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BINDING OF THE NICOTINIC CHOLINERGIC ANTAGONIST, DIHYDRO-β-ERYTHROIDINE, TO RAT BRAIN TISSUE[1]

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Abstract

The nicotinic cholinergic antagonist, dihydro-β-erythroidine, binds to two sites in rat cortical membranes with dissociation constants of 4 and 22 nM and respective apparent B_{max} values of 52 and 164 fmol/mg of protein. Binding to the higher affinity site, defined by the use of 2 nM [^{3}H]dihydro-β-erythroidine, was saturable, reversible, and susceptible to protein denaturation. Binding was highest in the thalamus and lowest in the spinal cord and showed preferential enrichment in a synaptosomal subfraction of rat brain. Nicotine displaced [^{3}H]dihydro-β-erythroidine in a stereospecific manner, the (−)-isomer being approximately 6 times more potent than the (+)-isomer. The alkaloid nicotinic agonists, cytisine and lobeline, were potent inhibitors of binding, while acetylcholine in the presence of the cholinesterase inhibitor di-isopropylfluorophosphate was equipotent with (+)-nicotine. Binding was also inhibited by the muscarinic ligands, arecoline, atropine, and oxotremorine. The nicotinic antagonists mecamylamine, hexamethonium, and pempidine were essentially inactive in displacing [^{3}H]dihydro-β-erythroidine. These findings indicate that dihydro-β-erythroidine binds to a nicotinic recognition site in rat brain which is neuromuscular, rather than ganglionic, in nature and that such binding is similar in several respects to that seen with nicotinic agonists. Whether such binding is to a nicotinic, as opposed to nicotinic cholinergic, recognition site or to a "common" nicotinic/muscarinic site is an issue that requires further study.

Acetylcholine (ACh) receptors in nervous tissue can be divided into muscarinic and nicotinic subclasses on the basis of the effects of the cholinergic agonists, muscarine and nicotine (Triggle, 1971). In the mammalian CNS, muscarinic cholinergic receptors (mAChRs) can be labeled with agonists such as *cis*-methyldioxolane (Vickroy et al., 1983) and antagonists such as quinuclidinyl benzilate (Yamamura and Snyder, 1974) and pirenzepine (Yamamura et al., 1983). Nicotinic cholinergic receptors (nAChRs) have been labeled with elapid neurotoxins such as α-bungarotoxin (McQuarrie et al., 1976; Oswald and Freeman, 1981) and *Naja naja siamensis* α-toxin (Speth et al., 1977) and the agonists, nicotine and ACh (Yoshida and Imura, 1979; Romano and Goldstein, 1980; Martin and Aceto, 1981; Marks and Collins, 1982; Schwartz et al., 1982; Costa and Murphy, 1983; Sloane et al., 1984).

There is considerable evidence to support the existence of putative nAChR in mammalian brain (Krnjevic, 1976), and *in vivo*, nicotine can alter spontaneous activity and brain excitability as well as being antinociceptive (Larson and Silvette, 1975; Costa and Murphy, 1983; Martin et al., 1983). However, the physiological relevance of neurotoxin-binding sites has been questioned (Brown and Fumagalli, 1977; Carbonetto et al., 1978), since α-bungarotoxin has no apparent nAChR antagonist activity in several mammalian neuronal systems. Furthermore, the classical nicotinic antagonists, decamethonium and hexamethonium, are poor inhibitors of toxin binding (McQuarrie et al., 1976; Schmidt, 1977; Morley et al., 1979). ACh itself is a weak inhibitor of bungarotoxin binding (Schmidt, 1977) and may be a noncompetitive antagonist of this ligand (Lukas and Bennett, 1979), perhaps reflecting toxin binding to the putative α-subunit of the mammalian nAChR (Mishina et al., 1984).

The relative ineffectiveness of the nAChR antagonists, mecamylamine and hexamethonium, in displacing [^{3}H]nicotine binding from rat brain membranes has led to the suggestion that either such binding is noncholinergic or that agonists may induce an agonist-selective nAChR-binding site which is insensitive to antagonists (Abood et al., 1980). It has been further suggested (Schwartz et al., 1982) that nicotinic agonists and antagonists label different membrane recognition sites. It is also apparent that α-bungarotoxin and nicotine label different sites in mouse brain membranes (Marks and Collins, 1982). To examine further the nature of putative nAChRs in mammalian brain tissue, the binding of dihydro-β-erythroidine (DBE; Fig. 1), a neuromuscular nicotinic antagonist (Curtis and Ryall, 1966; Nicoll, 1975; Ben Ari et al., 1976), to rat brain tissue was evaluated.

Materials and Methods

Rat brain tissue was routinely homogenized in 40 vol of ice-cold buffer (50 mM Tris-HCl, pH 7.4) using a Polytron (setting 5.5, 10 sec), the resultant homogenate being washed twice by centrifugation (48,000 × *g*; 4°C) with intermediate resuspension by the Polytron. The final

[1] We wish to thank Drs. Clement Stone and Richard Young for their enthusiasm in providing [^{3}H]dihydro-β-erythroidine, Dr. Sam Enna for helpful discussions, Dr. Steve Hurt for assessing the purity of bound ligand, and Dr. Doug Pettibone for help with the brain dissections.

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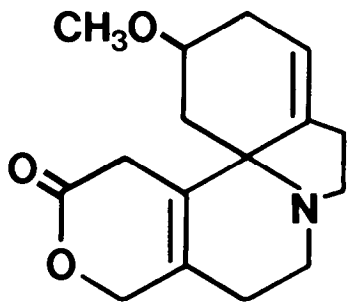

Figure 1. Structure of dihydro-β-erythroidine (DBE).

pellet was resuspended in 40 vol of buffer/gm of original wet weight (~2 mg of protein/ml), immediately before use.

Binding assays were conducted in a final volume of 2 ml at 4°C. For routine assays, tubes contained 0.1 ml of [^{3}H]DBE in a final concentration of 2 nM, 0.1 ml of (±)-nicotine dihydrochloride to a final concentration of 100 μM to determine nonspecific binding, and buffer in a final volume of 1 ml. When the effects of drugs on specific [^{3}H]DBE binding were examined, these were included to the final concentration indicated, in a volume of 0.1 ml. Incubation was initiated by the addition of 1 ml of tissue homogenate and was continued for 5 min at 4°C. The reaction was terminated by pouring the sample over Whatman GF/B glass fiber filters under vacuum, excess unbound ligand being removed with two 5-ml washes of ice-cold buffer. Filters were placed in scintillation vials to which were added 10 ml of Aquassure Scintillation Cocktail (New England Nuclear Corp., Boston, MA). After capping, the vials were shaken mechanically for 30 min and after equilibration were counted at an efficiency of approximately 40% by conventional liquid scintillation spectrometry.

Drugs were examined at five or more concentrations in triplicate in each experiment. The percentage of inhibition of specific binding was determined and IC_{50} values were calculated by log-probit analysis. Pseudo-Hill slope values (n_H) were determined for each of the displacement curves and, where n_H was not significantly different from unity, K_i values were derived from the relationship, $K_i = IC_{50}/1 + c/K_d$ (Cheng and Prusoff, 1973), where c = ligand concentration (2 nM) and K_d = dissociation constant (4 nM). Hill slope values were determined as described by Wold (1971), and the significance of their difference from unity was assessed by a two-tailed Student's t test (Bailey, 1959). In experiments in which the effects of the cholinesterase inhibitor, diisopropylfluorophosphate (DFP), on ACh displacement of [^{3}H]DBE binding were evaluated, DFP was included with the tissue to a final concentration of 100 μM as previously reported (Schwartz and Kellar, 1983). DFP had no significant effect on total and specific binding.

When association and dissociation rate constants were determined, incubations were carried out in bulk at 4°C, 2-ml aliquots being removed at the time intervals indicated. Dissociation was initiated by the addition of a small volume (0.05 ml) of (±)-nicotine in a final concentration of 100 μM. Radiolabeled DBE was included at a final concentration of 2 nM. Association and dissociation rate constants were determined as described by Kitagbi et al. (1977), assuming pseudo-first-order rate kinetics.

For Scatchard analysis, saturation isotherms were generated over the ligand concentration range 0.1 to 30 nM, nonspecific binding being determined in the presence of 100 μM (±)-nicotine. After transformation by the method of Scatchard (1949), data were evaluated by a nonlinear least squares regression analysis to determine the model for best fit (Draper and Smith, 1966), and K_d and apparent B_{max} values (Klotz, 1983) were determined as described by Zivin and Waud (1982).

In all routine studies, rat cortex minus cerebellum was used to study binding. For studies on the regional distribution of [^{3}H]DBE binding, rat brain was dissected according to the procedure of Glowinski and Iversen (1966) and membranes were prepared as described above. For subcellular distribution studies, the procedure of Whittaker (1969) was used to prepare nuclear (P_1), crude synaptosomal (P_2), microsomal (P_3), myelin (P_2A), synaptosomal (P_2B), and mitochondrial (P_2C) subfractions. Tissue protein content was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Authenticity of bound [^{3}H]DBE was determined by thin layer chromatography (TLC). Following incubation and filtration, filters together with bound ligand-receptor complexes were extracted into 5 ml of absolute ethanol. After filtration through glass wool to remove the denatured tissue residue, the resuspension was lyophilized, resuspended in ethanol, and analyzed on cellulose-TLC plates (Analtech, Avocel) using a butanol:acetic acid:water (25:4:10) developing system. Cold DBE was added as carrier and, following determination of the distribution of tritium using a TLC scanner (Packard Instruments, Downers Grove, IL), the plate was developed using Dragendorff reagent. Radioactivity thus detected was determined to be >90% authentic DBE.

[^{3}H]DBE (specific activity, 30 Ci/mmol) was obtained from New England Nuclear. The (−)- and (+)-isomers of nicotine were generous gifts from Dr. Dick Young, New England Nuclear, and DFP was obtained from Sigma Chemical Co., St. Louis, MO. The remainder of the compounds used were from the sample collection of the Merck Institute.

Results

Examination of several buffer systems and rat brain tissue preparations established that 50 mM Tris-HCl buffer (pH 7.4) at 4°C together with a washed cortical membrane preparation from rat brain gave optimal binding. Specific binding, defined as the difference in binding in the absence and presence of 100 μM (±)-nicotine dihydrochloride, was 45 to 55% of the total counts bound at a final [^{3}H]DBE concentration of 2 nM.

In initial experiments, centrifugation (Beckman J-21C) at 48,000 × g for 15 min (at 4°C) was used to isolate receptor-ligand complexes. Although reproducible binding was obtained, the high signal-to-noise ratio and the inconvenience of the methodology led to the development of a timed 5-min incubation at 4°C, isolating bound radioactivity by vacuum filtration over Whatman GF/B glass fiber filters. Filter binding was less than 1% of the total counts bound to the tissue (data not shown).

Binding of 2 nM [^{3}H]DBE to cortical membranes reached equilibrium between 1 and 3 min (Fig. 2) and remained at equilibrium for up to 20 min. Binding was rapidly reversed by the addition of an excess (100 μM final concentration) of (±)-nicotine (Fig. 2). Assuming pseudo-first-order reaction kinetics (Kitagbi et al., 1977), the dissociation rate constant (K_{+1}) was 9.4 × 10^9 $M^{-1}sec^{-1}$. From the ratio K_1/K_{+1}, a K_d (dissociation constant) value of 5.0 nM was derived. This value compared

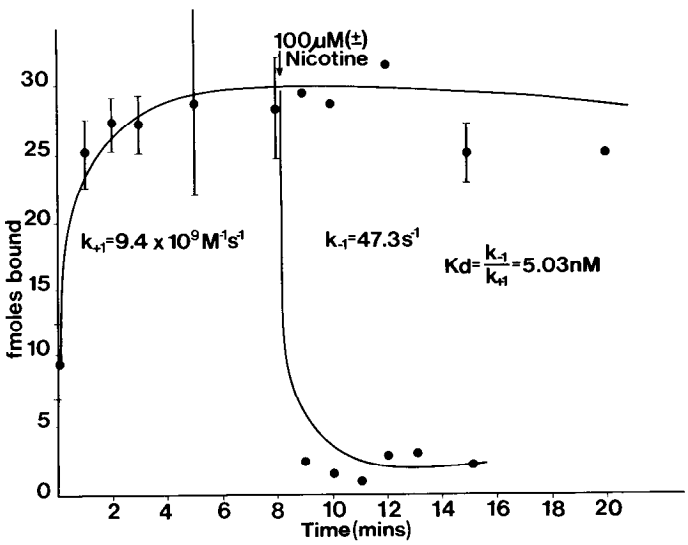

Figure 2. Association-dissociation curve for specific [^{3}H]DBE binding. Washed rat cortical membranes were incubated in bulk with 2 nM [^{3}H]DBE and aliquots (2 ml) removed at the times indicated. An excess of nicotine (100 μM final concentration) was added in a volume of 0.05 ml to examine reversibility of binding. Where indicated, results are the mean ± SD for four to six separate observations. Where only *single points* are shown, these represent the mean of two determinations. The results represent the aggregate of three separate experiments. Nonspecific binding was determined in the presence of 100 μM (±)-nicotine.

favorably with that obtained for a high affinity DBE-binding site using saturation analysis (Fig. 3). Over the ligand concentration range 0.1 to 30 nM, specific and total [^{3}H]DBE binding gave evidence of being saturable, whereas nonspecific binding was linear, showing no evidence of saturation. Scatchard (1949) analysis of the specific binding isotherm, using curve fitting analysis as described under "Materials and Methods," gave a biphasic plot (Fig. 3) indicative of two binding sites. A high affinity site with a K_d value of 4.02 ± 1.32 nM (mean ± SD; n = 4) and a lower affinity site with a K_d value of 21.6 ± 6.2 nM. The respective apparent B_{max} values (Klotz, 1983) for the two sites were 52.2 and 164 fmol/mg of protein. A typical saturation isotherm and Scatchard plot are shown in Figure 3. Specific binding was linear with tissue concentration up to 4 mg of protein/ml (data not shown) and was almost completely abolished by boiling. In unboiled membranes, specific [^{3}H]DBE binding was 9.2 fmol/mg of protein whereas in membrane boiled for 15 min, specific binding was 0.8 fmol/mg of protein.

Evaluation of the subcellular distribution of specific binding using 2 nM [^{3}H]DBE (Table I) showed a slight enrichment of binding in both the P_2 (crude synaptosomal) and P_2B (enriched synaptosomal) subfractions. Some enrichment of binding was also observed in the microsomal (P_3) subfraction. Approximately 42% of the total specific [^{3}H]DBE binding observed in the brain homogenate was in the crude synaptosomal fraction (Table I), whereas the P_2B fraction had more than twice the total amount of binding as the myelin (P_2A) subfraction and 4 times that observed in the mitochondrial (P_2C) fraction. Although the increases in the specific activity (femtomoles per milligram of protein) of specific [^{3}H]DBE binding in the P_2 and P_2B fractions are not especially striking, they were significantly different from that observed in the homogenate (Table I). Regional analysis of 2 nM [^{3}H]DBE binding showed a significant enrichment in thalamus which had 2.5 times the number of sites on a femtomole per milligram of protein basis that the hypothalamus had (Table II). Of the other brain regions studied, binding was lowest in the spinal cord, with binding in the remaining brain regions being caudate > hippocampus ≥ frontal cortex ≥ brainstem > cortex ≥ cerebellum. Negligible specific binding was observed in liver membranes (Table II).

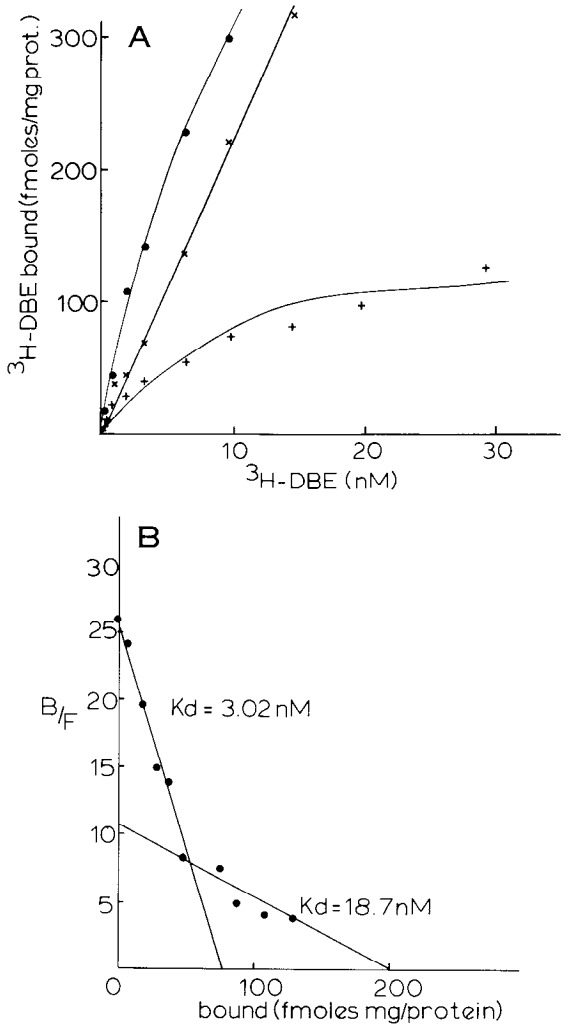

Figure 3. A, Saturation of isotherm of [^{3}H]DBE binding. ●—●, total binding: x—x, nonspecific binding; +—+, specific binding. All data were corrected for the amount of ligand bound. *B*, Scatchard plot of specific [^{3}H]DBE binding from *A*. Data were evaluated by a nonlinear least squares regression analysis to determine the model for best fit (Draper and Smith, 1966), and the K_d and apparent B_{max} values were determined as described by Zivin and Waud (1982).

TABLE I

Subcellular distribution of specific [^{3}H]DBE bound per milligram of protein

Results are the mean ± SD for four separate animal observations. Whole rat brain minus cerebellum was homogenized and subcellular fractions were prepared by the method of Whittaker (1969) as described under "Materials and Methods."

Fraction	[^{3}H]DBE Bound	Total Femtomoles Bound
	fmol/mg of protein	
Homogenate	5.3 ± 1.2	1165 ± 323 (100)[a]
P_1-Nuclear	3.9 ± 1.9	175 ± 97 (15)
P_2-Crude synaptosomal	8.2 ± 1.7[b]	484 ± 128 (42)
P_3-Microsomal	6.3 ± 0.3	303 ± 11 (26)
P_2A-Myelin	6.7 ± 2.3	35 ± 12
P_2B-Synaptosomal	8.1 ± 0.7[b]	83 ± 17
P_2C-Mitochondrial	5.7 ± 2.2	18 ± 8

[a] Numbers in parentheses, percentage of homogenate.

[b] Significantly different from homogenate ($p < 0.05$; Student's two-tailed t test; Bailey, 1959).

TABLE II

Regional distribution of binding specific [^{3}H]DBE binding

Results are the mean ± SD for three separate experiments using different tissue preparations. Tissue from up to six animals was pooled in each experiment. Brain regions refer to those described by Glowinski and Iversen (1966). Tissue was incubated at a final protein concentration of 0.5 to 1.2 mg/ml with 2 nM [^{3}H]DBE for 5 min at 4°C. Nonspecific binding was determined in the presence of 100 μM (±)-nicotine dihydrochloride as described under "Materials and Methods."

Region	[^{3}H]DBE Binding
	fmol/mg of protein
Thalamus	21.2 ± 2.9
Hypothalamus	8.8 ± 1.9
Caudate	6.3 ± 0.8
Hippocampus	4.7 ± 0.2
Frontal cortex	4.4 ± 0.6
Brainstem	3.5 ± 0.4
Cortex	2.4 ± 0.7
Cerebellum	2.1 ± 0.5
Spinal cord	1.6 ± 1.0
Liver	0.3 ± 0.1

Cholinergic agonists were considerably more potent than antagonists in displacing [^{3}H]DBE (Table III). The alkaloid, cytisine, a ganglionic agonist, was the most potent compound examined, having an IC_{50} value 10 times less than that of (−)-nicotine. Lobeline, another agonist alkaloid, was also potent in displacing specific [^{3}H]DBE binding, being 3 times more active

TABLE III

Pharmacology of [^{3}H]DBE binding to rat brain membranes

Results are the mean ± SD for three to eight separate observations. IC_{50} and n_H values were determined as described under "Materials and Methods" using washed rat brain membranes prepared from cortex minus cerebellum. The following compounds were considered inactive (IC_{50} > 100,000 nM): norepinephrine, serotonin, 2-chloroadenosine, pirenzepine, LSD, glutamate, diazepam, GABA, and scopolamine.

Compound	IC_{50} (nM)	n_H	K_i (nM)
Cytisine	5.7 ± 2.8	1.05 ± 0.05	3.8 ± 1.9
Lobeline	21.5 ± 8.0	1.51 ± 0.41	14.3 ± 5.3
(−)-Nicotine	67 ± 23	0.90 ± 0.58	44.7 ± 15.3
(+)-Nicotine	432 ± 68	1.29 ± 0.37	288 ± 45
Acetylcholine + DFP (100 μM)	465 ± 66	0.96 ± 0.06	312 ± 44
Acetylcholine	1,345 ± 314	2.09 ± 0.32[a]	
Arecoline	1,700 ± 400	1.49 ± 0.38[a]	
MK 212	1,720 ± 1,000	1.49 ± 0.61	1,147 ± 667
Anabasin	1,970 ± 600	1.04 ± 0.15	1,310 ± 400
Haloperidol	9,200 ± 4,900	1.11 ± 0.38	6,133 ± 3,267
Zimelidine	15,000 ± 1,800	1.01 ± 0.23	10,000 ± 1,200
Chlorpromazine	17,000 ± 7,000	0.94 ± 0.47	11,333 ± 4,667
Atropine	21,900 ± 12,000	0.71 ± 0.18[a]	
Desmethyl-imipramine	30,000 ± 9,000	0.61 ± 0.08[a]	
Apomorphine	31,000 ± 14,000	0.98 ± 0.17	20,667 ± 9,333
Oxotremorine	32,000 ± 4,000	1.03 ± 0.14	21,333 ± 2,667
Phencyclidine	59,800 ± 18,200	0.91 ± 0.38	39,867 ± 12,133
Morphine	71,000 ± 37,000	1.12 ± 0.52	47,333 ± 24,667
Hexamethonium	>100,000		
Mecamylamine	>100,000		
Pempidine	>100,000		

[a] $p < 0.05$ versus unity (Student's two-tailed t test; Bailey, 1959).

than (−)-nicotine. Binding of the DBE was stereospecific, the (+)-isomer of nicotine being 7 times less potent than (−)-nicotine (IC_{50} = 432 nM versus 67 nM; Table III). In the presence of DFP (100 μM), ACh had an IC_{50} of 465 nM, being approximately equipotent with (+)-nicotine in displacing [^{3}H]DBE. In the absence of DFP, ACh was some 3 times less potent than in the presence of the cholinesterase inhibitor (Table III). The n_H value of 2.09 compared with a value of 0.96 in the DFP-treated tissue is, therefore, probably a reflection of ACh metabolism. Arecoline, a cholinergic agonist with muscarinic and nicotinic activity (Krnjevic, 1976), had an IC_{50} value of 1.7 μM and was thus some 25 times less active than (−)-nicotine. Of interest was the fact that arecoline had an n_H value that differed significantly from unity (Table III). MK 212, a putative serotonomimetic, was also equipotent with arecoline in displacing DBE, whereas the piperidinyl analogue of nicotine, anabasine, had an IC_{50} value of 1.97 μM. The neuroleptics, haloperidol and chlorpromazine, were weak inhibitors of binding, as was the putative antidepressant, zimeldine. The muscarinic antagonist, atropine, which was approximately equipotent with the neuroleptics and zimeldine, had a Hill slope value which was significantly less than 1.0 (Table III), as did the tricyclic antidepressant, desmethylimipramine (DMI; Table III), which was some 450 times less potent than (−)-nicotine in displacing DBE binding. The dopamine agonist, apomorphine, and the muscarinic agonist, oxotremorine, were approximately equipotent with DMI yet had n_H values close to unity. The psychotomimetic, phencyclidine, which has been reported to interact with nAChR (Aguayo et al., 1981; Oswald et al., 1983), was a relatively weak inhibitor of DBE binding, as was morphine (Table III). The nicotine-cholinergic antagonists, mecamylamine, hexamethonium, and pempidine, and the muscarinic antagonists, scopolamine and pirenzepine, were essentially inactive (IC_{50} values >100,000 nM; Table III), as were the putative neurotransmitters/neuromodulators, norepinephrine, serotonin, glutamate, GABA, 2-chloroadenosine, lysergic acid diethylamide (LSD), and diazepam.

Discussion

The neuromuscular nAChR antagonist, DBE, was found to bind in a saturable, reversible manner to two high affinity binding sites in rat brain membranes. Examination of the higher affinity site using 2 nM [^{3}H]DBE showed that it was probably protein in nature, since denaturation of the tissue by boiling destroyed binding. In addition, the evidence accumulated indicated that this site showed some preferential localization in synaptosomal subfractions of rat brain (Table I), although it may be noted that the observed enrichment in binding reflects more properly the total number of sites rather than their density in terms of protein concentration, a finding which may reflect the fact that specific binding was only 50% of the total counts bound. It may also be noted, however, that whereas Schwartz et al. (1982) showed an enrichment of specific [^{3}H]ACh binding in the synaptosomal (P_2B) as opposed to myelin (P_2A) and mitochondrial (P_2C) subfractions, such binding was not significantly different from that seen in the initial homogenate.

The regional distribution of the higher affinity [^{3}H]DBE binding site was comparable to that reported for [^{3}H]nicotine (Yoshida and Imura, 1979; Martin and Aceto, 1981; Marks and Collins, 1982) and for [^{3}H]ACh (Schwartz et al., 1982). Thus, binding ranged from 21 fmol/mg of protein (thalamus) to 1.6 fmol/mg of protein (spinal cord). The actual amount of binding was similar to that observed by both Schwartz et al. (1982) and Marks and Collins (1982) but was somewhat lower than that observed by Yoshida and Imura (1979) and Martin and Aceto (1981), a finding that may reflect differences in the concentration of radioligands used. However, in both the present study and those of Yoshida and Imura (1979), Martin and Aceto (1981), and Schwartz et al. (1982), the thalamus and/or hypothalamus exhibited the highest regional density of putative nAChR-binding sites, a finding which is consistent with electrophysiological evidence for the existence of nicotinic cholinergic receptors in the thalamus (Krnjevic, 1976). In the study of Marks and Collins (1982), using [^{3}H]nicotine, regional binding was somewhat different in the present study and the others cited, inasmuch as binding was highest in the striatum. Whether the apparent differences in regional binding site density reflect true, as opposed to methodological, differences is an issue that requires resolution.

Binding of 2 nM [^{3}H]DBE to rat brain membranes exhibited stereospecificity, the (−)-isomer of nicotine being approximately 6 times more potent than (+)-nicotine (Table III). Stereoselectivity has also been observed for the isomers of nicotine using [^{3}H]nicotine (Romano and Goldstein, 1980; Costa and Murphy, 1983) and [^{3}H]ACh (Schwartz et al., 1982), although in these studies the (−)-isomer was 63, 63, and 22 times more potent, respectively, than (+)-nicotine.

The nicotine ganglionic blockers mecamylamine, hexamethonium, and pempidine had little activity at the [^{3}H]DBE-binding site (Table III), a finding in agreement with the lack of effect of these compounds on nicotine radioligand binding in general (Romano and Goldstein, 1980; Marks and Collins, 1982; Schwartz et al., 1982; Costa and Murphy, 1983; Sloan et al., 1984). These results contrast with the potency of arecoline, atropine, and oxotremorine (Table III), which are active at the mAChR. It may be noted, however, that the mAChR antagonists scopolamine and pirenzepine were inactive (Table III). Although the inactivity of the putative neurotransmitter/neuromodulators norepinephrine, serotonin, gluatamate, GABA, diazepam, and 2-chloroadenosine would suggest that the DBE-

binding site is selectively cholinergic, the present data do not resolve the issue as to why classical nicotinic antagonists do not displace nicotine agonist (Abood et al., 1980; Marks and Collins, 1982; Schwartz et al., 1982; Costa and Murphy, 1983; Sloane et al., 1984) and, in the present instance, antagonist binding.

The unusual pharmacology of the DBE-binding site, the unexpected sensitivity of the binding to a number of psychotropic agents, and the equivocal subcellular distribution raise the issue as to whether the DBE-binding site is an "acceptor" rather than a receptor. Furthermore, it is somewhat unusual for an antagonist recognition site to be more sensitive to agonists (nicotine, anabasine, lobeline) than to antagonists (mecamylamine, hexamethonium, pempidine), while the activity of mAChR ligands (arecoline, atropine, oxotremorine) is also somewhat unexpected.

It may be noted, however, that DBE is a neuromuscular, rather than a ganglionic-type, nicotinic antagonist (Krnjevic, 1976), and it is possible that the binding site in mammalian brain tissue is more related to the former than to the latter nicotinic receptor. This may in turn explain the lack of effectiveness of the ganglionic blockers on DBE binding. Attempts to study [^{3}H]mecamylamine binding in mammalian brain tissue have been unsuccessful (M. Williams and J. A. Totaro, unpublished results; S. J. Enna, personal communication), a finding which reinforces the possibility of differences between the putative nAChRs present in brain and those in ganglia. It is also possible, as has been noted from electrophysiological studies (Krnjevic, 1976), that neurons may have both mAChR and nAChR present on their cell surface, a finding that may reflect on the effectiveness of mAChR ligands at the DBE-binding site.

The binding of [^{3}H]DBE to rat brain membranes is similar in many respects to that seen with [^{3}H]nicotine (Marks and Collins, 1982; Costa and Murphy, 1983) and [^{3}H]ACh (Schwartz et al., 1982), suggesting that the recognition sites binding nicotinic agonists and nicotinic antagonists of the neuromuscular type are similar. Furthermore, the conclusion that nicotinic receptors in brain exist in distinct agonist and antagonist states (Abood et al., 1980; Schwartz et al., 1982) based on the ineffectiveness of ganglionic blockers in displacing agonist binding may be premature at the present time. The original observations of Abood et al. (1980) that nAChR may be noncholinergic may be relevant in this context if the nicotinic recognition site(s) in mammalian brain tissue are termed nicotinic rather than nicotinic cholinergic.

From a physiological standpoint, nicotine has pronounced effects in the CNS (Martin et al., 1983), and it has been reported (Costa and Murphy, 1983) that decreases in [^{3}H]nicotine binding in brain tissue following chronic cholinesterase inhibitor treatment can be correlated with a loss in antinociceptive activity. The displacement of DBE binding by the serotonomimetic, MK 212 (Clineschmidt, 1979; Williams and Risley, 1982), may also have physiological relevance since this compound can antagonize nicotine self-administration (H. M. Hanson, personal communication).

Whether the recognition site to which DBE binds is truly nicotinic in nature and is an "acceptor" or "receptor" is an issue that requires further study. Nonetheless, DBE is the first nicotinic antagonist which has been shown to bind to mammalian brain tissue. Further studies on its binding in both mammalian peripheral and invertebrate tissues such as *Torpedo* may shed light on the potential physiological significance of putative nicotinic recognition sites in mammalian brain tissue and their relevance to the pathophysiology of nicotine addiction.

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