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Brain Nicotinic Acetylcholine Receptors

Biochemical Characterization by Neosurugatoxin

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SUMMARY

Specific [^{3}H]nicotine binding to rat forebrain membranes was saturable and of high affinity, and it exhibited pharmacological specificity as well as stereoselectivity for nicotinic agents. There was a regional variation of specific [^{3}H]nicotine binding in rat brain. Low concentrations of neosurugatoxin markedly inhibited specific [^{3}H]nicotine binding in rat forebrain ($IC_{50} \equiv 78$ nM) and the competition curve by the toxin was biphasic (pseudo-Hill slope, 0.44). Approximately 50% of [^{3}H]nicotine binding in rat forebrain was inhibited by low concentrations (0.3–100 nM) of neosurugatoxin and the residual binding was inhibited by higher concentrations (0.3–10 μM). In the presence of 3 nM, 100 nM, and 1 μM neosurugatoxin, there was a concentration-dependent (28, 54, and 71%, respectively) loss of [^{3}H]nicotine-binding sites (B_{max}) in rat forebrain with little change in the dissociation constant (K_d). The blockade of brain [^{3}H]nicotine-binding sites induced by neosurugatoxin was not reversed by washing. Further, the toxin (10 μM) considerably accelerated the dissociation of [^{3}H]nicotine from its receptor sites initiated by nonlabeled (−)nicotine. These observations suggest that neosurugatoxin may allosterically regulate [^{3}H]nicotine binding rather than competing directly. In contrast to a marked inhibition of [^{3}H]nicotine binding, neosurugatoxin (100 nM–10 μM) had no effect on brain [^{3}H]quinuclidinyl benzilate binding. In conclusion, the present study has shown that [^{3}H]nicotine selectively labels nicotinic cholinergic receptors in rat brain and that neosurugatoxin is a potent noncompetitive antagonist of these receptors.

INTRODUCTION

It is known that nicotine has many pharmacological effects on the central nervous system in various species including humans. They include changes in behavioral and electrocortical activity (1–3), changes in body temperature (4), and twitching of the ears and salivation (5). Although most of these responses are probably mediated by the action of nicotine upon brain nicotinic cholinergic receptors (1, 3, 4), the pharmacological properties of these receptors are not totally clarified. This may be due to the absence of a potent antagonist which both biochemically binds receptor sites and blocks physiological responses. Radioactive α-bungarotoxin, a selective antagonist of neuromuscular nicotinic receptors, has been widely utilized by a number of investigators to identify central nicotinic receptors. However, there is an apparent dissociation between biochemical and physiological effects of this toxin. Furthermore, physiological and immunochemical studies have indicated that α-bungarotoxin may bind to sites distinct from nicotinic receptor sites in the mammalian brain (6–8). Consequently, Morley *et al.* (7, 8) have suggested that α-bungarotoxin is not the appropriate ligand for studying brain nicotinic receptors. The use of agonist ligands such as [^{3}H]nicotine and [^{3}H]acetylcholine to label nicotinic cholinergic receptors has been recently reported by several workers (9–13). In these studies, it has been shown that specific binding of both ligands to murine brain membranes was rapid, reversible, stereoselective, and of high affinity, while classical nicotinic antagonists, in contrast to agonists, were very poor inhibitors of agonist-binding sites.

NSTX,[1] isolated from Japanese ivory mollusc (*Babylonia japonica*) (14) has been suggested to be a specific antagonist of ganglionic nicotinic receptors (13, 15–17). Our previous study has demonstrated that low concentrations of NSTX decreased [^{3}H]nicotine-binding sites in rat forebrain membranes. The affinity of NSTX was at least 3 orders of magnitude higher than that of hexamethonium (13). Thus, NSTX might be a useful phar-

[1] The abbreviations used are: NSTX, neosurugatoxin; QNB, quinuclidinyl benzilate; DMPP, 1,1-dimethyl-4-phenylpiperazinium iodide; C_6, hexamethonium.

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macological probe for studies of nicotinic cholinergic receptors in the central nervous system. In the present study, we have characterized central nicotinic receptors by investigating in detail the inhibitory effect of NSTX on specific [^{3}H]nicotine binding in rat brain.

MATERIALS AND METHODS

Preparation of brain membrane fractions. Wistar rats (200–300 g) were sacrificed and the forebrain was dissected. To study the regional distribution of specific[^{3}H]nicotine binding, cerebral cortex, hippocampus, the thalamus (including hypothalamus), striatum, midbrain, cerebellum, and brainstem (pons + medulla oblongata) were dissected. The brain tissue was homogenized in 10 volumes of 20 mM Tris-HCl buffer (containing 118 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, and 1.2 mM $MgSO_4$, pH 7.5) in a Polytron. The crude membrane fraction of the rat brain was prepared according to the method described by Marks and Collins (10). Briefly, the homogenate was centrifuged at 38,000 × *g* for 20 min at 4° and the resulting pellet was suspended in distilled water. After a 1-hr incubation at 4°, the suspension was centrifuged and the pellet was washed by rehomogenization in 20 mM Tris-HCl buffer, followed by centrifugation. This pellet was finally resuspended in the buffer (15%, w/v) and utilized in [^{3}H]nicotine-binding assays.

[^{3}H]Nicotine-binding assays. In the routine assay of [^{3}H]nicotine binding, brain membrane fractions (800–900 μg of protein) were incubated with 20 nM (or 6 nM) [^{3}H]nicotine in a total volume of 250 μl of 20 mM Tris-HCl-containing buffer (composition identical to that of the buffer described above) at 37°. After 5 min, the reaction was terminated by dilution and the subsequent rapid filtration under vacuum through Whatman GF/B glass fiber filters. Each filter was rinsed four times with 4 ml of ice-cold buffer. The tissue-bound radioactivity was extracted from the filters overnight into 6 ml of scintillation fluid (2 liters of toluene, 1 liter of Triton X-100, 15 g of 2,5-diphenyloxazole, and 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene) and counted. Specific binding was defined as the difference in binding determined in the absence and presence of 10 μM (−)nicotine. The assays were conducted in duplicate. In a typical study, specific binding was more than 75% of total binding at 20 (35.1 Ci/mmol) or 6 nM (71.2 Ci/mmol) [^{3}H]nicotine.

In experiments where the dissociation kinetics of [^{3}H]nicotine were studied, rat forebrain membranes were incubated as described above with [^{3}H]nicotine. At the appropriate time, (−)nicotine (10 μM), NSTX (10 μM), or a mixture of (−)nicotine and NSTX was added. The incubation was terminated by filtration as previously described. Proteins were determined by the method of Lowry *et al.* (18), using bovine serum albumin as a standard. Muscarinic cholinergic receptors were measured using [^{3}H]QNB (19, 20).

Analysis of data. The analysis of binding data was performed as described previously (21–23). The dissociation constant (K_d) and maximal binding sites (B_{max}) for brain [^{3}H]nicotine binding were estimated by Scatchard analysis of the saturation data over the concentration range of 4–144 nM [^{3}H]nicotine (35.1 Ci/mmol). [^{3}H]Nicotine (3–32 nM) with higher specific activity (71.2 Ci/mmol) was used in an experiment for the determination of K_d and B_{max} in the absence and presence of NSTX (Table 2) and also in experiments of Figs. 4 and 6. The ability of cholinergic drugs to inhibit specific [^{3}H]nicotine binding was estimated by IC_{50} values which are the molar concentrations of unlabeled drugs necessary for competing for 50% of the specific radioligand binding (determined by log probit analysis). The pseudo-Hill slope (or coefficient) for an inhibition of [^{3}H]nicotine binding by cholinergic drugs was obtained from a pseudo-Hill plot. Statistical analysis of data was performed using a double-tailed Student's *t* test.

Materials. (±)[*pyrrolidinyl*-^{3}H]Nicotine (35.1 Ci/mmol), (±)[*N-methyl*-^{3}H]nicotine (71.2 Ci/mmol), and [^{3}H](−)QNB (40.2 Ci/mmol) were purchased from New England Nuclear Corp., Boston, MA. (+)Nicotine was kindly donated by Dr. T. Kisaki of Nippon Tobacco and Salt Corporation. Other drugs and materials were obtained from the pharmaceutical company of origin or commercial sources.

RESULTS

Central [^{3}H]nicotine binding. Specific binding of [^{3}H]nicotine to rat forebrain membranes increased linearly with increasing protein concentrations over the range of 300–1000 μg of protein per assay (data not shown). Assays were routinely conducted by using 800–900 μg of protein per assay. Fig. 1 illustrates specific binding of [^{3}H]nicotine at the concentrations of 4–144 nM in rat forebrain. Scatchard analysis (Fig. 1, inset) of the specific [^{3}H]nicotine-binding data suggested the existence of complex binding behavior. For binding analysis, the complexity was assumed to result from the existence of multiple populations of nicotine-binding sites. The [^{3}H]nicotine-binding data were fitted to a two-site mass action equation by nonlinear least squares regression analysis using the computer program NONLIN (23). Regression analysis gave values of 8.9 and 44.9 nM for the dissociation constant and values of 18.7 and 60.3 fmol/mg of protein for the capacities of these two binding sites, respectively. [^{3}H]Nicotine rapidly associated with its binding sites in rat forebrain and reached steady state by 2 min at 37° (Fig. 2). [^{3}H]Nicotine binding remained at steady state for at least 20 min and dissociation of the binding could be monitored by the addition of 10 μM (−)nicotine ($t_{1/2}$ = 0.4 min). The association rate constant (k_{+1}) was calculated to be 6.54 ± 1.12 × 10^7 M^{-1}, min^{-1}

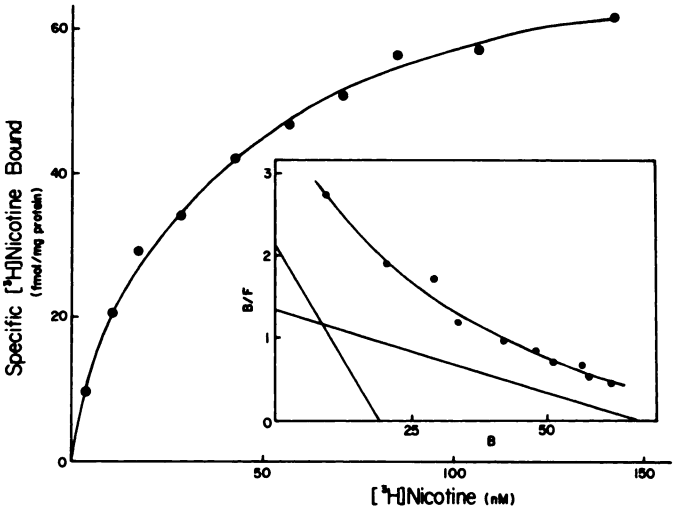

FIG. 1. *Specific [^{3}H]nicotine binding to rat forebrain as a function of increasing concentrations of [^{3}H]nicotine*

Specific [^{3}H]nicotine binding was experimentally determined as the difference between total and nonspecific binding in parallel assays in the absence and presence of 10 μM (−)nicotine. Each point represents the average of six to nine determinations. The nonspecific binding fits to the linear equation: $y = 0.23x - 1.40$ (correlation coefficient, r = 0.99), where y = fmol/mg of protein and x = [^{3}H]nicotine concentrations (nanomolar). The inset shows a Scatchard plot derived from the specific [^{3}H]nicotine-binding data. Ordinate, bound over free [^{3}H]nicotine (microliters per milligram of protein). Abscissa, [^{3}H]nicotine bound (femtomoles per milligram of protein). The nonlinear least squares regression analysis of [^{3}H]nicotine-binding data revealed that a two-site model gave a slightly improved fit over a one-site model as demonstrated by a decrease in the sum (20 vs. 24) of residuals (a measure of the variability between the fitted regression and the actual data).

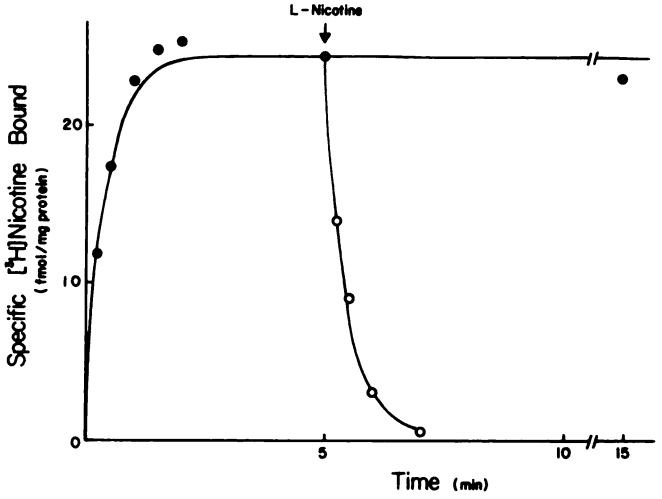

FIG. 2. *Time course of association and dissociation of specific [³H] nicotine binding to rat forebrain*

[³H]Nicotine (20 nM) binding was quantified as a function of time from the addition of ligand. At the arrow, 10 μM (−)nicotine was added to a parallel set of tubes and the dissociation of the [³H]nicotine·receptor complex was monitored. Ordinate, specific [³H]nicotine bound (femtomoles per milligram of protein). Abscissa, time (minutes). Each point represents the average of two to four determinations.

and the dissociation rate constant (k_{-1}) was calculated to be 1.70 ± 0.12 min^{-1} (mean ± SE, n = 3).

Characteristics expected of physiological nicotinic receptors include stereoselectivity and pharmacological specificity. The competition profiles observed for cholinergic drugs competing for brain [³H]nicotine binding are shown in Fig. 3 and calculated IC_{50} values and pseudo-Hill slopes are shown in Table 1. Cholinergic agonists competed with [³H]nicotine for the binding sites in the order (−)nicotine > DMPP > lobeline > (+)nicotine > carbamylcholine ≫ oxotremorine. Thus, [³H]nicotine-binding sites exhibited 1000–7000 times higher affinity for nicotinic agonists than a muscarinic agonist, oxotremorine. Stereoselectivity was demonstrated by the 25-fold greater potency of (−)nicotine compared with its (+) isomer. In addition, the effect of acetylcholine on [³H] nicotine binding was determined in forebrain tissues which had been incubated twice for 5 min with diisopropyl fluorophosphate (10 μM). Acetylcholine was a potent competitor of [³H]nicotine-binding sites as demonstrated by the IC_{50} value of 175 ± 23 nM (n = 5). Cholinergic antagonists showed much weaker binding affinity than nicotinic agonists. The potencies of cholinergic drugs in competing for [³H]nicotine binding to rat brain resemble values determined for binding to mouse brain (10).

Displacement of [³H]nicotine binding by NSTX. NSTX at the concentrations of 0.3 nM–10 μM decreased the specific binding of [³H]nicotine (20 nM) in rat forebrain (Fig. 3) and the toxin was much more potent than classical cholinergic antagonists such as hexamethonium (Table 1). At lower (2 and 6 nM) and higher (86 and 144 nM) concentrations of [³H]nicotine, NSTX (300 nM) showed a similar inhibition of the specific binding as seen at 20 nM of the radioligand (extent of inhibition by NSTX at 2, 6, 86, and 144 nM [³H]nicotine was 51.9 ± 6.5, 58.1 ± 4.3, 51.2 ± 4.6, and 51.1 ± 3.4%, respectively, n = 3–5). This finding may indicate a similar inhibition by NSTX of two [³H]nicotine-binding sites identified in Fig. 1.

Interestingly, NSTX demonstrated a biphasic displacement curve on brain [³H]nicotine binding: the inhibition at low concentrations (0.3–100 nM) was followed by a further inhibition at relatively higher concentrations (0.3–10 μM). The pseudo-Hill slopes for the inhibition of [³H]nicotine binding by NSTX and cholinergic drugs were calculated (Table 1). Although pseudo-Hill slopes

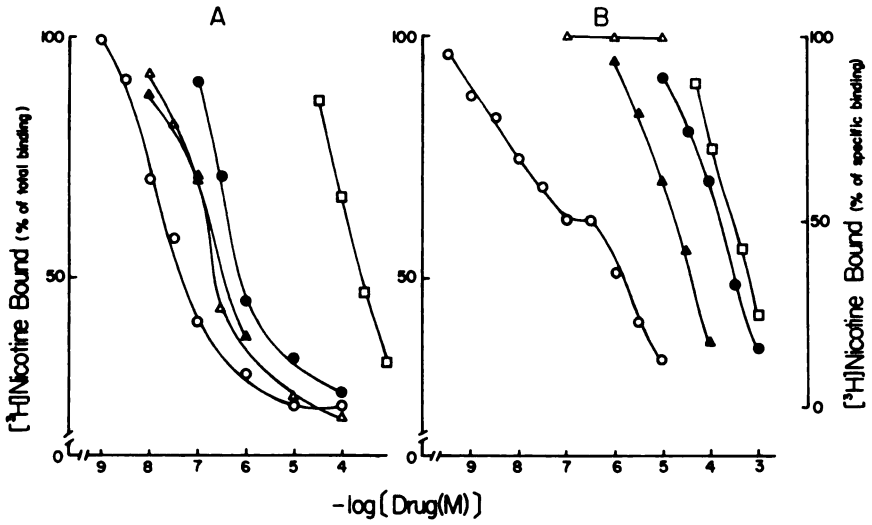

FIG. 3. *Inhibition of specific [³H]nicotine binding to rat forebrain by neosurugatoxin and cholinergic drugs*

The inhibition of [³H]nicotine binding by cholinergic agonists and antagonists was determined by incubating [³H]nicotine (20 nM) with three to ten concentrations of cholinergic agonists (A: ○, (−)nicotine; △, lobeline; ▲, DMPP; ●, (+)nicotine; □, oxotremorine) and antagonists (B: ○, NSTX; ▲, (+)tubocurarine; ●, hexamethonium; □, atropine; △, α-bungarotoxin). The scale on the left side is [³H]nicotine bound expressed as a percentage of the total [³H]nicotine binding in the absence of any displacing agent. The scale on the right is [³H]nicotine bound expressed as a percentage of the specific [³H]nicotine binding which was displaced by 10 μM (−)nicotine. All solutions of drugs were freshly made before use. Each point represents the average of three to eight determinations.

TABLE 1

Inhibition of specific [^{3}H]nicotine binding by cholinergic drugs in rat forebrain

Drug inhibition studies were conducted as described in Fig. 3. The IC_{50} values and Hill slopes were calculated as described in Materials and Methods. The values are means ± standard error of three to eight determinations. The effect of noncholinergic drugs on brain [^{3}H]nicotine binding was examined. Strychnine and piperidine inhibited the [^{3}H]nicotine binding, and their IC_{50} values were 27,900 ± 1,100 and 8,980 ± 1,060 nM, respectively. Other agents that were ineffective or that gave less than 10% inhibition at 100 μM: adrenaline, phentolamine, propranolol, pentobarbital, phenobarbital, caffeine, picrotoxin, morphine, pentylenetetrazol, *l*-glutamate, aminopyrine, procaine, amitryptyline, spiroperidol, flunitrazepam, γ-aminobutyric acid, dopamine, imidazole, bicuculline, adenosine, meprobamate, mephenesine, and indomethacin.

Drugs	IC_{50} values	Hill slopes (or coefficients)
	nM	
Cholinergic agonists		
(−)Nicotine	17 ± 4	0.78 ± 0.07
(+)Nicotine	428 ± 81	1.04 ± 0.21
Lobeline	126 ± 8	0.89 ± 0.07
DMPP	100 ± 12	0.85 ± 0.09
Acetylcholine	175 ± 23	0.80 ± 0.09
Carbamylcholine	963 ± 64	0.84 ± 0.02
Oxotremorine	119,800 ± 9,100	0.96 ± 0.12
Cholinergic antagonists		
Neosurugatoxin	78 ± 9	0.44 ± 0.08
(+)Tubocurarine	12,900 ± 2,000	0.93 ± 0.11
Hexamethonium	152,000 ± 24,000	0.89 ± 0.06
Pentolinium	105,000 ± 21,000	0.89 ± 0.21
Decamethonium	11,000 ± 1,200	1.00 ± 0.20
Mecamylamine	474,000 ± 172,000	0.39 ± 0.05
Atropine	259,000 ± 11,000	0.91 ± 0.14
α-Bungarotoxin	>10,000	

for most cholinergic drugs were close to 1, the value for biphasic inhibition by NSTX was 0.44. Thus, we can define high and low affinity components of [^{3}H]nicotine binding for NSTX as binding abolished or remaining, respectively, in the presence of 100 nM NSTX. There were equivalent quantities of these two components in rat forebrain areas. This high affinity component of [^{3}H]nicotine binding for NSTX was inhibited 50% by about 3 nM, and the low affinity component was inhibited 50% by about 2 μM.

The radioligand-binding technique can be used to determine whether a ligand interacts with receptor sites in a competitive or noncompetitive manner. Equilibrium binding isotherms were determined for [^{3}H]nicotine binding in the presence of various concentrations of NSTX, and the results of these experiments are shown in Table 2. Because fewer concentrations of [^{3}H]nicotine were utilized in this experiment than in the experiment shown in Fig. 1, two binding sites for [^{3}H]nicotine by the nonlinear least squares regression analysis were not determined. In the presence of 3 nM, 100 nM, and 1 μM NSTX, there was a concentration-dependent (28, 54, and 71%, respectively) loss of [^{3}H]nicotine-binding sites (B_{max}) in rat forebrain with no significant change in K_d. On the other hand, hexamethonium (300 μM) significantly increased the K_d value for brain [^{3}H]nicotine binding without a change in the B_{max} value.

TABLE 2

Effect of neosurugatoxin and hexamethonium on K_d and B_{max} values of specific [^{3}H]nicotine binding to rat forebrain

Scatchard analysis for specific [^{3}H]nicotine (3–32 nM) binding to rat forebrain in the absence (control) and presence of 3, 100, and 1000 nM NSTX was performed. [^{3}H]Nicotine with higher specific activity (71.2 Ci/mmol) was used in this experiment. The values are means ± standard error of five to seven determinations. Asterisks show a significant difference from the control value (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Drugs	Specific [^{3}H]nicotine binding	
	K_d	B_{max}
	nM	*fmol/mg protein*
Control	15.3 ± 1.4	83.7 ± 4.9
NSTX		
3 nM	17.5 ± 1.9	59.9 ± 10.4*
100 nM	13.6 ± 1.6	38.6 ± 3.9**
1000 nM	15.0 ± 1.9	24.5 ± 2.7***
C_6 (300 μM)	48.4 ± 5.1***	88.1 ± 9.0

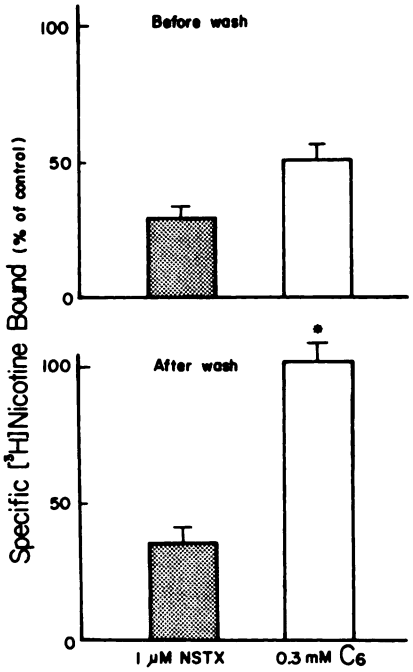

FIG. 4. *Effect of neosurugatoxin and hexamethonium on specific [^{3}H]nicotine binding to rat forebrain*

Rat forebrain membranes were preincubated with 1 μM NSTX, 300 μM hexamethonium, or without added antagonists (control) for 5 min at 37°. Ordinate, specific [^{3}H]nicotine binding expressed as a percentage of the specific binding in the absence of any antagonists. Top, specific [^{3}H]nicotine binding was assayed in the preincubated membranes as described in Materials and Methods. Bottom, the preincubated membranes were washed three times in 20 mM Tris-HCl buffer (pH 7.5) by centrifugation and resuspension. The washed membranes were then assayed for [^{3}H]nicotine binding. Each column represents the average (±standard error) of four to six determinations. The asterisk shows a significant difference from the value in the hexamethonium-treated membranes before washing ($p < 0.01$).

In the presence of 1 μM NSTX or 300 μM hexamethonium, 50–70% of specific [^{3}H]nicotine binding to rat forebrain membranes was inhibited as shown in Figs. 3 and 4 (upper panel). When membranes previously exposed to 300 μM hexamethonium for 5 min were washed

extensively and subsequently assayed for [^{3}H]nicotine binding, the specific binding was restored to the level of the untreated membranes. By contrast, when membranes exposed to 1 μM NSTX for 5 min were washed under the same conditions as the hexamethonium-treated membranes, the binding of [^{3}H]nicotine was not restored but was apparently blocked irreversibly (Fig. 4, lower panel).

In contrast to its marked inhibition of [^{3}H]nicotine binding, NSTX at 100 nM–10 μM had no effect on brain [^{3}H]QNB binding (Fig. 5). In the same experiment, atropine was a potent inhibitor of the [^{3}H]QNB-binding sites.

Regional variation of [^{3}H]nicotine-binding sites. The relative density of specific [^{3}H]nicotine binding was determined in seven brain areas of the rat. The relatively higher levels of [^{3}H]nicotine binding were found in the thalamus, cerebral cortex, and striatum; the lowest were in the cerebellum (Table 3). The regional distribution of [^{3}H]nicotine-binding sites in rat brain agrees well with the distribution of [^{3}H]acetylcholine-binding sites determined in the presence of atropine (11).

To determine the regional distribution of high and low affinity components of [^{3}H]nicotine binding for NSTX in rat brain, the inhibition by 100 nM NSTX of specific [^{3}H]nicotine binding in each region was compared. This concentration of the toxin was shown to occupy most of the high affinity component with little influence on the low affinity component in rat forebrain (Fig. 3). As shown in Table 3, NSTX showed a greater inhibition of specific [^{3}H]nicotine binding in the cerebral cortex, hippocampus, thalamus, and striatum than the midbrain, cerebellum, and brainstem. In three brain regions (striatum, thalamus, and brainstem) examined, NSTX led to a biphasic inhibition of [^{3}H]nicotine binding with pseudo-Hill slopes less than 1, as observed in the forebrain. The relative proportion of high and low affinity components

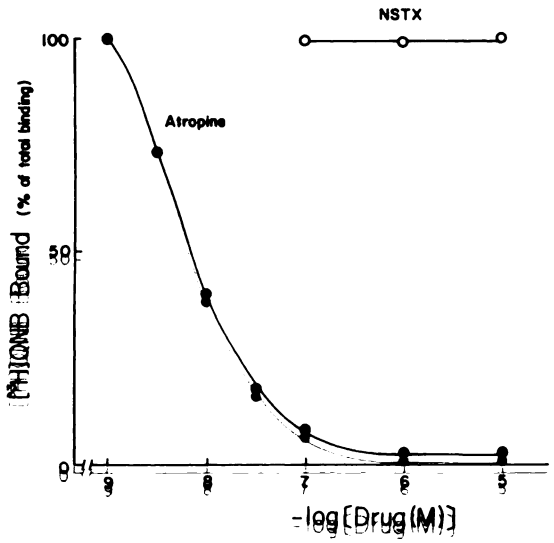

FIG. 5. *Effect of neosurugatoxin and atropine on brain [^{3}H]QNB binding*

Rat forebrain membranes (approximately 150 μg protein per assay) were incubated with 0.25 nM [^{3}H]QNB in a total volume of 2 ml of 50 mM Na/K phosphate buffer (pH 7.4) for 60 min at 37°. Ordinate, [^{3}H]QNB bound expressed as a percentage of the total [^{3}H]QNB binding in the absence of any displacing agent. Each point represents the average of three determinations.

TABLE 3

Regional distribution of specific [^{3}H]nicotine binding in rat brain and inhibition by 100 nM neosurugatoxin

The determination of specific [^{3}H]nicotine (17 nM) binding in the absence and presence of 100 nM NSTX was performed as described in Materials and Methods. The values are means ± standard error of three to nine determinations.

Brain region	Specific [^{3}H]nicotine binding	Inhibition by 100 nM NSTX
	fmol/mg protein	%
Cerebral cortex	21.3 ± 1.4	49.5 ± 2.0
Hippocampus	9.3 ± 0.8	52.1 ± 3.7
Thalamus	33.4 ± 2.1	47.9 ± 1.6
Striatum	20.0 ± 0.4	47.4 ± 1.2
Midbrain	16.9 ± 0.8	37.6 ± 1.4
Cerebellum	8.9 ± 0.8	38.4 ± 4.1
Brainstem	12.8 ± 0.6	39.9 ± 2.2

for NSTX (as defined by their sensitivity to 100 nM NSTX) appeared to differ among these brain areas. The striatum and thalamus possessed a similar number of the two components, while in the brainstem there was a greater proportion (60–70%) of the low affinity component. The concentration of NSTX reducing 50% of these two components in each brain region was of a similar magnitude as observed in the forebrain (3–5 nM for the high affinity component; 1–2 μM for the low affinity component).

Dissociation of [^{3}H]nicotine binding by NSTX. The time course of dissociation at 25° of the specifically bound [^{3}H]nicotine in the presence of (−)nicotine and NSTX in rat forebrain was examined. The dissociation rate monitored following addition of 10 μM (−)nicotine was essentially linear when plotted on a semilogarithmic scale (ln $[B/B_e]$ versus time), indicating a first order process (Fig. 6). When 10 μM NSTX instead of (−)nicotine was used to monitor dissociation of the specifically bound [^{3}H]nicotine, on the other hand, the dissociation rate was biphasic (Fig. 7). The half-lives for the fast and slow components were about 2.5 and 12 min, respectively.

If NSTX noncompetitively inhibited [^{3}H]nicotine binding rather than competing directly as seen in Table 2, then one would expect an influence of NSTX upon [^{3}H]nicotine dissociation. Accordingly, we determined the effect of NSTX on the dissociation of [^{3}H]nicotine from its receptor sites initiated by nonlabeled (−)nicotine. Nicotine (10 μM) in the presence of 10 μM NSTX produced near doubling of the dissociation rate of [^{3}H]nicotine (Fig. 6). Thus, NSTX considerably accelerated the dissociation of [^{3}H]nicotine from its receptor sites by (−)nicotine.

DISCUSSION

The major findings of the present study are that: 1) [^{3}H]nicotine selectively labels nicotinic cholinergic receptors in rat brain; 2) NSTX may be a very potent inhibitor of these nicotinic receptors; 3) there is a regional variation of [^{3}H]nicotine-binding sites in rat brain; and 4) there is a pharmacological resemblance between brain and ganglionic nicotinic receptors.

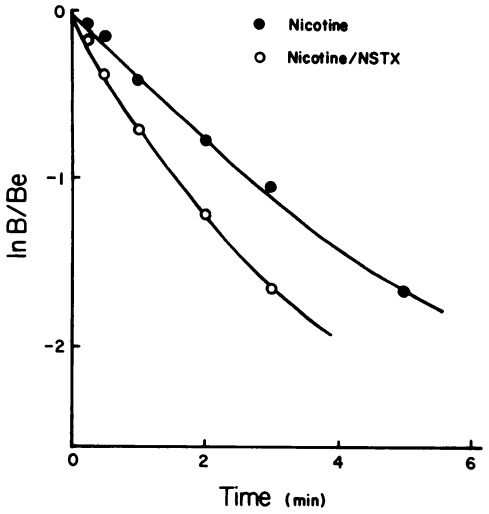

FIG. 6. *Dissociation of [^{3}H]nicotine from rat forebrain membranes at 25° and the effect of neosurugatoxin*

The dissociation of specifically bound [^{3}H]nicotine was measured at 25° following incubation with 6 nM [^{3}H]nicotine to equilibrium (5 min). At time zero, 10 μM (−)nicotine or a mixture of (−)nicotine (10 μM) and NSTX (10 μM) was added to the incubation mixture and the reactions were terminated by a rapid filtration at various times. Ordinate, ln B/B_e (B = the amount of [^{3}H]nicotine specifically bound at time t; B_e = the amount bound at equilibrium). Abscissa, time (minutes). Linear regression analysis of the semilogarithmic data yielded a straight line (dissociation rate constant: 0.33 (●) and 0.60 min^{-1} (○); correlation coefficient, r = 0.97 and 0.96). Each point represents the average of five determinations.

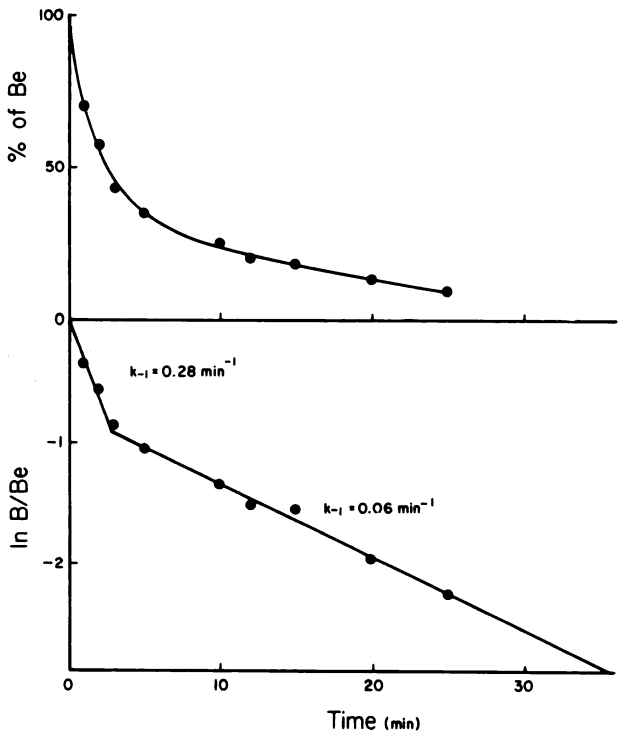

FIG. 7. *Time course of dissociation for specific [^{3}H]nicotine binding to rat forebrain by neosurugatoxin*

The dissociation of specifically bound [^{3}H]nicotine was measured at 25° following incubation with 20 nM [^{3}H]nicotine to equilibrium (5 min). At time zero, 10 μM NSTX was added to the incubation mixture and the reactions were terminated by a rapid filtration at various times. Ordinate, percentage of B_e (upper panel) and ln B/B_e (lower panel) (B = the amount of [^{3}H]nicotine specifically bound at time t; B_e = the amount bound at equilibrium). Abscissa, time (minutes). Linear regression analysis of the semilogarithmic data yielded two straight lines of slopes (dissociation rate constant: 0.28 and 0.06 min^{-1}; correlation coefficient, r = 0.99 and 0.99). Each point represents the average of three determinations.

The specific binding of [^{3}H]nicotine to rat forebrain membranes was saturable, of high affinity, and reversible. Also, the brain [^{3}H]nicotine-binding sites exhibited pharmacological specificity as well as stereoselectivity for nicotinic agonists. (−)Nicotine, DMPP, and lobeline were 3 orders of magnitude more potent as competitive agents than a muscarinic agonist, oxotremorine. The maximal binding capacity, pharmacological specificity, and regional distribution of brain [^{3}H]nicotine binding obtained in the present study agree well with those of [^{3}H]acetylcholine binding in rat brain in the presence of atropine (11), indicating that both ligands may bind to the same nicotinic receptor sites. Scatchard plots of [^{3}H]nicotine-binding data were curvilinear (Fig. 1). Nonlinear Scatchard plots of brain nicotinic receptor binding were previously reported by other investigators (9, 12). These observations have suggested that there exists more than one population of binding sites for nicotinic agonists in rat brain.

NSTX has been shown to be a selective antagonist of central nicotinic receptor sites because this toxin markedly inhibited specific [^{3}H]nicotine binding to rat forebrain with no effect on the binding of [^{3}H]QNB to muscarinic receptors. However, the antagonism by NSTX does not appear to be competitive in that the toxin reduced in a concentration-dependent manner the number of [^{3}H]nicotine-binding sites with little change in the apparent affinity. This conclusion might be supported by the physiological observation in the guinea pig isolated ileum that low concentrations of NSTX caused a marked depression of maximal contractile response to nicotine, suggesting a noncompetitive antagonism (13). By contrast, hexamethonium inhibited the brain [^{3}H]nicotine binding in a competitive manner, as demonstrated by a reduction only in the apparent affinity for [^{3}H]nicotine-binding sites. The blockade of [^{3}H]nicotine-binding sites induced by NSTX was not reversed by washing, whereas the blockade of binding sites by hexamethonium was easily reversible under these conditions. These results indicate that, unlike hexamethonium, NSTX may irreversibly inactivate the nicotinic receptor sites in rat brain. Thus, the nonequilibrium nature by NSTX may be due to an irreversible (or slowly dissociating) blockade of [^{3}H]nicotine-binding sites. Schwartz and Kellar (24) reported that a nonequilibrium blockade of brain [^{3}H]acetylcholine-binding sites in the presence of atropine was seen by a reduction of the disulfide bond with dithiothreitol and it was not reversed by washing. Although the mechanism of nicotinic receptor inactivation by NSTX in rat brain is not clear, it seems likely that this toxin reacts with the nicotinic receptors or some adjacent grouping to form a stable bond. In experiments where the dissociation of [^{3}H]nicotine binding was studied, NSTX considerably accelerated the disso-

ciation of [^{3}H]nicotine from its receptor sites by (−)nicotine. The alteration in the dissociation kinetics of [^{3}H]nicotine in the presence of NSTX supports the concept that this toxin may allosterically regulate [^{3}H]nicotine binding rather than competing directly.

The pattern of inhibition of [^{3}H]nicotine binding by NSTX was distinctive, exhibiting high (nanomolar) affinity and low (micromolar) affinity components for NSTX, and there was a similar density of these two components in rat forebrain. In this connection, it is interesting to note that, when NSTX was used to dissociate the specifically bound [^{3}H]nicotine in rat forebrain, the fast and slow dissociation components were observed. The distribution of high and low affinity components of [^{3}H]nicotine binding for NSTX in different brain regions was studied. NSTX at the concentration which occupied most of the high affinity component caused less inhibition of [^{3}H]nicotine binding in the midbrain, cerebellum, and brainstem than in the cerebral cortex, hippocampus, thalamus, and striatum. Further, the displacement curves of specific [^{3}H]nicotine binding by NSTX have shown that the brainstem may contain a greater proportion of low affinity sites than do the thalamus and striatum. Consequently, a regional study of [^{3}H]nicotine-binding sites indicates that there may be some difference in the relative density of high and low affinity components of [^{3}H]nicotine binding for NSTX between brain areas. Although the physiological relevance of these two components is not clear, our preliminary experiment has shown that chronic diisopropyl fluorophosphate treatment tended to cause a selective loss of high affinity components of [^{3}H]nicotine binding for NSTX in rat forebrain (25), suggesting a differential regulation of these components by central cholinergic activity.

Previous studies have demonstrated that extracts of the Japanese ivory mollusc (*B. japonica*) which contained NSTX exerted a potent blocking action at nicotinic receptors in autonomic ganglia of cats and rats, with little effect on skeletal muscle receptors (15–17). In addition, the antinicotinic effect of NSTX in the guinea pig ileum was at least 3 orders of magnitude greater than that of hexamethonium (13). These physiological data indicate that NSTX may be a neurotoxin with a selective affinity for ganglionic nicotinic receptors. In the present study, it has been shown that, in contrast to a marked inhibition by NSTX, α-bungarotoxin was not competitive at [^{3}H]nicotine-binding sites in rat brain. Similar observations with α-bungarotoxin were previously described in brain membranes (9–11). Taken together, the results of our binding experiments with two selective neurotoxins may provide the biochemical evidence for a closer pharmacological resemblance of brain nicotinic receptors to the ganglionic type of nicotinic receptors rather than to the neuromuscular receptors. This finding may be supported by electrophysiological and behavioral observations that central effects of nicotinic agonists were effectively antagonized by ganglionic blocking agents but not by α-bungarotoxin (6, 7, 26, 27).

We and others showed that classical nicotinic antagonists such as hexamethonium and mecamylamine were poor inhibitors at [^{3}H]nicotine- and [^{3}H]acetylcholine-binding sites in brain membranes (9–11, 13). In our study, hexamethonium was approximately 3 orders of magnitude less potent in competing with [^{3}H]nicotine binding than was NSTX, an observation which agrees with physiological data in the guinea pig ileum (13). Moreover, in our recent experiment, a subcutaneous injection of NSTX at the dosage of 4 nmol/kg markedly reduced (90%) the nicotine-induced antinociceptive effect in mice, and the similar inhibitory effect was observed by the injection of mecamylamine of 5 μmol/kg.[2] Accordingly, the observed low affinity of classical nicotinic antagonists for central nicotinic receptor sites appears to be correlated with their potency at influencing the physiological responses elicited by nicotine. In conclusion, we have demonstrated that NSTX is a selective, albeit noncompetitive, antagonist of central [^{3}H]nicotine-binding sites and, thus, the toxin could be a useful pharmacological probe for studies of nicotinic cholinergic receptors.

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