Characterization of Epibatidine Binding to Medial Habenula: Potential Role in Analgesia

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ABSTRACT

The objective of the present study was to characterize a recently described binding site in the habenula, which has high affinity for \(^{3}H\)epibatidine and low affinity for nicotine and acetylcholine. We report that the extension of this binding area in coronal and horizontal sections corresponds to the anatomical extension of the medial habenula. The affinity \((K_{d})\) of the medial habenula receptors for \(^{3}H\)epibatidine was estimated to be 0.5 nM using an autoradiographic saturation assay, whereas the affinity of the binding site for nicotine and acetylcholine was estimated to be 5 and 8 \(\mu M\), respectively. The receptor density \((B_{\text{max}})\) in the medial habenula was estimated to be about 1100 fmol/mg wet weight using \(^{3}H\)epibatidine. The subunit composition of the “epibatidine receptor” was investigated by the ability of different compounds with affinity to various subtypes of nicotinic receptors to displace \(^{3}H\)epibatidine bound to the receptor. The results suggest that the receptor contains \(\alpha_{3}\) subunits but that it is unlikely to be an \(\alpha_{3}\beta_{4}\) nicotinic receptor. Systemic administration of epibatidine has analgesic effects in rats. Here we report that \(2 \times 1 \mu l\) of 10 nM epibatidine, resulting in a \(2 \times 10^{-6}\) mol dose, administered directly to the medial habenula by bilateral stereotactic injection had an analgesic effect measured in the hot-plate test. This dose of epibatidine increased hot-plate latency significantly, whereas 2 \(\times 2\) fmol of epibatidine or 2 \(\times 10^{-6}\) mol of nicotine were without effect. This leads us to suggest that the medial habenular epibatidine binding site might be a valuable target for the development of non-opioid analgesics.

Epibatidine is a cholinergic agonist with affinity for neuronal nicotinic receptors (nAChRs) in the low picomolar range (Houghtling et al., 1995). Besides binding to the \(\alpha_{4}\beta_{2}\) subtype, epibatidine also binds to several other nAChRs composed of different \(\alpha\) and \(\beta\) subunits (Parker et al., 1998). Epibatidine thus is a rather nonselective agonist at most nAChR subtypes. The pharmacological interest in epibatidine arises from the finding that the compound has considerable analgesic potency (Badio and Daly, 1994) and, although its unselective stimulation of nAChRs and its narrow therapeutic index make epibatidine too poisonous to be used clinically, this observation opens up the possibility of powerful analgesia without the use of opiates. The \(\alpha_{4}\beta_{2}\) nAChR subtype is the most abundant in the rat brain, and nicotine, which is slightly analgesic (Qian et al., 1993), binds with some selectivity to this receptor subtype. Accordingly, drug candidates with some analgesic potency and selectivity for this subtype have been developed (Bannon et al., 1998). However, the nonselective agonistic activity of epibatidine makes it possible that other nAChR subtype alleles also could be involved in the analgesic effect. We have previously described a receptor population in the habenula with high affinity for \(^{3}H\)epibatidine but low affinity for nicotine and acetylcholine because neither 2 nor 10 \(\mu M\) concentrations of the respective drugs could displace the bound \(^{3}H\)epibatidine (Plenge and Mellerup, 1998). This indicates that this binding site is not an \(\alpha_{4}\beta_{2}\) nAChR receptor, a result supported by recent studies with \(\alpha_{4}\)- as well as \(\beta_{2}\)-knockout mutant mice in which \(^{3}H\)epibatidine binding is preserved in the habenula but absent in \(\alpha_{4}\beta_{2}\)-rich areas, such as cortex and thalamus (Zoli et al., 1998; Marubio et al., 1999).

Using autoradiographic methods, the present study was directed toward determining kinetic parameters for the “epibatidine receptor” in the habenula. Using \(^{3}H\)epibatidine, \(K_{D}\) and \(B_{\text{max}}\) were estimated. By displacing \(^{3}H\)epibatidine bound to the receptor with acetylcholine and nicotine, the affinity of these two substances for the receptor was determined. We studied the anatomical extension of \(^{3}H\)epibatidine binding in the habenula in both coronal and horizontal sections. The “nonacetylcholine” displaceable \(^{3}H\)epibatidine binding to medial habenula was sought and characterized using a considerable number of “nicotinic” compounds with selectivity for various nAChR subtypes.

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ABBREVIATIONS: nAChR, neuronal acetylcholine receptor; mHb, medial habenula; CNS, central nervous system; ABT-594, (R)-5-(2-azetidinylmethoxy)-2-cloropyridine.
Various lines of evidence suggest that the habenular complex is involved in central pain processing (Andres et al., 1999). Habenular neurones respond to noxious stimuli (Benabid and Jeaugey, 1989; Dafny and Qiao, 1990; Nagao et al., 1993), and the expression of the immediate early gene c-fos is induced in this structure after peripheral noxious stimulation (Dai et al., 1993; Smith et al., 1997; Michl et al., 2001). Analgesia can be achieved by electrical or chemical stimulation of the habenula (Cohen and Melzack, 1986, 1993; Mahieux and Benabid, 1987; Terenzi and Prado, 1990; Terenzi et al., 1990). In addition, the habenula has been shown to be involved in the modulation of acupuncture analgesia (Takeshige et al., 1993). On this background, we decided to study the analgesic effect in rats of direct bilateral stereotactic application of epibatidine to the habenular complex using the hot-plate test.

### Materials and Methods

**Autoradiography.** Sections of rat brains containing habenula [approximately −2.6 to −4.1-mm caudal to bregma (Paxinos and Watson, 1986)] were cut in coronal orientation at 20 μm and thaw-mounted on SuperFrost/Plus slides (Menzel-Glaser, Braunschweig, Germany). On each slide, three to four sections from different rat brains were mounted. Slides were stored at −20°C until processed. Binding assays were performed in buffer A (120 mM NaCl, 5 mM KCl, 50 mM Tris, pH 7.5) at 2°C. The slides were hydrated in buffer A for 15 min before being incubated with [3H]epibatidine (Amersham Biosciences Europe GmbH, Horsholm, Denmark; 53 Ci/mmol) and A for 15 min before being incubated with [3H]epibatidine (Amersham Biosciences Europe GmbH, Horsholm, Denmark; 53 Ci/mmol) and other compounds for 1 h at 4°C. Nonspecific binding was determined both with 1 μM nicotine. To reduce/eliminate nonspecific binding of [3H]epibatidine, slides were subsequently washed two times for 30 min at 0°C in buffer A. We have determined the half-life of [3H]epibatidine bound to rat brain membrane receptors to be 4 h at 0°C; thus, elimination of nonspecific binding by this washing procedure only results in an approximate loss of 16% of the specifically bound [3H]epibatidine. Slides were dried and exposed to Hyperfilm [3H] (Amersham Biosciences Europe GmbH). The films were exposed from 3 to 6 months in autoradiography cassettes at −20°C before being developed. Developed films were analyzed and quantitated in a computer-assisted video densitometer (Scion-Image, Scion Corporation, Frederick, MD) using the standard curve generated from [3H] standards. (Amersham Biosciences Europe GmbH). These standards have their radioactive concentration expressed in becquerels per milligram wet weight, allowing calculation of the receptor density when the specific activity of the ligand is known.

Affinities of different compounds to the [3H]epibatidine binding receptor in habenula were determined on brain sections prepared as described above. The sections were incubated for 1 h at 4°C with 0.2 nM [3H]epibatidine and the compound to be tested. In most cases, three concentrations, 0.1, 1.0, and 10 μM, were used. In the experiments in which the affinities of nicotine and acetylcholine were studied, concentrations from 0.5 to 1000 μM were used to displace [3H]epibatidine in a concentration of 0.1 nM. When studying the affinity of acetylcholine, 1 mM diisopropylfluorophosphate was added to block acetylcholinesterase. For comparison, the affinity of nicotine displacing [3H]epibatidine bound to nACh receptors in superior cervical ganglia was determined. Superior cervical ganglia from four rats (eight pieces) were frozen, aligned, and imbedded in a drop of Cryo-Gel (Ax-Lab, Copenhagen, Denmark). Sections (15 μm) of the Cryo-Gel lump containing cross-sections of several ganglia were thaw-mounted on SuperFrost/Plus glass slides.

Different pharmaceutical companies provided compounds with selectivity for different nicotinic receptors. The affinities of these compounds were determined in the respective laboratories using cell membranes from rat brains or cell cultures and different ligands. The binding data for the drugs described in Fig. 3 are indicated in Table 1.

The anatomical extension of the [3H]epibatidine binding area in habenula was determined in rat brains cut both horizontally and coronally. The brain sections were incubated with 0.2 nM [3H]epibatidine and treated as described above.

**Binding characteristics** ($K_d$ and $B_{max}$) for epibatidine to the receptor in the habenula were determined by incubating brain sections with 11 different concentrations of [3H]epibatidine, increasing by a factor of 2 from 5 to 5000 pM. Two independent experiments were performed. Nonspecific binding was determined both with 1 μM nicotine and 1 μM epibatidine.

**Intracerebral Injections.** Double guide cannulas (22-gauge, 1.2-mm distance between guiding cannulas, cut 5.0 mm below ped- estal), double internal cannulas (33-gauge, 6-mm length), double dummy cannulas (5-mm length), and double connector assemblies were purchased from Plastics One, Inc. (Roanoke, VA). Approximately 3 weeks before the first hot-plate test, the rats (male, Wistar, 240–260 g; purchased from M&B Breeding Center, Lille Skensved, Denmark) were anesthetized with 1.5% halothane given by nose cone and warmed on a heating pad to a body temperature of 33°C.

### Table 1

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<tr>
<td>a7 compound</td>
<td>3-(2-Benzothienyl)-8-methyl-8-azabicyclo[3.2.1]oct-2-ene</td>
<td>&gt;30</td>
<td>&gt;30</td>
<td>0.067</td>
<td>N.D.</td>
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<tr>
<td>α4β2 compound</td>
<td>1-[5-(2-Methyl-1-propoxy)-3-pyridyl]-homopiperazine</td>
<td>0.002</td>
<td>0.01</td>
<td>&gt;30</td>
<td>N.D.</td>
</tr>
<tr>
<td>Mixed affinity</td>
<td>(S)-5-(2-Azetidinyl-methoxy)-2-chloropyridine</td>
<td>0.0004</td>
<td>0.0008</td>
<td>0.64</td>
<td>0.011</td>
</tr>
<tr>
<td>Most selective</td>
<td>3-(3-Bromo-2-thienyl)-8-methyl-8-azabicyclo[3.2.1]oct-2-ene</td>
<td>2.2</td>
<td>2.8</td>
<td>0.082</td>
<td>0.007</td>
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N.D., not done.
Denmark) were implanted with a double guiding cannula directed at the medial habenula (mHb) (anterior-posterior − 3.3 mm and mediolateral-lateral ± 0.6 mm from bregma; dorsal-ventral − 3.8 mm from dura). Between injections, the guiding cannulas were kept open with double dummy cannulas, covered with dust caps. At the time of testing, double injection cannulas, which protruded 1 mm into the tissue ending near the habenula, were inserted into the guide cannulas, while one experimenter gently restrained the rat. For 1 min, 2 × 1 µl of a drug solution in physiological saline were infused bilaterally with the help of two Hamilton syringes connected to the injection cannulas. The injection cannulas were left in place for 4 min more to allow diffusion into the tissue and prevent backflow into the guiding cannulas. Hot-plate testing was carried out 10 and 20 min after injection.

**Hot-Plate Testing.** Analgesia was tested on a hot-plate (Harvard Apparatus, Inc., Holliston, MA) set at 50°C. For each trial, the timer was started simultaneously with the rat being placed on the hot-plate. Two experimenters closely observed the rat, and the timer was stopped and the rat removed from the hot-plate at the first hind paw licking reaction. Animals that had not shown a hind paw licking reaction were removed from the hot-plate after 30 s. Each animal was injected with drug or the saline vehicle, in counterbalanced order, at a 1-week interval. The experimenters were blinded to the test time, at a 1-week interval. The experimenters were blinded to the treatment the animals received.

**Statistical Analysis.** Latency to the first hind paw licking reaction after saline injection was compared with latency after drug injection using paired t tests for the two test times.

## Results

The anatomical part of the habenula, which binds [3H]epibatidine not being displaced by 1 µM nicotine, was visualized in a series of both coronal and horizontal brain sections. Figure 1A shows representative coronal sections in frontal to caudal direction, labeled with 0.2 nM [3H]epibatidine. In the frontal part, the binding area is almost circular, whereas it becomes elongated in the more caudal sections with a ditch in the upper part. Also, in the caudal part, the beginning fasciculus retroflexus is seen protruding down. In the more caudal sections, the receptor seems to be unevenly distributed because lighter and darker patches are seen within the area with binding sites. Figure 1B shows the two parallel binding areas in horizontal sections. Taken together, the [3H]epibatidine binding area coincides with the medial habenula.

The affinity of the non-nicotine displaceable [3H]epibatidine binding was estimated using 11 [3H]epibatidine concentrations from 5 to 5000 µM and either 1 µM nicotine to displace α4β2 binding or 1 µM epibatidine to determine nonspecific binding. The autoradiograms from brain sections incubated with 1 µM epibatidine were without any binding, demonstrating that the washing procedure used eliminated nonspecific binding. The binding that appeared on the autoradiograms thus was specific binding of [3H]epibatidine to nACh receptors. Saturation analysis of non-nicotine displaceable binding to mHb revealed a saturable binding with a K_D value of about 0.5 nM (K_D = 0.47 nM and 0.57 nM in two experiments). The receptor concentration, B_max value, was estimated to be about 1100 fmol/mg wet weight (930 and 1200 fmol/mg wet weight in two experiments).

Using a [3H]epibatidine concentration of 0.1 nM, the affinity of nicotine and acetylcholine for the binding site was estimated on a series of slides incubated with nicotine and acetylcholine in concentrations increased by a factor of 2 from 0.5 to 1000 µM. Figure 2 shows the binding to mHb with and without the lowest concentrations of acetylcholine. It is evident that the binding of [3H]epibatidine to cortical and thalamic receptors disappears already with 0.5 µM acetylcholine, whereas some binding in mHb persisted even with 64 µM acetylcholine; in parallel experiments, the same was found to apply for nicotine. From the displacement curves, the binding site affinities (K_i value) for nicotine and acetylcholine were estimated to be 5 and 8 µM, respectively, for displacement of [3H]epibatidine bound to the epibatidine receptor in mHb. In a similar experiment performed on sections of superior cervical ganglia, nicotine was found to displace [3H]epibatidine bound to the ganglionic α3β4 nACh receptor with a K_i value of 1.3 nM.

To explore the subunit composition of the [3H]epibatidine binding receptor in mHb, about 70 different experimental

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**Fig. 1.** [3H]Epibatidine binding to rat brain medial habenula. A, coronal brain section incubated with 0.2 nM [3H]epibatidine and 1 µM nicotine. B, horizontal brain section incubated with 0.2 nM [3H]epibatidine without or with 1 µM nicotine.

**Fig. 2.** [3H]Epibatidine (0.1 nM) binding to coronal rat brain sections containing medial habenula displaced with increasing concentrations of acetylcholine.
compounds with known selectivity for different nACh receptor subunit combinations were obtained from various pharmaceutical companies and screened with respect to a possible affinity for the epibatidine receptor. The compounds were tested in three concentrations, 0.1, 1.0, and 10 μM, together with 0.2 nM [3H]epibatidine, with the objective of finding a compound that displaced [3H]epibatidine binding to mHb without displacing binding to cortex and thalamus. The results obtained can be illustrated with the displacement pattern of the four different compounds shown in Fig. 3. Table 1 shows the affinity as determined by the pharmaceutical companies of the four compounds to nACh receptors composed of α7, α4β2, and α3β4 subunits, respectively. To ease the discussion of the four compounds, we have named them: α7 compound, α4β2 compound, “mixed affinity compound”, and “most selective compound”. Figure 3, bottom, shows two autoradiograms of mHb-containing brain sections, which had been incubated without or with 1 μM nicotine; as expected, nicotine displaced the [3H]epibatidine binding to cortex and thalamus but not the binding to mHb. Figure 3, top, shows brain sections incubated with [3H]epibatidine and the four different compounds. The compound with selectivity for α7 receptors displaced neither the [3H]epibatidine binding to cortex and thalamus (α4β2) nor the binding to mHb. The compound with high affinity and selectivity for nicotinic receptors composed of α4β2 subunits readily displaced [3H]epibatidine binding to cortex and thalamus without displacing binding to mHb, even at 10 μM. The mixed affinity compound had high affinity to both α4β2 and α3β4 nicotinic receptors (Table 1). This compound readily displaced the α4β2 binding to cortex and thalamus without displacing the binding to mHb, even at 10 μM. The most selective compound also had high affinity to receptors composed of α3β4 subunits but low affinity to α4β2 subunit containing receptors (Table 1). As seen in Fig. 3, even at 10 μM, the most selective compound only partly displaced the [3H]epibatidine binding to mHb, as well as to α4β2 in thalamus and cortex. This failure to displace [3H]epibatidine bound to mHb is in contrast to the result seen with the mixed affinity compound, whereas the weak affinity for an α4β2 subunit containing receptors reflects the binding data seen in Table 1. The remaining [3H]epibatidine labeling in thalamus and mHb, respectively, at the three concentrations (0.1, 1.0, and 10 μM) after displacement by the most selective compound was 3.9, 3.0, and 1.6 Bq/mg wet weight in the thalamus and 101, 50, and 9.7 Bq/mg wet weight in the mHb (n = 4).

To study in vivo the effect of a selective stimulation of the [3H]epibatidine binding receptors in mHb, a stereotactic method was developed by which minute amounts of epibatidine could be injected in the vicinity of habenula using guiding canulas to direct double injection canulas to the right position. Diffusion and washout of [3H]epibatidine from the injection site was studied by injections of 1 μCi of [3H]epibatidine in a volume of 1 μl, followed by decapitation from 5 to 40 min after injection. Figure 4A, bottom, is an autoradiogram of a coronal brain section at approximately bregma −3.30, showing the distribution of [3H]epibatidine 20 min (left) and 30 min (right) after bilateral intracerebral injection in the vicinity of the habenula. Figure 4A, top, is a Nissl-stained coronal section at approximately bregma −3.30 from the same rat, overlaid onto the autoradiogram. High concentrations of [3H] radioactivity did not spread far from the injection site, and medial habenula is clearly labeled, especially 20 min after the injection. Figure 4B shows the washout curve of [3H]epibatidine from the injection area. From the slope of the curve, the half-life was estimated to be 9 min with one determination at each time point. After rats had been used in the experiments, the position of the cannulas was verified on Nissl-stained brain sections.

Figure 5 shows hot-plate latency in two different experiments after injection of epibatidine or nicotine into the habenula. In one experiment, the animals were injected bilaterally 3 and 4 weeks after surgery with 2 × 1 μl of 2 nM epibatidine and saline in counterbalanced order, and during weeks 5 and 6, with 2 × 1 μl of 10 nM epibatidine and vehicle. In the second experiment, the animals were injected bilaterally 3 and 4 weeks after surgery with 2 × 1 μl of 10 nM epibatidine and saline in counterbalanced order, and during weeks 5 and 6 with 2 × 1 μl of 10 nM nicotine and vehicle. The two experiments included 8 and 14 rats, respectively. Latency to hind paw licking reaction was assessed 10 and 20 min after injection. Latencies during drug trial and corresponding vehicle trials were compared by paired t tests. Animals that did not respond within 30 s during the vehicle test were excluded from statistical analysis. Figure 5 shows that 10 fmol (1 μl, 10 nM) of epibatidine increased the hot-plate latency in both experiments whereas neither 2 fmol of epibatidine nor 10 fmol of nicotine had any effect.

**Discussion**

Nicotinic cholinergic receptors in the CNS and ganglia are composed of α and β subunits, and until now, nine different α subunits and three different β subunits have been characterized. Many of these subunits form functional acetylcholine sensitive ion channels both as homo- and heteromers when expressed in *Xenopus* oocytes. They can be stimulated with nicotinic agonists like epibatidine (Paterson and Nordberg, 2000), which has high affinity to several of these combinations (Parker et al., 1998). In the rat brain in vivo, neuronal
cells in the medial habenula have been found to express mRNA for many of the different subunits, \( \alpha_3, \alpha_4, \alpha_5, \alpha_6, \) and \( \alpha_7, \) and \( \beta_2, \beta_3, \) and \( \beta_4 \) (Sheffield et al., 2000). Thus, at least theoretically, the number of possible different nACh receptors in mHb is rather large. However, only a few of the possible combinations have until now been shown with certainty to be functional in the rat, i.e., \( \alpha_4\beta_2, \) which is widespread in the CNS, and \( \alpha_3\beta_4, \) which was first described in ganglia (Flores et al., 1996) and then also in the CNS (Quick et al., 1999) and the medial habenula (Sheffield et al., 2000). Many other combinations may be functional in vivo, but due to lack of compounds with selectivity for the different subunit combinations, their existence has been difficult to establish.

A possible new nACh receptor with high affinity for epibatidine, and low affinity for acetylcholine and nicotine was found using autoradiographic methods (Plenge and Mellerup, 1998). The receptor has a very limited distribution in the rat brain, as it was only found in a measurable concentration in the medial habenula and a few other brain structures (nucleus interpeduncularis, fasciculus retroflexus, and the pineal gland). In the present study, we have expanded the investigation of this receptor while concentrating on the mHb. The medial habenula was chosen because of its high receptor concentration, which suggests a function for the receptor in this brain structure. Furthermore, the literature suggests a role for the habenular complex in the modulation of pain perception (Cohen and Melzack, 1993; Andres et al., 1999). A more precise outline of the epibatidine recep-

Fig. 4. A, bottom, autoradiogram (bregma −3.30) of the distribution of 1 \( \mu l \) of \(^{3}H\)epibatidine (1 \( \mu Ci \)) 20 min (left) and 30 min (right) after a bilateral intracerebral injection in the vicinity of habenula. Top, Nissl-stained coronal section at bregma −3.30 containing medial habenula from the same rat, overlaid onto the autoradiogram. B, washout of \(^{3}H\)epibatidine in vivo from the brains of rats. Rats were sacrificed from 5 to 40 min after a bilateral intracerebral injection of 1 \( \mu l \) of \(^{3}H\)epibatidine (1 \( \mu Ci \)). The half-life for washout was estimated to be 9 min.

Fig. 5. Latency to hind paw licking in a hot-plate analgesia test at 50°C. Rats received a bilateral intracerebral injection near the habenula with 2 \( \times \) 1 \( \mu l \) of physiological saline containing epibatidine or nicotine in the stated amounts or the saline vehicle. Hot-plate latency was tested 10 and 20 min after the injection. Increase in latency was significant \((P < 0.05)\) 20 min after injection of 2 \( \times \) 10 fmol of epibatidine. ■, vehicle; □, drug.
tor distribution (Fig. 1, A and B) coincides with the shape and extension of mHB. The coronal sections of mHB labeled with \(^{3}H\)epibatidine (Fig. 1A) indicate that the receptor concentration within mHB varies because lighter and darker patches are seen in the autoradiogram. The receptor concentration in mHB is high (in the presence of 1 \(\mu\)M nicotine, it is estimated to be about 1100 fmol/mg wet weight), with an affinity to \(^{3}H\)epibatidine of about 0.5 nM. This affinity corresponds to the value for the \(\alpha_3\beta_4\) nACh receptor of 0.3 nM found by Parker et al. (1998). However, in the same study, the affinity of acetylcholine and nicotine was determined to be 0.5 and 0.3 \(\mu\)M, respectively, which was far from our \(K_i\) estimates of 8 and 5 \(\mu\)M. Sheffield et al. (2000) suggest that the \(\alpha_3\)-containing nACh receptors in the mHB consist of more additional subunits than \(\beta\)4 because data indicate the possibility of both \(\alpha_4\) and \(\alpha_5\) inclusion in an \(\alpha_3\beta_4(\beta_2)\) receptor. The affinities of these combinations for epibatidine, nicotine, and acetylcholine are at present not described, and it is therefore unknown whether one of the combinations is a likely candidate for the epibatidine receptor described here. Figure 2 documents the difference between the high affinity of \(\alpha_4\beta_2\) receptors and the much lower affinity of the \(^{3}H\)epibatidine binding receptor in mHB for acetylcholine because 0.5 \(\mu\)M acetylcholine displaced all bound \(^{3}H\)epibatidine except from the mHB, where some binding persisted even in the presence of 64 \(\mu\)M acetylcholine.

Another way of investigating whether the epibatidine receptor in the mHB is an \(\alpha_3\beta_4\) receptor was to measure, with autoradiographic methods, the affinity of nicotine when displacing \(^{3}H\)epibatidine bound to ganglionic \(\alpha_3\beta_4\) nACh receptors. This was done on sections from superior cervical ganglion, and nicotine was found to displace \(^{3}H\)epibatidine with an affinity (\(K_i\) value) of 1.3 \(\mu\)M, which is different from the 5 \(\mu\)M mentioned above, thus, making it further unlikely that the epibatidine receptor in the mHB is composed of \(\alpha_3\beta_4\) subunits alone.

Several compounds with known affinity to different nicotinic receptors were tested for their ability to displace \(^{3}H\)epibatidine binding to the mHB. Our objective was to search for a compound with selectivity for the epibatidine receptor in the mHB, i.e., displacement of \(^{3}H\)epibatidine binding to the mHB without displacement of the binding to other brain structures. A perfect compound with this capacity was not found, but different valuable indications were obtained (see Fig. 3 and Table 1). Among the compounds were some with high selectivity and high affinity for \(\alpha_7\) and \(\alpha_4\beta_2\) nicotinic receptors, respectively. Their systematic chemical names and affinities to different nicotinic receptors are presented in Table 1. Neither type of compound was able to displace \(^{3}H\)epibatidine binding to the mHB even at 10 \(\mu\)M; “\(\alpha_7\)” compounds did not displace \(^{3}H\)epibatidine at all, whereas “\(\alpha_4\beta_2\)” compounds readily displaced the \(^{3}H\)epibatidine binding to all other brain structures. This apparent lack of \(\alpha_4\beta_2\) receptors in mHB is consistent with the finding that \(^{3}H\)epibatidine binds to the mHB in mice in which the \(\alpha_4\)- or \(\beta_2\)-nACh subunits have been knocked out (Zoli et al., 1998; Marubio et al., 1999).

Table 1 and Fig. 3 also show results obtained with two other substances, named by us mixed affinity compound and most selective compound. Both compounds have the same high affinity to the \(\alpha_3\beta_4\) receptor subtype, whereas their affinity to the \(\alpha_4\beta_2\) receptor differs; the mixed affinity compound has very high affinity, whereas the most selective compound has low affinity. Autoradiograms obtained with the mixed affinity compound (Fig. 3) showed that already at 0.1 \(\mu\)M the \(^{3}H\)epibatidine binding to \(\alpha_4\beta_2\) receptors was displaced as expected. At 1 \(\mu\)M, the binding to the mHB fades, disappearing completely at 10 \(\mu\)M. Autoradiograms obtained with the most selective compound showed a completely different picture. Even at 10 \(\mu\)M, not all the \(^{3}H\)epibatidine bound to \(\alpha_4\beta_2\) receptors in the thalamus and cortex had vanished, but neither had the binding to mHB. Because the two compounds in question have about the same affinity to \(\alpha_3\beta_4\) receptors (the most selective compound actually having slightly higher affinity; see Table 1), this result must be interpreted as showing that the \(^{3}H\)epibatidine binding receptor in mHB most likely is not an \(\alpha_3\beta_4\) receptor. The partial displacement of the \(^{3}H\)epibatidine bound to the mHB by compounds with high affinity for \(\alpha_3\beta_4\) may indicate that the epibatidine receptor contains \(\alpha_3\) subunits because epibatidine binds to this subunit (Warpman et al., 1998). However, when seen in the light of the results discussed above, the receptor in the mHB most likely is composed of more nACh subunits than \(\alpha_3\beta_4\). A suggestion could be \(\alpha_3\beta_2\delta_5\) or \(\alpha_3\beta_4\delta_5\) receptors, which have been expressed in a cell line (Wang et al., 1998).

The strong analgesic effect of epibatidine has spurred interest in analgesia obtained via stimulation of nACh receptors, and the \(\alpha_4\beta_2\) receptor has been proposed to be responsible for this effect. The drug ABT-594 is a fairly selective \(\alpha_4\beta_2\) ligand and has analgesic properties in different pain models after systemic administration (Bannon et al., 1998). Also, an injection of minute amounts of ABT-594 into nucleus raphe magnus has an antinociceptive effect on rats in a hot-plate assay, probably by gating transmission of afferent nociceptive inputs from reaching higher centers. Following this idea, and because various lines of evidence suggest an involvement of the habenular complex in pain processing (Cohen and Melzack, 1993), a role for the habenular epibatidine receptors in signal gating might be suggested.

A rat model was developed in which the effect of minute amounts of epibatidine injected bilaterally into brain areas near the mHB could be studied. Figure 4A confirms that epibatidine injected near mHB labels the epibatidine receptors in mHB. In preliminary experiments, amounts of epibatidine up to 500 fmol were injected bilaterally near the habenula to evaluate the toxicity of epibatidine after this form of administration. None of the doses tested seemed to affect the spontaneous behavior of the rats, e.g., no signs of sedation, hyperactivity, or motor disturbances were observed. Based on the affinity of epibatidine (0.5 nM at 4°C) for the epibatidine receptor in the mHB and the washout half-life (9 min) of \(^{3}H\)epibatidine injected locally into the brain, it was decided to test the analgesic effect of about 10 fmol (1 \(\mu\), 10 nM) of epibatidine injected near the mHB. As seen in Fig. 5, 10 fmol of epibatidine indeed proved to have some analgesic effect, 10 and 20 min after injection, whereas neither 2 fmol of epibatidine nor 10 fmol of nicotine (which we considered would stimulate \(\alpha_4\beta_2\) receptors) had any effect.

Compounds with selectivity for \(\alpha_3\beta_4\) receptors are not likely to be useful analgesics because ganglionic nACh receptors primarily are of this subtype and, therefore, both agonists and antagonists would have profound physiological effects. Compounds with selectivity for \(\alpha_4\beta_2\) receptors have
analgesic effects as shown by Bannon et al. (1998), but due to the widespread distribution of the α4β2 receptor in the CNS, it may be anticipated that other, maybe less desirable effects may appear, when stimulating this receptor. The moderate analgesic effect observed in our experiments is most likely due to stimulation of the epibatidine receptors in the mHb because the estimated concentration of epibatidine 10 and 20 min after an injection of 10 fmol is in the right concentration range compared with the receptor Kᵰ of 0.5 nM found in the saturation measurements. The effect is not likely to be caused by stimulation of α4β2 receptors because injection of 10 fmol of nicotine was without analgesic effect. The epibatidine receptor in the mHb thus may represent a possibility for obtaining analgesia selectively. Due to the very limited distribution of the receptor in the CNS and probable absence from ganglia, it could be a pharmacologically interesting target. The development of a selective drug with the right balance of agonistic activity remains, however, a challenge for future research.

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