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[^{3}H]Acetylcholine and [^{3}H](−)Nicotine Label The Same Recognition Site In Rat Brain

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SUMMARY

High affinity binding sites for [^{3}H]acetylcholine and [^{3}H](−)nicotine in rat brain were compared with respect to key characteristics, any one of which should distinguish them if they are different. The density of binding sites for each ligand varied approximately 4-fold in five areas of rat forebrain, but in each of these areas and in human cerebral cortex as well, the densities of [^{3}H] acetylcholine- and [^{3}H](−)nicotine-binding sites were indistinguishable. The affinity of [^{3}H](−)nicotine was higher than that of [^{3}H]acetylcholine, but nicotinic cholinergic drugs competed for the sites labeled by the two ligands with similar affinities; and in each case, the site labeled displayed marked stereoselectivity for the enantiomers of nicotine. The binding of [^{3}H]acetylcholine and [^{3}H](−)nicotine was decreased to the same extent by preincubation of tissues with dithiothreitol, and the binding was restored by subsequent treatment with 5,5′-dithiobis-2-nitrobenzoic acid, indicating that a disulfide bond is required at or near the binding site for each ligand. Treatment of rats with nicotine for 10 days increased the density of binding sites for both ligands, and treatment with the cholinesterase inhibitor soman for 9 days decreased the density of binding sites for both ligands. Taken together, these results indicate that [^{3}H]acetylcholine and [^{3}H](−)nicotine bind to the same nicotinic cholinergic recognition site in rat brain.

In mammalian brain, binding sites with characteristics of agonist recognition sites of nicotinic cholinergic receptors can be labeled with [^{3}H]ACh (1–3) or [^{3}H]nicotine (4–6). α-Bungarotoxin, an important ligand for studying nicotinic cholinergic receptors in muscle and electric tissue, also binds to sites in mammalian brain; however, these sites appear to be different from those labeled by [^{3}H]ACh or [^{3}H]nicotine in brain (1, 5, 7, 8).

The [^{3}H]ACh and the high affinity [^{3}H]nicotine recognition sites in brain appear to be similar with respect to their high affinities for nicotinic agonists, their low affinities for most nicotinic antagonists (1, 4–6), their brain region distribution (7), and, in some studies, their regulation by repeated or chronic administration of nicotine and cholinesterase inhibitors (2, 3, 6, 9). However, several differences in the pharmacology, regional distribution in adult and developing brain, and *in vivo* regulation of [^{3}H]ACh- and [^{3}H]nicotine-binding sites have also been reported which suggested that these ligands might not label the same recognition site (10–18), and further, that receptors with high affinity for nicotine in brain might be noncholinergic (10, 13–15).

Differences in these basic characteristics of the recognition sites for [^{3}H]ACh and [^{3}H]nicotine could indicate that receptors with high affinity for nicotine in brain are not cholinergic—or at least, not identical to the sites that bind [^{3}H]ACh with high affinity. However, it is possible that the reported differences in the [^{3}H]ACh- and [^{3}H]nicotine-binding sites stem from variations in the assay conditions used by different laboratories and, in most cases, from the use of racemic [^{3}H]nicotine rather than the naturally occurring (−)-isomer as the binding ligand.

Identification of the receptor in brain that binds nicotine with high affinity is important because it could have implications for the mechanisms of action of nicotine on neurotransmission in brain and for mechanisms related to nicotine dependence. In addition, recently the densities of both nicotinic [^{3}H]ACh and [^{3}H](−)nicotine recognition sites have been found to be markedly decreased in brains from patients who have died with Alzheimer's disease (19–23). Thus, identification of [^{3}H](−)nicotine recognition sites in brain as cholinergic or noncholinergic could aid in understanding some of the neuronal deficits in this disease.

We have compared the binding characteristics and regulation of [^{3}H]ACh and [^{3}H](−)nicotine recognition sites in rat brain under identical assay conditions.[1] In addition, we have measured the binding of these two ligands in human cerebral cortex. The results indicate that [^{3}H]ACh and [^{3}H](−)nicotine label the same site in brain.

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[1] A preliminary report of this work has been presented (24).

ABBREVIATIONS: [^{3}H]ACh, [^{3}H]acetylcholine; DTT, dithiothreitol; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; DBE, dihydro-β-erythroidine.

Materials and Methods

Tissues. Male Sprague-Dawley rats (250–300 g) were housed in a light- and temperature-controlled room (12-hr light cycle; 22°) and had free access to food and water. They were killed by decapitation and the brains were dissected on ice, frozen on dry ice, and stored at −70° until assayed. Human cerebral cortex was obtained from the Brain Resource Center of the Johns Hopkins University School of Medicine. These tissues were from neurologically normal patients (mean age 64 years) and were frozen within 13 hr of death.

Binding assays. Binding sites for both [^{3}H]ACh and [^{3}H](−)nicotine were measured as described previously for [^{3}H]ACh (1). Briefly, tissues were suspended in 50 mM Tris-HCl buffer containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 1.5 μM atropine sulfate (pH 7.4 at 0°) and homogenized with a Brinkmann Polytron (setting 6, 10 sec). The homogenates were washed twice by centrifugation at 48,000 × *g* for 10 min with intermediate homogenization in fresh buffer. Diisopropyl fluorophosphate (200 μM), a cholinesterase inhibitor, was added to the final volume of homogenates prior to their addition to assay tubes. Aliquots of homogenate equivalent to 5–10 mg of original tissue weight (250–500 μg of protein) were added to assay tubes containing the indicated concentrations of [^{3}H]ACh or [^{3}H](−)nicotine and, where indicated, drugs. Assays were carried out in sextuplicate, with half of the tubes containing 100 μM carbachol to determine nonspecific binding. After incubating at 0° for 45 min, the reaction was stopped by addition of 4 ml of cold buffer and rapid vacuum filtration through Whatman GF/C filters that had been treated with 0.05% polyethyleneimine to reduce displaceable binding to the filters (1). The filters were washed three times with cold buffer, placed in liquid scintillation fluid (Safety Solve; RPI, Mount Prospect, IL), and counted by liquid scintillation spectrometry. Specific binding was defined as the difference between total binding and nonspecific binding.

***In vitro* modification of disulfide bonds.** To study *in vitro* effects of reduction of disulfide bonds, rat cerebral cortex was homogenized in buffer and washed once by centrifugation. The homogenates were then preincubated in buffer (pH 8.0) for 30 min at 0° in the absence or presence of 3 mM DTT, which reduces disulfide bonds. The homogenates were washed by centrifugation in fresh buffer and then preincubated a second time at 0° for 30 min in the absence or presence of DTNB, which reoxidizes sulfhydryl groups to disulfide bonds. The homogenates were then washed twice by centrifugation in fresh buffer, and [^{3}H]ACh- and [^{3}H](−)nicotine-binding sites were measured as above.

***In vivo* regulation.** To study *in vivo* regulation, rats were given subcutaneous injections of nicotine bitartrate dihydrate (1.8 mg/kg = 0.59 mg/kg free base = 3.6 μmol/kg) dissolved in water twice daily for 10 days, or soman (60 μg/kg), a cholinesterase inhibitor, once daily for 9 days. Control rats received injections of water in equivalent volumes (1 ml/kg). The rats were sacrificed 1 day after the last injections and the brains were dissected and frozen as above until assayed.

Data analyses. Binding constants for [^{3}H]ACh and [^{3}H](−)nicotine were derived from linear regression analyses of Scatchard plots and Hill plots, both of which indicated that each ligand was binding to a single class of sites. IC_{50} values of drugs competing for [^{3}H]ACh- and [^{3}H](−)nicotine-binding sites in the cerebral cortex were calculated from Hill plots and converted to K_I values using the Cheng-Prusoff equation (25). This conversion is accurate only when Hill coefficients are close to 1. In these studies the Hill coefficients of competing drugs were 0.7 to 1.1, and because the Hill coefficients from both [^{3}H]ACh and [^{3}H](−)nicotine saturation binding studies were very close to 1 and the Scatchard plots did not reveal evidence of more than one class of sites, the conversion was considered to be useful for purposes of comparison. Protein was measured by the method of Lowry *et al.* (26). Statistical comparisons between two means were made by Student's *t* test.

Materials. [^{3}H]ACh (80 Ci/mmol) with a purity of 97% or higher was synthesized by acetylation of [^{3}H]choline (80 Ci/mmol); New England Nuclear Corp.) as described previously (1). [^{3}H](−)Nicotine (71–76 Ci/mmol) was purchased from New England Nuclear Corp. Nicotine bitartrate dihydrate was purchased from K & K Laboratories Inc. (Plainview, NY). DBE and mecamylamine were kind gifts from Merck Sharp & Dohme Research Laboratories, and (+)nicotine was a kind gift from Dr. B. R. Martin (Medical College of Virginia). Soman was supplied by the United States Army Medical Research and Development Command, Fort Detrick, MD. All other drugs and chemicals were from commercial sources.

Results

Binding of [^{3}H]ACh and [^{3}H](−)nicotine in brain. In rat cerebral cortex, specific binding of both [^{3}H]ACh and [^{3}H](−)nicotine is 70–80% of the total binding at concentrations that occupy up to 67% of the receptor-binding sites (Fig. 1). Hill coefficients (n_H) of the binding of both [^{3}H]ACh and [^{3}H](−)nicotine are close to 1 (Fig. 1, a and b, *insets*), suggesting that each ligand binds to a single class of sites under these assay conditions. The K_d for [^{3}H]ACh is approximately 11 nM and that for [^{3}H](−)nicotine is approximately 3.5 nM (Fig. 2, Table 1). Whereas the affinity of (−)nicotine is approximately 3 times higher than that of the endogenous ligand, the densities of the recognition sites for the two ligands in rat cerebral cortex

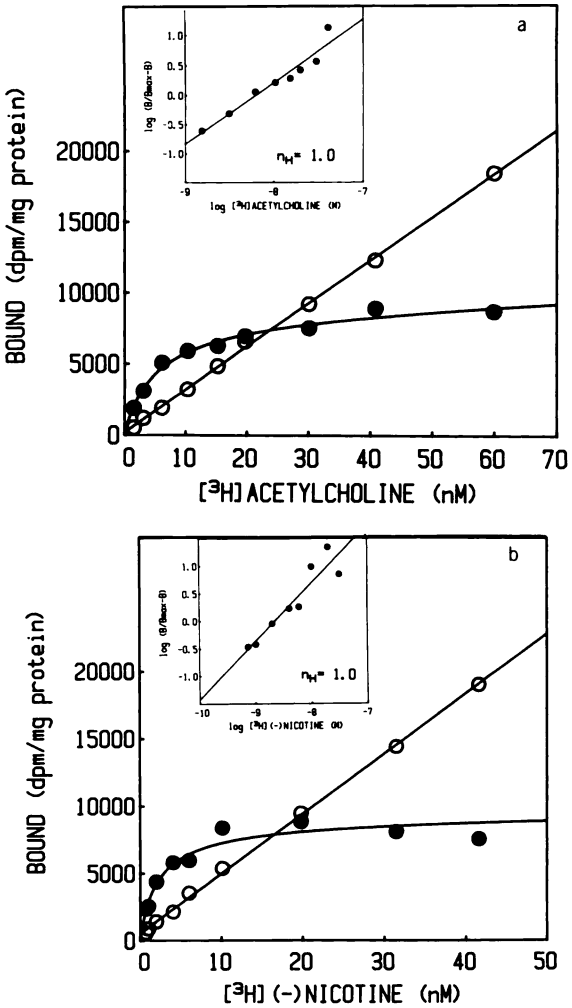

Fig. 1. Saturation analyses of [^{3}H]ACh (a) and [^{3}H](−)nicotine (b) in rat cerebral cortex. Nonspecific binding (○) was determined in the presence of 100 μM carbachol. Specific binding (●) is the difference between total binding and nonspecific binding. *Insets* are Hill plots of the specific binding of [^{3}H]ACh (a) and [^{3}H](−)nicotine (b).

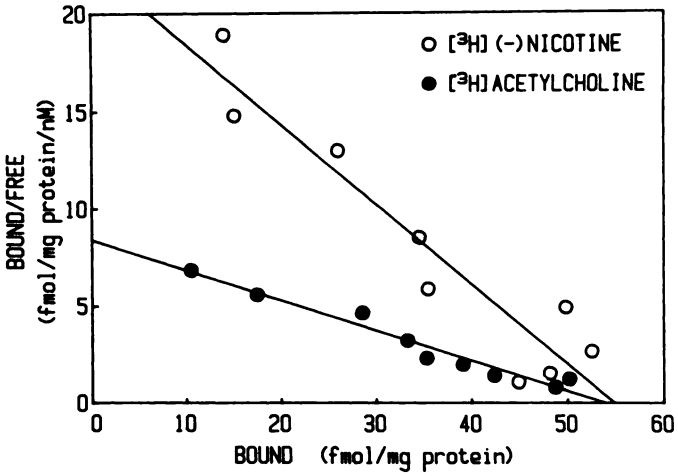

Fig. 2. Scatchard plots of [³H]ACh- and [³H](−)nicotine-specific binding in rat cerebral cortex. Data are representative of seven assays.

are virtually identical (Fig. 2, Table 1). In other rat brain regions and in human cerebral cortex as well, the affinity of [³H](−)nicotine is 2–3 times higher than that of [³H]Ach (Table 1). But, again, in each rat brain region examined and in human cerebral cortex, the number of recognition sites for the two ligands is nearly identical (Table 1). The correlation coefficient of the number of binding sites for the two ligands in five areas of rat brain and in human cerebral cortex is 0.99 (Fig. 3); and, more important, the regression line has a slope close to 1 and passes very near the origin (Fig. 3), indicating that the absolute number of sites for each ligand (as well as their rank-order) is indistinguishable. In all brain regions examined, the Hill coefficients for each ligand are close to 1; but compared to rat cerebral cortex, the affinity of both ligands appeared to be slightly lower in rat striatum and hippocampus and possibly slightly higher in human cortex (Table 1).

Pharmacology of [³H]ACh and [³H](−)nicotine recognition sites. The affinities of a series of nicotinic cholinergic drugs for [³H]ACh and [³H](−)nicotine recognition sites in rat cerebral cortex were derived from competition experiments. All of the agonists tested, as well as DBE and *d*-tubocurarine, could compete for 100% of the sites labeled by both ligands. The K_i values of each drug at the sites labeled by [³H]ACh and [³H](−)nicotine were similar (Table 2). The correlation coefficient of the K_i values for the sites labeled by [³H]ACh and [³H](−)nicotine was 0.99 and the slope of the regression line was 1 (Fig. 4). The K_i values for (−)nicotine and acetylcholine, derived from competition against [³H]ACh and [³H](−)nicotine, respectively, agreed closely with the K_d values for the two ³H-ligands obtained directly from Scatchard plots and Hill plots (cf. Tables 1 and 2). (−)Nicotine is 30–50 times more potent than (+)nicotine in competing for [³H]ACh and [³H](−)nicotine recognition sites (Table 2), indicating that both sites display a high degree of stereoselectivity for the enantiomers of nicotine. As reported previously (1, 4–6), most nicotinic cholinergic antagonists have low affinity for both [³H]ACh and [³H](−)nicotine recognition sites; however, DBE is an exception in both cases (Table 2).

Effects of disulfide bond modification. [³H]ACh recognition sites in brain contain disulfide bonds that are essential for the binding of [³H]ACh. Following preincubation with DTT, which reduces disulfide bonds to sulfhydryl groups, the apparent density of [³H]ACh recognition sites is decreased; and following a subsequent preincubation with DTNB, which reoxidizes sulfhydryl groups, the density of sites is restored (27). The effects of disulfide bond reduction with DTT on [³H]ACh and [³H](−)nicotine binding in rat cortex appear to be virtually identical. In both cases, preincubation with 3 mM DTT decreased binding by approximately 50% (Fig. 5); and, in both cases, reoxidation of the sulfhydryl groups by a second preincubation with 1 mM DTNB restored binding to control values (Fig. 5). Preincubation with DTNB alone did not affect the binding of either ligand (data not shown).

***In vivo* regulation.** *In vivo* regulation of [³H]ACh and [³H](−)nicotine recognition sites in rat brain was compared by measuring the effects of repeated administration of nicotine and the cholinesterase inhibitor soman on binding of the two ligands. Treatment of rats with nicotine for 10 days increased [³H]ACh and [³H](−)nicotine binding in the cerebral cortex by 37 and 29%, respectively (Fig. 6a). In the striatum, binding of both ligands was increased by 20–25% (Fig. 6b). As found previously for [³H]ACh recognition sites (2, 3), the increased binding of [³H](−)nicotine was due to an increase in the density of recognition sites (Table 3). Neither the affinity of [³H](−)nicotine for the sites nor the Hill coefficient of binding was affected by the nicotine treatment (Table 3), suggesting that the increased density of sites was due to up-regulation of a homogeneous population of recognition sites.

Treatment of rats with soman for 9 days decreased the density of both [³H]ACh and [³H](−)nicotine recognition sites in the cerebral cortex without affecting the affinity of the sites for either ligand (Table 4).

Discussion

The densities of [³H]ACh and [³H](−)nicotine recognition sites are indistinguishable in the limited number of brain areas

TABLE 1

Binding constants of [³H]ACh and [³H](−)nicotine in rat brain regions and human cerebral cortex

Values were derived from Scatchard plots and are the means ± standard errors of three to seven determinations.

Rat brain region	B_{max}		K_d		n_H	
	[³H]ACh	[³H](−)Nicotine	[³H]ACh	[³H](−)Nicotine	[³H]ACh	[³H](−)Nicotine
	fmol/mg or protein		*nM*			
Thalamus	91.4 ± 4.8	93.5 ± 6.3	8.6 ± 0.4	3.5 ± 0.3	0.97 ± 0.04	0.99 ± 0.04
Striatum	88.5 ± 8.8	80.9 ± 5.3	19.1 ± 1.7	6.5 ± 0.6	0.96 ± 0.02	0.97 ± 0.01
Cerebral cortex	55.4 ± 2.4	57.0 ± 4.0	10.9 ± 0.9	3.6 ± 0.4	0.98 ± 0.02	0.94 ± 0.02
Hypothalamus	29.5 ± 5.0	32.7 ± 4.6	7.6 ± 1.7	3.9 ± 0.4	0.73 ± 0.05	1.1 ± 0.09
Hippocampus	25.4 ± 6.9	23.5 ± 3.2	16.3 ± 1.5	6.7 ± 0.5	0.98 ± 0.08	0.93 ± 0.02
Human cortex[a]	14.1 ± 2.7	16.1 ± 2.7	5.2 ± 0.9	1.9 ± 0.4	1.02 ± 0.05	0.92 ± 0.04

[a] Values for human cortex have been published previously (20).

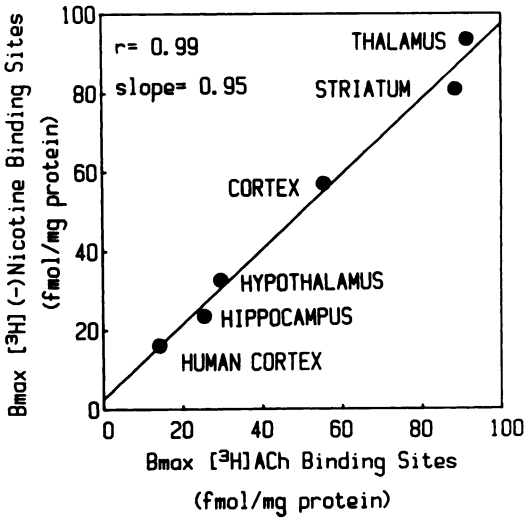

Fig. 3. Correlation between the densities (B_{max}) of [^{3}H]ACh- and [^{3}H](−)nicotine-binding sites in several areas of rat brain and in human cerebral cortex. The values for the B_{max} of [^{3}H]ACh and [^{3}H](−)nicotine binding are the means of three to seven assays. The correlation coefficient and slope were determined from linear regression analysis.

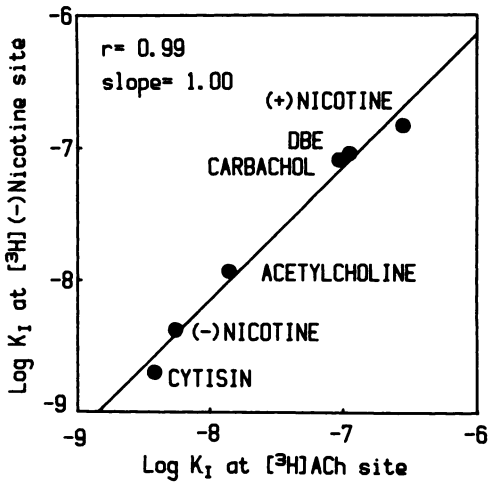

Fig. 4. Correlation between the affinities of the [^{3}H]ACh- and [^{3}H](−)nicotine-binding sites in rat cerebral cortex for nicotinic cholinergic drugs. The K_i values for each drug were derived from competition experiments against [^{3}H]ACh and [^{3}H](−)nicotine. Each value is the mean of three to six assays. The correlation coefficient and slope were determined from linear regression analysis.

TABLE 2

Inhibition of [^{3}H]ACh and [^{3}H](−)nicotine binding in rat cerebral cortex by cholinergic drugs

Competing drugs were incubated with 10 nM [^{3}H]ACh or 4 nM [^{3}H](−)nicotine. K_i values are the means ± standard errors of three to six determinations.

Drug	K_i	
	[^{3}H]ACh	[^{3}H](−)Nicotine
	nM	
Cholinergic agonists		
Cytisin	3.8 ± 0.9	2.0 ± 0.2
(−)Nicotine	5.5 ± 2.3	4.2 ± 1.3
Acetylcholine	13.9 ± 5.1	11.7 ± 1.8
Carbachol	93.4 ± 27.6	81.2 ± 26.2
(+)Nicotine	282.0 ± 187	148.0 ± 42
Cholinergic antagonists		
Dihydro-β-erythroidine	111.9 ± 28.7	90.7 ± 9.6
d-Tubocurarine	28,500 ± 9,240	17,900 ± 3,880
Mecamylamine	>1,000,000	>1,000,000

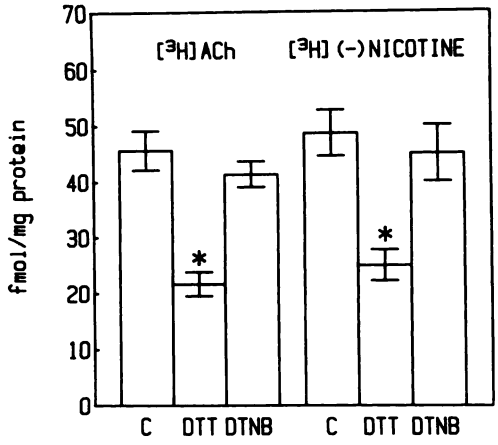

Fig. 5. Effects of reduction of disulfide bonds with DTT and reoxidation of sulfhydryl groups with DTNB on [^{3}H]ACh and [^{3}H](−)nicotine binding in rat cerebral cortex. Cortex homogenates were preincubated for 30 min at 0° in the absence or presence of 3 mM DTT and then washed by centrifugation and preincubated a second time in the absence or presence of 1 mM DTNB. The homogenates were then washed again and binding of [^{3}H]ACh (10 nM) and [^{3}H](−)nicotine (4 nM) was measured. Preincubation with DTNB alone had no effect on the binding of either ligand. Values are the mean ± standard error from five experiments. *, $p < 0.05$ compared to control homogenates (*C*), which were preincubated in the absence of modifying agents.

measured here. This supports an extensive autoradiographic study by Clarke *et al.* (7) which showed that the pattern of binding of the two ligands is similar throughout rat brain. The affinities of nicotinic cholinergic drugs for [^{3}H]ACh and [^{3}H](−)nicotine binding sites are indistinguishable, the binding of both ligands depends on intact disulfide bonds, and the *in vivo* regulation of the binding sites for both ligands by direct and indirect acting cholinergic drugs is virtually identical. Thus, we conclude that [^{3}H]ACh and [^{3}H](−)nicotine almost certainly label the same recognition site in rat brain. Recently, a similar conclusion has been reached by Marks *et al.* (8) based on studies of rat and mouse brain. In addition, in human brains from Alzheimer's disease patients there is an equivalent loss of [^{3}H]ACh and [^{3}H](−)nicotine recognition sites in the cerebral cortex (19, 20).

In agreement with two recent reports (8, 28), [^{3}H](−)nicotine appears to label only a single, high affinity class of recognition sites in rat brain. In contrast, racemic [^{3}H]nicotine labels both a high affinity class of sites and a more numerous lower affinity class of sites in brain (4–6). This alone could explain many of the previously reported differences between [^{3}H]ACh and [^{3}H]nicotine binding characteristics. In addition, Lippiello and Fernandes (28) reported that the appearance of a lower affinity class of binding sites in brain might result from the action of proteolytic enzymes in the tissue homogenate. (In this regard, it should be noted that diisopropyl fluorophosphate, which was used to inhibit cholinesterase enzymes in all of the experiments reported here, is also an inhibitor of serine-type proteolytic enzymes.) We did not investigate the binding of [^{3}H](−)nicotine at concentrations higher than 50 nM; but at this concentration, more than 93% of the high affinity sites are occupied, and we saw no indication of a second class of sites.

The site labeled by [^{3}H]ACh and [^{3}H](−)nicotine has the characteristics of an agonist recognition site of a nicotinic

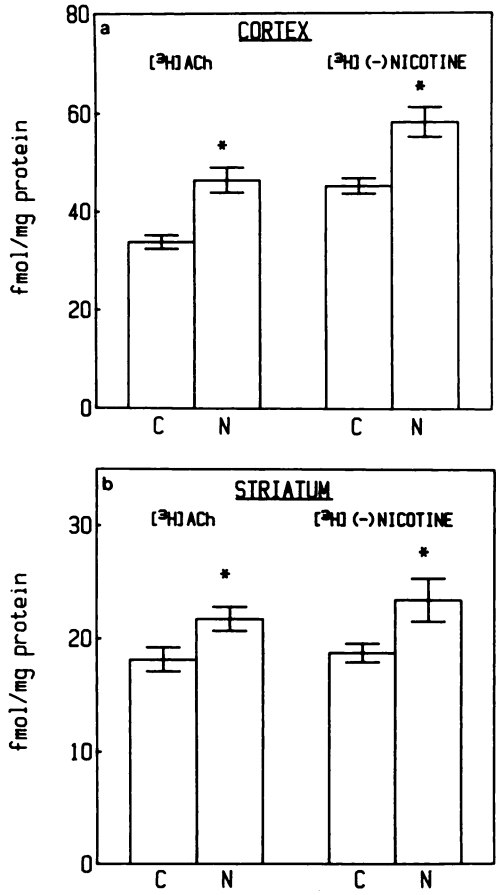

Fig. 6. Effects of repeated administration of nicotine on [³H]ACh and [³H](−)nicotine binding in rat cerebral cortex (a) and striatum (b). Rats were injected subcutaneously with nicotine bitartrate dihydrate (3.6 μmol/kg) twice daily for 10 days and sacrificed 1 day after the last injection. Binding sites in the cerebral cortex were measured using 10 nM [³H]ACh and 4 nM [³H](−)nicotine. Binding sites in the striatum were measured using 6.6 nM [³H]ACh and 2.9 nM [³H](−)nicotine. Values are the mean ± standard error of five control (*C*) and six nicotine-treated (*N*) rats. *, $p < 0.05$ compared to control values.

TABLE 3

Effect of repeated treatment with nicotine on [³H](−)nicotine binding constants in rat cerebral cortex

Rats received subcutaneous injections of nicotine bitartrate dihydrate (3.6 μmol/kg) twice daily for 10 days and were sacrificed 1 day after the last injection. Controls received water injections. Values are the means ± standard errors from three rats and were derived from Scatchard plots and Hill plots of saturation binding assays.

Treatment	B_{max}	K_d	n_H
	fmol/mg of protein	*nM*	
Control	55.3 ± 4.9	1.7 ± 0.2	0.95 ± 0.01
Nicotine	79.0 ± 1.3[a]	1.9 ± 0.3	1.02 ± 0.02

[a] $p < 0.05$ compared to controls.

cholinergic receptor. The up-regulation of nicotinic cholinergic recognition sites in rat and mouse brain following repeated treatment with nicotine has been reported previously and has been ascribed to protracted desensitization of the receptors following stimulation by nicotine (2, 3, 9). The decrease in the density of nicotinic cholinergic recognition sites in rat brain following treatment with soman is similar to that found following treatment with two other cholinesterase inhibitors, diisopropyl fluorophosphate (2, 3) and disulfoton (6). This decrease is consistent with down-regulation of nicotinic receptors as a consequence of increased synaptic availability of acetylcholine following cholinesterase inhibition, and it is one indication that these recognition sites are innervated by cholinergic axons.

TABLE 4

Effect of chronic cholinesterase inhibition with soman on [³H]ACh and [³H](−)nicotine binding constants in rat cerebral cortex

Rats received subcutaneous injections of soman (60 μg/kg) once daily for 9 days and were sacrificed 1 day after the last injection. Controls received water injections. Values are the means ± standard errors from four rats and were derived from Scatchard plots of saturation binding assays.

Treatment	B_{max} (fmol/mg protein)		K_d (nM)	
	[³H]ACh	[³H](−)Nicotine	[³H]ACh	[³H](−)Nicotine
	fmol/mg of protein		*nM*	
Control	65.8 ± 4.6	62.5 ± 3.6	12.2 ± 1.8	3.6 ± 0.3
Soman	46.9 ± 1.4[a]	51.8 ± 2.3[a]	11.6 ± 1.9	3.7 ± 0.3

[a] $p < 0.05$ compared to controls.

In contrast to the detailed information about nicotinic cholinergic receptors in muscle and electric organs (see, Refs. 29–32), the structure of mammalian brain nicotinic receptors is not known, nor is it known whether the receptors in brain operate ion channels as they do in muscle. However, *in situ* hybridization studies indicate that rat and mouse brain express RNA homologous to cDNA clones that encode an α-subunit of peripheral-type nicotinic cholinergic receptors. In particular, an α-subunit probe of a neural-type receptor derived from a rat pheochromocytoma cell line (PC12) hybridized in brain much more strongly than did an α-subunit probe of a muscle receptor (33, 34).

It is not yet known whether the encoded protein in mammalian brain that is homologous to the α-subunit of peripheral neural-type nicotinic receptors is part of either the high affinity agonist-binding site or the α-bungarotoxin-binding site. There is little correlation between the brain regional distributions of these sites (1, 7, 8), indicating that they are on different proteins. Thus, the relationship between the high affinity agonist-binding site and the α-bungarotoxin-binding site is not known. However, based on multiple subtypes of other receptors, there is certainly room in mammalian brain for two nicotinic cholinergic receptor subtypes.

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