1999).

[³H]Epibatidine Binding to Tissue Homogenates

C57BL/6J mice were sacrificed by cervical dislocation and whole brains were quickly removed and placed in 4-ml ice-cold hypotonic buffer (NaCl, 14 mM; KCl, 0.15 mM; CaCl₂, 0.2 mM; MgSO₄, 0.1 mM; HEPES ½ Na, 2.5 mM; pH=7.5). Each brain was homogenized using a glass/Teflon tissue grinder and the homogenate was centrifuged at 20,000×g for 20 min. Following centrifugation, the supernatant was discarded, and the pellet was suspended in 4 ml of hypotonic buffer. The centrifugation/suspension cycle was repeated four times. Following the final centrifugation step, the supernatant was discarded, and the pellet was overlaid with 1 ml of hypotonic buffer and stored frozen until assay.

On the day of assay, the samples were thawed; the pellet was suspended in the overlying buffer, and the homogenate was centrifuged at 20,000×g for 20 min. The supernatant was discarded, and the pellet was suspended in water for assay. Saturation curves for binding were determined by incubating samples with [³H]epibatidine (specific activity=48 Ci/mmol; Perkin-Elmer NEN, Shelton, CT, USA) for 2.5 h at 22°C in incubation buffer (NaCl, 140 mM; KCl, 1.5 mM; CaCl₂, 2 mM; MgSO₄, 1 mM; HEPES ½ Na, 25 mM; bovine serum albumin, 0.1%; pH=7.5). In the lower concentration range (0.005–1 nM), incubation volume was 500 μ l, while in the higher concentration range (0.25–32 nM) incubation volume was 65 μ l. Different incubation conditions were used to reduce problems with ligand depletion encountered with this high-affinity ligand. Concentrations were chosen such that the three highest concentrations in the low range and the three lowest concentrations in the high range were the same to assure that binding in the two incubation conditions was similar. Inhibition of high-affinity binding sites by cytosine (3×10^{-10} to 1×10^{-5} M) and *d*-tubocurarine (1×10^{-8} to 1×10^{-2} M) was measured using 0.4 nM [³H]epibatidine, and inhibition of low-affinity binding by cytosine (1×10^{-8} to 5×10^{-4} M) and by *d*-tubocurarine (1×10^{-8} to 1×10^{-2} M) was measured using 10 nM [³H]epibatidine. Blanks were established by including nicotine (10 μ M or 1 mM used with the 0.4 nM and 10 nM [³H]epibatidine concentrations, respectively). Samples were collected by filtration at 4°C onto two glass fiber filters that had been soaked in 0.5% polyethylenimine (top filter type GB100, Microfiltration Systems, Dublin, CA, USA; bottom filter type A/E, Gelman Sciences, Ann Arbor, MI, USA) using a 96 place manifold (Inotech Biosystems, Rockville, MD, USA). Samples were washed five times with ice-cold incubation buffer without added albumin. Filters were placed in 5-ml scintillation vials and after addition of 1.5 ml of BudgetSolve (RPI, Arlington Heights, IL, USA) counted at 45% efficiency using a Packard 1600 liquid scintillation counter.

Saturation binding data were fit to a two site model: $B = B_{\max-H} \times \text{Epi} / (K_{D-H} + \text{Epi}) + B_{\max-L} \times \text{Epi} / (K_{D-L} + \text{Epi})$, where B is binding at each Epi concentration; $B_{\max-H}$ and $B_{\max-L}$ are maximal binding site density to the high- and low-affinity sites with apparent binding affinities of K_{D-H} and K_{D-L} , respectively.

Inhibition curves were also fit to a two-site model: $B = B_{HA} / (1 + I / IC_{50-HA}) + B_{LA} / (1 + I / IC_{50-LA})$, where B is binding at each inhibitor concentration; I , B_{HA} , and B_{LA} are binding site densities inhibited with IC_{50-HA} and IC_{50-LA} , respectively. Nonlinear curve fitting was accomplished using Sigma Plot.

Autoradiography Mice

($\beta 2^{+/+}$, $\beta 2^{+/-}$, and $\beta 2^{-/-}$) were sacrificed by cervical dislocation; the brains were rapidly removed and quickly frozen by immersion in isopentane (-35°C). Brains were stored at -70°C until sectioning (14- μM thickness) using an IEC cryostat. Sections were thaw mounted on Fisher SupraFrost Plus microscope slides. Slides containing the sections were stored at -70°C until use.

On the day of the assay, samples were warmed to room temperature under desiccation, rehydrated before use, and treated with 1 mM phenylmethylsulfonyl fluoride during rehydration. High-affinity binding was measured using [^{125}I]epibatidine (2,200 Ci/mmol, Perkin-Elmer NEN, Shelton, CT, USA). The ligand was mixed with unlabeled iodoepibatidine (a generous gift from Kenneth Kellar, Georgetown University) to reduce the specific activity to 220 Ci/mmol. The final total epibatidine concentration was 0.2 nM. Incubations under these conditions were done in the binding buffer to which 5 mM EDTA, 5 mM ethylene glycol tetraacetic acid, and 10 $\mu\text{g/ml}$ each of aprotin, leupeptin, and pepstatin had been added. Samples were incubated for 2 h at 22°C with either no addition, addition of 100 nM cytosine, or addition of 100 nM cytosine plus 50 nM α -conotoxin MII. Binding to low- and high-affinity sites was measured using [^3H]epibatidine (48 Ci/mmol, Perkin-Elmer NEN, Shelton, CT, USA). Final epibatidine concentration was 10 nM. Incubation under these conditions was done in binding buffer with no further additions. Samples were incubated at 22°C for 2 h with either no addition, addition of 1 μM α -bungarotoxin, or addition of 1 μM α -bungarotoxin plus 300 μM *d*-tubocurarine. Following the incubation, the slides were washed by immersion in ice-cold binding buffer (2 $\times$ 30 s), ice-cold 0.1 $\times$ binding buffer (2 $\times$ 10 s), and ice-cold 5 mM HEPES, pH 7.5 (2 $\times$ 5 s each). Samples were then dried under a gentle stream of air and desiccated overnight before exposure to Kodak MR film (^{125}I) or Amersham β -max film (^3H). Samples adjacent to those used for high-affinity binding were used to measure the binding of [^{125}I] α -conotoxin MII (0.5 nM). Incubation conditions were the same as those used for [^{125}I]epibatidine. Slides were washed by immersion in binding buffer (22°C , 1 $\times$, 30 s), binding buffer (4°C , 1 $\times$ 30 s), 0.1 $\times$ binding buffer (4°C , 2 $\times$ 5 s), and 5 mM HEPES, pH 7.5 (4°C , 2 $\times$ 5 s). Samples were air-dried and desiccated overnight before exposure to Kodak MR film. Digital images were captured, and pseudocolor images were generated using NIH Image.

Results

The biphasic nature of the binding of [^3H]epibatidine to whole mouse brain membranes was reported by Marks et al. (1999). Figure 1 illustrates the biphasic nature of [^3H] epibatidine binding to membranes derived from whole mouse brain and the inhibition of [^3H]epibatidine binding by cytosine and *d*-tubocurarine. The saturation curve for [^3H]epibatidine binding can be resolved into two components with apparent K_D values of 0.02 and 6.4 nM, which are illustrated by the *red* and *green lines* in Fig. 1a. The biphasic nature of the binding is clearly demonstrated by the nonlinear Scatchard plot shown in Fig. 1b. The inhibition of high-affinity [^3H]epibatidine binding by cytosine is also biphasic as illustrated in Fig. 1c. In whole brain, the component with high affinity for cytosine accounts for 85% of total binding. The estimated K_I for cytosine at the site with higher affinity is 0.3 nM. The component with lower affinity for cytosine accounts for the remaining 15%. The estimated K_I at this site is 13 nM. The proportion of cytosine-sensitive and cytosine-resistant [^3H]epibatidine binding sites varies markedly among brain regions (Marks et al. 1998). The cytosine inhibition of low-affinity [^3H]epibatidine binding sites was calculated and is illustrated in the inset to Fig. 1c. The K_I for cytosine at the low-affinity [^3H]epibatidine sites is estimated to be 0.6 μM . The inhibition of high-affinity [^3H]epibatidine binding by *d*-tubocurarine is also biphasic with proportions of high- and low-sensitivity sites very similar to those measured with cytosine. However, the affinity of *d*-tubocurarine for these sites is much lower than that for cytosine

(K_I values of 2.2 and 62 μM , respectively). The *d*-tubocurarine inhibition of low-affinity [^3H]epibatidine binding sites was calculated and is illustrated in the inset to Fig. 1d. In contrast to the large difference in affinity for the high- and low-affinity [^3H]epibatidine binding sites exhibited by cytisine, the K_I value for *d*-tubocurarine estimated for the lower-affinity [^3H]epibatidine binding sites (4 μM) is very similar to that calculated for high-affinity [^3H]epibatidine binding. This property makes *d*-tubocurarine an effective reagent to measure low-affinity [^3H]epibatidine binding by differential inhibition as illustrated by the black curve in Fig. 1d. Therefore, both [^3H]epibatidine saturation binding and differential inhibition of the high- and low-affinity binding sites reveal that [^3H]epibatidine binds to several diverse sites.

In order to analyze further the diversity of epibatidine binding sites, the effect of deletion of specific nAChR subunits on these sites has been evaluated using autoradiography. The autoradiograms shown in Fig. 2 illustrate the effect of differential inhibition by cytisine and α -conotoxin MII as well as of the deletion of the $\beta 2$ nAChR subunit on high-affinity [^{125}I]epibatidine binding at the level of the nucleus accumbens and caudate putamen. High-affinity binding in these brain regions is heterogeneous as illustrated by the effects of cytisine and α -conotoxin MII in wild-type $\beta 2^{+/+}$ mice. The binding of [^{125}I]epibatidine is substantially inhibited when 100 nM cytisine is included in the incubation, although significant signal persists in the caudate putamen, nucleus accumbens, and olfactory tubercles. Binding is further reduced, but not completely eliminated, by addition of 50 nM α -conotoxin MII. Deletion of the $\beta 2$ subunit eliminates most [^{125}I]epibatidine binding in these sections. However, piriform cortex and medial septum of $\beta 2^{-/-}$ samples retain low levels of [^{125}I] epibatidine binding, indicating the presence of non- $\beta 2$ sites. [^{125}I]Epibatidine binding in $\beta 2^{+/-}$ mice was intermediate between that of wild-type and null mutants. The pattern of inhibition was similar to that of wild type, consistent with a gene dose-dependent expression of these sites. Deletion of the $\beta 2$ subunit eliminated specific binding of [^{125}I] α -conotoxin MII, consistent with the results seen with inhibition of [^{125}I]epibatidine binding by this toxin.

The autoradiograms shown in Fig. 3 illustrate the effects of differential inhibition of binding by α -bungarotoxin and *d*-tubocurarine and the effects of $\beta 2$ gene deletion on binding of 10 nM [^3H]epibatidine at the level of the thalamus, medial habenula, and dorsal hippocampus. Total [^3H]epibatidine binding is heterogeneous as illustrated by the differential inhibition by several competitive ligands and by gene deletion. In wild-type ($\beta 2^{+/+}$) mice, incubation in the presence of α -bungarotoxin significantly reduces labeling in hippocampus, demonstrating that [^3H]epibatidine will bind to $\alpha 7$ -nAChR at this high concentration. Addition of 300 μM *d*-tubocurarine generally reduces binding and the resulting pattern resembles that seen for samples incubated with a lower concentration of [^3H] epibatidine (0.4 nM). Deletion of the $\beta 2$ subunit reduces [^3H]epibatidine binding throughout the brain, but significant α -bungarotoxin-sensitive [^3H]epibatidine binding is noted in hippocampus, cerebral cortex, and hypothalamus. The only detectable [^3H]epibatidine binding persisting in the presence of 300 μM *d*-tubocurarine in these sections of $\beta 2^{-/-}$ mice is found in the medial habenula and fasciculus retroflexus and almost certainly due to $\beta 4^*\text{nAChR}$.

Epibatidine binding in the $\beta 2^{+/-}$ mice is intermediate between that of wild-type and knockout mice. This figure also illustrates that deletion of $\beta 2$ has no effect on the binding of [^{125}I] α -bungarotoxin binding as expected. Note the similarity of the patterns of [^3H]epibatidine and [^{125}I] α -bungarotoxin binding in the $\beta 2^{-/-}$ mice.

Discussion

The results presented here summarize how pharmacological and genetic approaches have been used to investigate the diversity of epibatidine binding sites in rodent brain and to begin to establish the subunit composition of the nAChR subtypes measured with epibatidine. The results presented in this article provide a general description of nAChR subtypes identified by epibatidine binding. Greater detail is provided in previous publications cited where appropriate. Figure 4 presents these results diagrammatically.

Epibatidine was first isolated by John Daly from the skin of the Ecuadorean tree frog, *Epipedobates tricolor* (Daly et al. 1978) and was identified as a very potent nAChR agonist. The extraordinary affinity of epibatidine has made it a particularly useful reagent with which to identify multiple nAChR binding sites (Badio and Daly 1994; Houghtling et al. 1994, 1995; Davila-Garcia et al. 1997; Marks et al. 1998, 1999; Zoli et al. 1998; Whiteaker et al. 2000; Perry and Kellar 1995, 2002). Because of epibatidine's potency, it has been possible to label not only high-affinity sites that are amenable to measurement with other ligands such as nicotine and cytosine but also to label sites with lower affinity that evade measurement with other ligands as illustrated in Fig. 1.

Gene knockout (null mutant) mouse strains have been developed for all nine nAChR subunit genes that are expressed in mammalian brain. These knockout strains have been very useful for determining subunits that are required to form specific binding sites. Examination of the effect of $\beta 2$, $\beta 4$, or $\alpha 7$ subunit deletion established that both high- and low-affinity epibatidine binding sites require expression of either $\alpha 7$, which assembles into homomeric pentamers, or $\beta 2$ or $\beta 4$, which are required for the assembly of heteromeric receptors (Marks et al. 2006).

The higher-affinity sites ($K_D \approx 0.02$ nM) account for approximately one half of total epibatidine binding and are relatively well characterized. At least three families of nAChR subtypes are included in the higher affinity [^3H] epibatidine binding sites. Each of these families is heterogeneous. The major subtype, accounting for approximately 90% of high-affinity [^3H] epibatidine binding (45% of total), is sensitive to inhibition by cytosine and is eliminated by deletion of $\beta 2$ as illustrated in Fig. 2. The cytosine-sensitive site is also eliminated by deletion of the $\alpha 4$ (Marks et al. 2007), establishing this site as an $\alpha 4\beta 2^*$ -nAChR. This site can also be measured by direct binding of [^3H]cytosine or [^3H]nicotine (Anderson and Arneric 1994). Consistent with the effects of gene deletion on cytosine-sensitive epibatidine binding, it has previously been demonstrated that [^3H] nicotine binding is eliminated in $\alpha 4$ (Marubio et al. 1999) and $\beta 2$ (Picciotto et al. 1995) null mutant mice. Furthermore, gene deletion and immunoprecipitation establish that some of these sites also contain the $\alpha 5$ subunit (Brown et al. 2007).

In contrast to the complete dependence of cytosine-sensitive [^3H] epibatidine binding sites on expression of the $\beta 2$ subunit, only some of the cytosine-resistant sites require expression of $\beta 2$, while the remainder require expression of $\beta 4$. Most of the cytosine-resistant $\beta 2^*$ -nAChR sites bind α -conotoxin MII with high affinity and correspond to $\alpha 6\beta 2^*$ -nAChR (see Fig. 2). These receptors are heterogeneous and include $\alpha 6\alpha 4\beta 2\beta 3^-$, $\alpha 6\beta 2\beta 3^-$, and $\alpha 6\beta 2$ -nAChR subtypes as demonstrated by assays of ligand binding (Salminen et al. 2005) and nAChR-mediated dopamine release (Salminen et al. 2007). However, a minor subset of α -conotoxin MII binding, particularly in the medial habenula and interpeduncular nucleus, is likely $\alpha 3\beta 2^*$ -nAChR (Whiteaker et al. 2002; Grady et al. 2009). Relatively few $\alpha 6\beta 2^*$ -nAChR are measurable in whole-brain preparations, but this subtype is expressed in significant levels in dopaminergic and visual pathways (Champtiaux et al. 2003; Salminen et al. 2005; Gotti et al. 2005a,b; Marritt et al. 2005; Cox et al. 2008).

The $\beta 4^*$ -nAChR also have restricted distribution. However, $\beta 4^*$ -nAChR are highly expressed in several brain regions including medial habenula, interpeduncular nucleus, inferior colliculus, and accessory olfactory bulb (Whiteaker et al. 2000). Most of these receptors are $\alpha 3\beta 4^*$ -nAChR (Marks et al. 2002; Grady et al. 2009), and ganglionic versions of this subtype often include the $\alpha 5$ subunit (Conroy and Berg 1995; Mao et al. 2006). It has recently been shown that $\beta 3$ can assume the role of the accessory subunit in this subtype in the interpeduncular nucleus (Grady et al. 2009).

The lower-affinity [^3H]epibatidine binding sites ($K_D \approx 5$ nM) have been less well characterized. These sites are nicotinic as demonstrated by their elimination by deletion of the $\beta 2$, $\beta 4$, or $\alpha 7$ subunit (Marks et al. 2006). These lower-affinity sites represent approximately 50% of total [^3H]epibatidine binding measured at high (30–40 nM) ligand concentrations.

It has previously been shown that [^{125}I] α -bungarotoxin binding is eliminated by deletion of $\alpha 7$ (Orr-Urtreger et al. 1997), which also selectively eliminates the α -bungarotoxin-sensitive component of lower-affinity [^3H] epibatidine binding. Thus, $\alpha 7$ -nAChR sites can be measured with [^3H]epibatidine and represent approximately one third of low-affinity sites (16% of the total). Deletion of either $\beta 2$ or $\beta 4$ (or $\alpha 4$) has no measurable effect on these sites.

The remaining lower-affinity binding sites represent a significant fraction of total binding (33% of total, two thirds of the low-affinity sites). Most of these sites require expression of $\beta 2$ (Marks et al. 2006) and $\alpha 4$ (Marks et al. 2007). These low-affinity [^3H]epibatidine binding sites are expressed throughout the brain, and their distribution resembles that of high affinity $\alpha 4\beta 2^*$ -nAChR [^3H]epibatidine binding sites (see Fig. 3). These non- $\alpha 7$ sites have not been well characterized. It is not yet established whether they are assembled functional receptors, partially assembled receptors, or dead-end assembly/folding intermediates. It was speculated that these binding sites correspond to receptors with lower sensitivity to activation by agonists and corresponding to $\alpha 4\beta 2$ -nAChR with a stoichiometry of $(\alpha 4)_3(\beta 2)_2$. However, studies of binding to heterologously expressed receptors with this stoichiometry establish that cytosine binds to them with high affinity (Moroni et al. 2006).

In summary, pharmacological, genetic, and immunochemical methods have successfully been used to identify many diverse epibatidine binding sites reflecting a diverse population of nAChR. These approaches confirm the often-made assertion that $\alpha 4\beta 2^*$ -nAChR and $\alpha 7^*$ -nAChR are the most highly expressed subtypes but also indicate that there are a large number of less common subtypes with diverse subunit composition. The tendency for these unusual subtypes to be expressed in discrete brain nuclei suggests that they may play roles in specific nicotinic responses in these areas as indicated by the important role of $\alpha 6\beta 2^*$ -nAChR in dopaminergic pathways and $\alpha 3\beta 4^*$ -nAChR in the habenulo-interpeduncular nucleus pathway. Indeed, $\alpha 6\alpha 4\beta 2(\beta 3)$ -nAChR seem to be particularly important in regulating nicotine intake (Pons et al. 2008), and $\alpha 3\beta 4^*$ -nAChR are involved in nicotine-induced convulsions (Adams et al. 2004; Salas et al. 2004). Identification and functional characterization of native nAChR subtypes will be a continuing effort.

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Figure 1. [³H]Epibatidine binding to whole mouse brain membranes. The saturation curve for [³H]epibatidine binding is shown in **a**. The *black circles* represent the data points. Total binding, represented by the *black curve*, was resolved into high-affinity (*red*) and low-affinity (*green*) components. **b** displays the Scatchard plot for the data in **a**. Inhibition of [³H] epibatidine binding (0.4 nM, *open circles*; 12.5 nM *open squares*) by cytosine is shown in **c**. The *solid red curve* displays the biphasic fit for inhibition using 0.4 nM [³H]epibatidine and the *dotted red line* illustrates the estimated cytosine-resistant component of this binding. The *inset* to **c** shows the inhibition of the lower-affinity [³H]epibatidine binding sites. Inhibition of [³H]epibatidine binding (0.4 nM, *open circles*; 12.5 nM *open squares*) by *d*-

tubocurarine is shown in **d**. The *solid red curve* displays the biphasic fit for inhibition using 0.4 nM [^3H] epibatidine and the *dotted red line* illustrates the estimated *d*-tubocurarine-resistant component of this binding. The *inset* to **d** shows the inhibition of the lower-affinity [^3H]epibatidine binding sites. This figure was modified from Marks et al. (1999)

Figure 2.

Effect of deletion of the $\beta 2$ subunit on subsets of high-affinity [^{125}I]epibatidine binding sites and [^{125}I]- α -conotoxin MII binding. [^{125}I] Epibatidine (0.2 nM) was incubated with sections derived from $\beta 2^{+/+}$, $\beta 2^{+/-}$, and $\beta 2^{-/-}$ mice in the absence of added ligands, in the presence of 100 nM cytosine, and in the presence of 100 nM cytosine plus 50 nM α -conotoxin MII as indicated. Near adjacent sections were incubated with 0.5 nM [^{125}I] α -conotoxin MII.

Labels: CP, caudate putamen; Cx, cerebral cortex; nAcc, nucleus accumbens; OT, olfactory tubercles; Pir, piriform cortex

Figure 3.

Effect of deletion of the $\beta 2$ subunit on subsets on [³H]epibatidine binding sites and [¹²⁵I] α -bungarotoxin binding. [¹²⁵I]Epibatidine (0.2 nM) was incubated with sections derived from $\beta 2^{+/+}$, $\beta 2^{+/-}$, and $\beta 2^{-/-}$ mice in the absence of added ligands, in the presence of 1,000 nM α -bungarotoxin, and in the presence of 300 μ M *d*-tubocurarine as indicated. Near adjacent sections were incubated with 1 nM [¹²⁵I] α -bungarotoxin. Labels: *Cx*, cerebral cortex; *Hp*, hippocampus; *Ht*, hypothalamus; *mHb*, medial habenula; *Th*, thalamus

Figure 4.
Contribution of nAChR subunits to epibatidine binding sites. Subunit composition of epibatidine binding sites determined by pharmacological, genetic, and immunochemical approaches. This figure has been expanded from Marks et al. (2006)