Autoradiographic Localization of Putative Nicotinic Receptors in the Rat Brain Using ^{125}I-Neuronal Bungarotoxin

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Neuronal bungarotoxin (NBT), a snake venom neurotoxin, selectively blocks nicotinic receptors in many peripheral and central neuronal preparations. α-Bungarotoxin (αBT), on the other hand, a second toxin isolated from the venom of the same snake, is an ineffective nicotinic antagonist in most vertebrate neuronal preparations studied thus far. To examine central nicotinic receptors recognized by NBT, we have characterized the binding of ^{125}I-labeled NBT (^{125}I-NBT) to rat brain membranes and have mapped the distribution of ^{125}I-NBT binding in brain sections using quantitative light microscopic autoradiography.

The binding of ^{125}I-NBT was found to be saturable, of high affinity, and heterogeneously distributed in the brain. Pharmacological studies suggested that more than one population of sites is labeled by ^{125}I-NBT. For example, one component of ^{125}I-NBT binding was also recognized by αBT, while a second component, not recognized by αBT, was recognized by the nicotinic agonist nicotine. The highest densities of these αBT-insensitive, nicotine-sensitive sites were found in the fasciculus retroflexus, the lateral geniculate nucleus, the medial terminal nucleus of the accessory optic tract, and the olivary pretectal nucleus. αBT-sensitive NBT binding sites were found in highest density in the lateral geniculate nucleus, the subthalamic nucleus, the dorsal tegmental nucleus, and the medial mammillary nucleus (lateral part).

The number of brain regions with a high density of ^{125}I-NBT binding sites, blocked either by αBT or by nicotine, is low when compared with results obtained using other approaches to studying the central distribution of nicotinic receptors, such as labeling with ^{3}H-nicotine or labeling with cDNA probes to mRNAs coding for putative receptor subunits. It is proposed that ^{125}I-NBT labels a subpopulation of nicotinic receptors in the rat brain, and that, given its ability to block nicotinic receptors in a variety of neuronal preparations, NBT will be a useful probe for studying the functional properties of these sites.

It is now clear that nicotinic, as well as muscarinic, receptors mediate the effects of ACh in the CNS. Nicotinic receptors have been linked to a variety of central cholinergic effects, including locomotor activity (Clarke and Kumar, 1983), antinociception (Sahley and Berntson, 1979; Tripathi et al., 1982), and intracranial self-stimulation (Pradhan and Bowling, 1971). Nicotinic agonists elicit excitatory electrophysiological responses that are blocked by classical ganglionic nicotinic antagonists (e.g., mecamylamine) in many different brain regions, including the substantia nigra pars compacta and the ventral tegmental area (Clarke et al., 1985a; Grenhoff et al., 1986), the caudate-putamen (Misgeld et al., 1980), the interpeduncular nucleus (Brown et al., 1983), the medial habenula (McCormick and Prince, 1987), the locus coeruleus (Egan and North, 1986), and the hypothalamus (Hatton et al., 1983). Recent postmortem studies of brains removed from patients who had suffered from Alzheimer's disease have suggested that nicotinic receptors are selectively depleted in certain regions of the CNS (Nordberg and Winblad, 1986; Shimohama et al., 1986; Whitehouse et al., 1986), consistent with the idea that a change in central nicotinic neurotransmission might underlie some of the neurological deficits that accompany this condition.

Several approaches have been used previously to study the neuroanatomical distribution of central nicotinic receptors. Immunohistochemical localization of putative nicotinic receptors in the rat brain has been accomplished using monoclonal antibodies raised against nicotinic receptors immunoaffinity purified from the chick brain (monoclonal antibody 270; Deutch et al., 1987; Swanson et al., 1987). cDNAs coding for putative α and β subunits of central nicotinic receptors have been cloned, and in situ hybridization techniques have been used to localize the corresponding mRNAs in the brain (Boulter et al., 1986; Goldman et al., 1986, 1987; Wada et al., 1989). An autoradiographic approach using ^{14}C-labeled 2-deoxyglucose has been employed to determine which areas in the brain become metabolically active following parenteral administration of nicotine (Pazdernik et al., 1982; London et al., 1988). Finally, binding studies have attempted to label nicotinic receptors autoradiographically by utilizing radiolabeled analogs of the following nicotinic ligands: ACh (Rainbow et al., 1984; Clarke et al., 1985b), nicotine (Clarke et al., 1984, 1985b; London et al., 1985), and α-bungarotoxin (αBT; Arimatsu et al., 1978; Hunt and Schmidt, 1978; Segal et al., 1978; Clarke et al., 1985b).

Of these different methodologies, *in vitro* autoradiography is most appropriate for obtaining quantitative measures of receptor density, because binding sites may be labeled directly. With all studies using this approach, of course, one must be concerned as to whether the binding sites being measured using a particular ligand correspond, in fact, to functional receptors. For example, tritium-labeled nicotinic agonists label binding sites with nanomolar affinities, while micromolar concentrations of these agonists are required to evoke physiological responses. Such findings raise questions as to whether the labeled sites are, in fact, nicotinic receptors, though these high-affinity sites could represent desensitized states of the receptors (reviewed by Lindstrom et al., 1987; Schmidt, 1988). A second problem encountered when using tritium-labeled compounds is that it is often difficult to obtain accurate quantitative data, due to quenching of β particles by white matter (Kuhar and Unnerstall, 1985).

αBT is a potent antagonist of nicotinic synaptic transmission at the neuromuscular junction and has been useful in characterizing nicotinic receptors in muscle (reviewed by Popot and Changeux, 1984). However, interpretation of experiments using ¹²⁵I-αBT to study nicotinic receptors in the brain is made difficult by repeated observations that αBT fails to antagonize nicotinic function in almost all neuronal preparations that have been examined (reviewed by Schmidt et al., 1980; Lindstrom et al., 1987; Loring and Zigmond, 1988), though a few exceptions have been reported (Marshall, 1981; Zatz and Brownstein, 1981; Farley et al., 1983; de la Garza, 1987). Therefore, there is clearly a need for a radiolabeled antagonist of high specific activity that recognizes functional nicotinic receptors with high affinity.

In 1979, a peptide toxin distinct from αBT was purified from the venom of the snake *Bungarus multicinctus* (Ravdin and Berg, 1979). This toxin, which has been referred to as bungarotoxin 3.1 (Ravdin and Berg, 1979), toxin F (Loring et al., 1983), and κ-bungarotoxin (Chiappinelli, 1983), was found to block potently nicotinic activity in chick and rat autonomic ganglia (Ravdin and Berg, 1979; Chiappinelli, 1983; Loring et al., 1983, 1984; Chiappinelli and Dryer, 1984; Sah et al., 1987), with IC₅₀ values of <100 nM. In contrast, this toxin is very poor at blocking nicotinic receptors at the neuromuscular junction and, thus, has a selectivity for neuronal versus muscle nicotinic receptors that is opposite to that of αBT. Bungarotoxin 3.1, toxin F, and κ-bungarotoxin are now known to be the same toxin (Loring et al., 1986), and it has been suggested that the name "neuronal bungarotoxin" (NBT) be used to refer to this molecule (Lindstrom et al., 1987).

In the chick ciliary ganglion, NBT antagonizes the depolarizations caused by application of nicotinic agonists, as well as the nicotinic responses evoked by presynaptic stimulation (Loring et al., 1984). Thus, it is likely that NBT acts at postsynaptic nicotinic receptors in this preparation. In addition, because NBT's long-lasting blocking effects in autonomic neurons are prevented by co-applying the rapidly reversible antagonists dihydro-β-erythroidine (Sah et al., 1987) or *d*-tubocurare (Chiappinelli, 1983), it appears that NBT acts at the same site on the receptor as do classical competitive nicotinic antagonists.

In order to study its binding in autonomic ganglia, NBT has been labeled with ¹²⁵I (¹²⁵I-NBT). It was found that a portion of high-affinity ¹²⁵I-NBT binding sites is recognized by αBT, while a second component is selective for NBT (Chiappinelli, 1983; Loring et al., 1984; Sah et al., 1987). Because αBT fails to block nicotinic activity in autonomic ganglia, it has been proposed that it is this second component that corresponds to functional

nicotinic receptors (Loring et al., 1984; Loring and Zigmond, 1988). Interestingly, electron microscopic studies of chick ciliary ganglia and cultured rat sympathetic neurons have established that the NBT-selective sites are preferentially concentrated at synaptic regions (Loring and Zigmond, 1987; Loring et al., 1988).

In addition to blocking nicotinic transmission in peripheral autonomic ganglia, NBT has been shown to block nicotinic responses in the chick (Loring et al., 1989) and rat (Lipton et al., 1987; Aizenman et al., 1990) retina. These results raised the possibility that NBT might be useful more generally in studying central nicotinic transmission. In support of this idea, we have discovered that NBT blocks the nicotine-evoked release of endogenous dopamine from slices of the rat striatum (Schulz and Zigmond, 1989). Blockade by NBT of physiological responses mediated by neuronal nicotinic receptors in other central preparations has also been reported recently (de la Garza et al., 1989; Vidal and Changeux, 1989; Wong and Gallagher, 1989).

In the present study, we have used light-microscopic autoradiography to examine the distribution of ¹²⁵I-NBT binding in the rat brain. Studies in membrane preparations, which were done in order to determine whether ¹²⁵I-NBT is an appropriate high-affinity ligand for nicotinic receptors in the rat brain, are also reported.

Certain of these data have been presented to the Society for Neuroscience (Schulz et al., 1988).

Materials and Methods

NBT was purified from *Bungarus multicinctus* venom (a gift from Biotoxins Inc., St. Cloud, FL) as described previously (Loring et al., 1986). A chloramine-T procedure (Loring and Zigmond, 1987) was used to label the toxin with ¹²⁵I. The resulting specific activity of ¹²⁵I-NBT ranged from 700 to 1300 Ci/mmol.

Binding to membrane preparations. Preliminary receptor binding studies utilized membranes prepared from rat corpora striata, because we had previously characterized the functional properties of NBT using this region of the brain (Schulz and Zigmond, 1989). Adult male Sprague–Dawley rats were decapitated, and the striata were rapidly dissected from the brain on ice. Tissue was either used immediately or stored frozen at $-20°C$, with no differences in binding observed for the 2 procedures. The tissue was homogenized in 20 vol of ice-cold buffer using a Brinkmann Polytron (Westbury, NY) at setting 6 for 20 sec and washed twice by centrifuging at $25,000 \times g$, and the pellet was resuspended at a tissue concentration of 60 mg wet weight/ml. The buffer contained 120 mM NaCl, 50 mM Tris (pH, 7.4), and 2 mg/ml bovine serum albumin (BSA). Fifty-microliter aliquots of tissue suspension were preincubated with 50 μl of buffer alone or with a competing ligand for 30 min in 12 × 75-mm polypropylene culture tubes (Fisher Scientific, Pittsburgh, PA). The binding reaction was then initiated by adding 50 μl ¹²⁵I-NBT at a concentration 3 times the desired final concentration. Nonspecific binding was defined as binding in the presence of 0.5 μM nonradioactive NBT. The tubes were incubated at 37°C for 1 hr (unless noted otherwise), and the reaction was terminated by dilution with 4 ml cold buffer and immediate filtration onto Whatman GF/C filters that had been presoaked in cold buffer. Filters were washed twice more with 4 ml cold buffer containing 0.1 mg/ml BSA and counted in a Beckman 300 gamma counter at 60% efficiency. Quadruplicate determinations were made for each condition within a given experiment. Protein content was determined by the method of Lowry et al. (1951) using BSA as the standard.

Autoradiography experiments. Adult male Sprague–Dawley rats were anesthetized with chloral hydrate (35%, 0.14 ml/100 gm) and perfused with phosphate-buffered isotonic sucrose. Brains were frozen onto cryostat chucks, and 20-μm sections were cut, thaw mounted onto glass slides, and stored at $-20°C$ until they were used. The procedure for incubating and washing tissue sections was adapted from earlier studies that had used ¹²⁵I-αBT as a radioligand (Arimatsu et al., 1978; Clarke et al., 1985b). Sections were preincubated with or without nonradioactive compounds at room temperature for 30 min in Tris-buffered saline with BSA (see above). The sections were then incubated at 37°C in

Table 1. Quantitative analysis of regional ^{125}I-NBT binding in the rat brain

| Brain area	Specific[125] I-NBT binding (amol/mm²)		
	Total	In presence of 0.5 μM αBT	In presence of αBT + 100 μM nicotine
Amygdala (medial nuclei)	19.4 ± 5.3	15.3 ± 3.5	16.1 ± 4.4
Caudate-putamen	16.0 ± 2.1	13.5 ± 2.0	10.6 ± 0.9
Central gray	14.3 ± 1.6	* 10.8 ± 2.7	10.7 ± 1.6
Corpus callosum	9.3 ± 0.8	7.6 ± 0.7	8.3 ± 1.3
Cortex (frontal lobe)	17.5 ± 1.8	14.6 ± 1.6	13.2 ± 1.6
Cortex (occipital lobe)	15.4 ± 1.0	13.7 ± 2.2	11.9 ± 1.7
Dorsal tegmental nucleus	64.9 ± 16.4	* 12.5 ± 1.9	* 6.1 ± 0.7
Fasciculus retroflexus	17.6 ± 1.4	17.6 ± 2.0	† 8.7 ± 1.1
Habenula (lateral nucleus)	14.1 ± 1.9	12.0 ± 1.5	13.2 ± 1.1
Habenula (medial nucleus)	28.3 ± 3.7	* 22.1 ± 3.5	* 10.6 ± 1.1
Hilus of dentate gyrus	30.4 ± 4.5	* 13.8 ± 2.3	12.9 ± 0.9
Hippocampus (CA1)	17.0 ± 0.5	14.3 ± 1.7	14.2 ± 1.5
Hypothalamus (ML)	33.8 ± 4.6	* 11.5 ± 1.7	11.2 ± 1.3
Hypothalamus (MM)	14.7 ± 1.2	11.8 ± 2.1	11.3 ± 1.9
Inferior colliculus	15.6 ± 0.8	13.7 ± 2.5	9.9 ± 2.1
Interpeduncular nucleus	14.2 ± 1.9	12.2 ± 2.0	9.0 ± 1.3
Lateral geniculate nucleus (dorsal)	25.0 ± 2.8	21.7 ± 1.3	† 10.6 ± 1.3
Lateral geniculate nucleus (ventral)	27.1 ± 2.5	† 15.8 ± 1.3	* 11.0 ± 2.1
Medial geniculate nucleus	15.1 ± 0.6	9.2 ± 3.8	8.0 ± 2.6
Medial terminal nucleus	37.3 ± 1.3	30.8 ± 3.1	† 9.1 ± 1.3
Olivary pretectal nucleus	28.2 ± 1.1	24.1 ± 1.1	† 10.5 ± 1.5
Septum (lateral nucleus)	11.6 ± 1.7	9.4 ± 1.3	9.0 ± 0.9
Substantia nigra	15.0 ± 1.3	* 10.8 ± 1.6	9.8 ± 0.8
Subthalamic nucleus	34.0 ± 3.9	† 11.1 ± 2.0	8.8 ± 1.4
Superior colliculus (SuG)	41.9 ± 4.9	* 24.2 ± 3.9	* 10.0 ± 2.1
Superior colliculus (non-SuG)	20.2 ± 0.8	* 13.4 ± 2.9	9.9 ± 1.3
Thalamus (VPM-VPL)	13.9 ± 2.0	12.3 ± 1.8	12.1 ± 0.2
Ventral tegmental area	13.9 ± 1.3	* 9.3 + 1.2	7.5 ± 0.4

Twenty-micron sections were incubated for 1 hr at 37°C in 2.0 nM ^{125}I-NBT. Values represent mean ± SEM for 3–4 rats in units of amol/mm². Nonspecific binding (i.e., binding in the presence of 0.5 μM unlabeled NBT) has been subtracted. ML, medial mammillary nucleus (lateral part); MM, medial mammillary nucleus (medial part); VPM-VPL, ventroposteromedial and ventroposterolateral nuclei; SuG, superficial gray layer of the superior colliculus.

*, $p < 0.05$; †, $p < 0.0025$ by 1-tailed paired t test. The symbols are placed between pairs of values for which significant differences were detected.

buffer containing 2.0 nM ^{125}I-NBT. Certain sections were incubated in the presence of 0.5 μM nonradioactive NBT, 0.5 μM αBT (Biotoxins Inc., St. Cloud, FL), 100 μM nicotine (Sigma Chemical Co., St. Louis, MO), 100 μM d-tubocurare (CalBiochem, San Diego, CA), or 100 μM dihydro-β-erythroidine (a gift from Merck Sharpe and Dohme, West Point, PA). These concentrations were found to be saturating when used as competing ligands in membrane binding studies (data not shown). After the incubation period, sections were washed twice for 30 min at 4°C, first in buffer containing BSA and then in protein-free buffer. Washes of 30 min were found to minimize nonspecific binding, while causing no changes in the amount of specific binding observed when shorter (1 or 5 min) wash periods were used. In some preliminary experiments, binding was quantitated by wiping each tissue section off the slide with a piece of filter paper and counting the paper in a gamma counter. However, in most experiments, the sections were blow dried at ambient temperature after washing and apposed to LKB Ultrafilm for 5–10 d. Sections were co-exposed with Amersham ^{125}I microscales (20 μm thickness, ranging in activity from 0.2 to 180 nCi/mg) in order to convert optical density values on the film to units of radioactivity per mg tissue. A fourth-order polynomial function was used to relate optical density and radioactivity. In one experiment, the activity/optical density profiles of brain paste standards containing ^{125}I were compared with those of the ^{125}I microscales, in order to determine an accurate conversion factor to be used in calculating receptor density values in tissue. The results were in close agreement with those obtained by Davenport and Hall

(1988); that is, tissue equivalent activities were approximately half those of the polymer microscales.

Quantitative analyses of the resulting autoradiograms were carried out using the MCID computerized image analysis system (Imaging Research Inc., St. Catharine's, Ontario). This system allows one to read the optical density of a square-shaped area, with the size of the square determined by the experimenter. In larger brain regions, several such randomly assigned areas were read, until well over 50% of a given brain region had been analyzed. The mean of these optical density (OD) values was determined for a particular section, and a mean value for a single animal was obtained by examining at least 3 different sections. The mean ± SEM values presented in Table 1 represent values determined by combining these results from 3–4 different rats. With smaller brain regions (e.g., medial habenula), a single square of appropriate size was centered over each of the nuclei, and the bilateral optical densities were determined. The same size was used consistently for a given region. Variations in the data that could result from differences in the rostral-caudal localization of sections were minimized by assigning adjacent or near-adjacent sections to the different incubation conditions. All quantitative values are expressed as mean ± SEM, with units for all data obtained from autoradiographic experiments expressed in amol/mm², as suggested previously (Clark and Hall, 1986; Davenport and Hall, 1988). Brain regions were identified in accordance with the atlas of Paxinos and Watson (1986). Analyses of variance were performed and revealed that there were regional differences in receptor densities. Paired
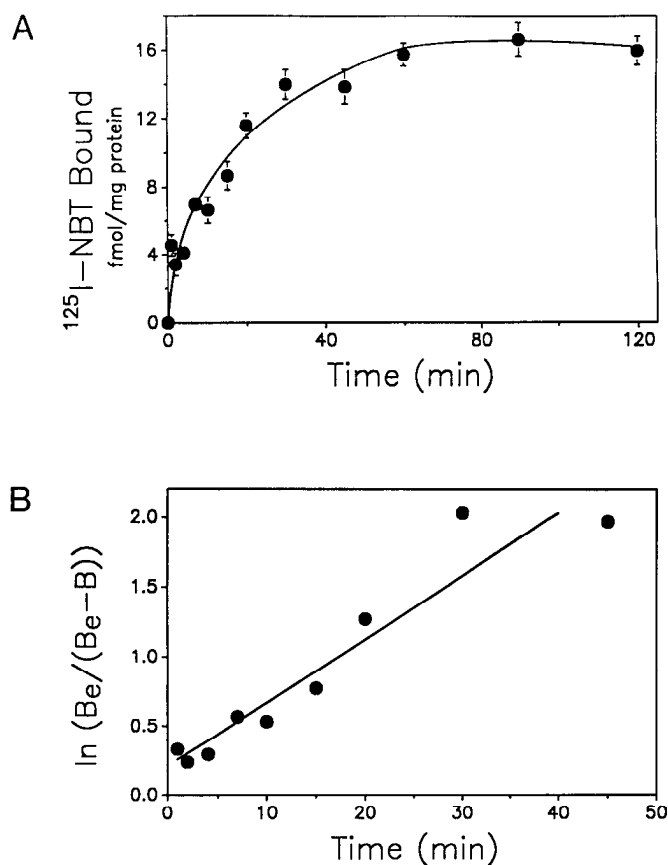
A

B
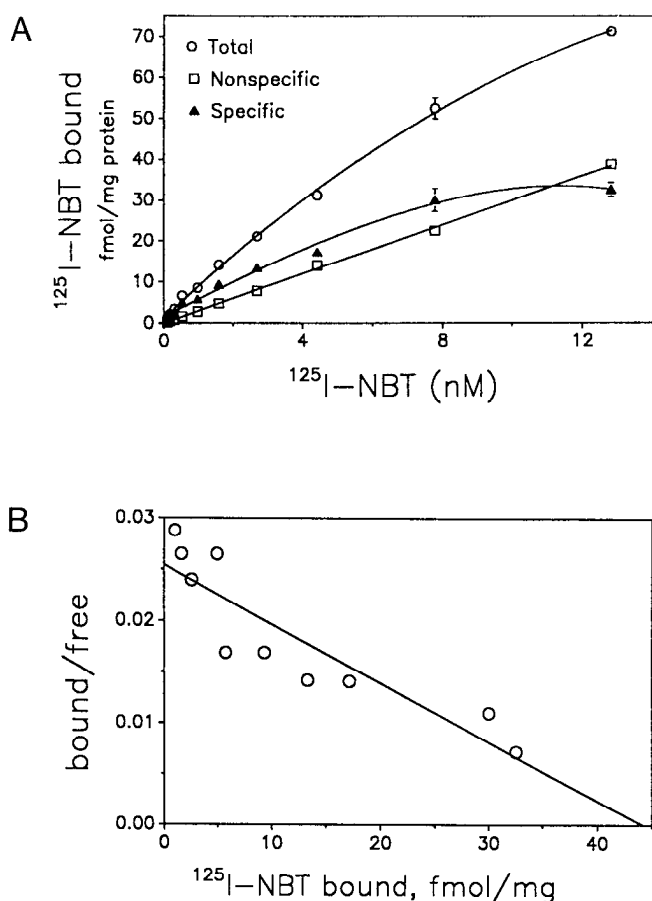
Figure 1. Concentration dependence of binding of ^{125}I-NBT to rat striatal membranes. *A*, Total (*circles*), specific (*triangles*), and nonspecific (*squares*) binding, with each point representing mean ± SEM for quadruplicate determinations. *B*, Scatchard transformation of data in *A*, yielding of K_d of 6.7 nM and a B_{max} of 44 fmol/mg protein.

A

B

Figure 2. Association of ^{125}I-NBT binding to striatal membranes. *A*, Washed membranes from rat striatum were incubated in 2.0 nM ^{125}I-NBT for the indicated periods of time at 37°C. The binding reaction was terminated by dilution with cold buffer, followed by rapid filtration. Each *point* represents mean ± SEM for specific binding determined from quadruplicate determinations of both total and nonspecific binding. *B*, Linear transformation of the data from *A*, yielding a k_{obs} value of 0.04 min^{-1}.

t tests (1-tailed) were then used to determine which regions contained significant densities of certain receptor subtypes.

Results

Because we had previously demonstrated that NBT is a functional nicotinic antagonist in rat striatal tissue, initial characterizations of ^{125}I-NBT binding were performed using striatal membranes. Saturation data from a typical single experiment are shown in Figure 1*A*, and a Scatchard transformation is depicted in Figure 1*B*. Binding of ^{125}I-NBT was found to be saturable and of high affinity, with 5 separate experiments yielding an average receptor density of 39 ± 6 fmol/mg protein and an affinity constant of 4.6 ± 0.6 nM (all values given are means ± SEM). The ratio of total to nonspecific binding at ½ the K_d was approximately 2.7:1, though autoradiographic experiments revealed that this ratio varies for different brain regions. The Hill coefficient for binding was 0.95 ± 0.08, indicating no cooperativity. While appearing somewhat curvilinear, the data on computer analysis (Lundeen and Gordon, 1986) were best fit by a straight line.

The kinetic properties of ^{125}I-NBT binding at 37°C were determined. Typical experiments showing the association and dissociation of binding are depicted in Figures 2*A* and 3*A*, respectively, with linear transformations of these data shown in Figures

2*B* and 3*B*. As shown in Figure 2*A*, binding reached equilibrium by about 1 hr. Following determination of k_{obs} and k_{-1} values, the mean value for the association constant (k_{+1}) was found to be 0.010 ± 0.001 nM^{-1} min^{-1}. Interruption of equilibrium, either by addition of excess nonradioactive NBT (0.5 μM) or by 40-fold dilution with buffer, demonstrated that binding was reversible, though the dissociation was not clearly monophasic (Fig. 3*A*). After 4 hr, 20% of the binding seen at equilibrium remained, while by 24 hr, dissociation was virtually complete. If a single component of dissociation is assumed, the k_{-1} value calculated from these data is 0.020 ± 0.003 min^{-1}, yielding a K_d of 2 nM. This is in fairly close agreement with the value obtained from Scatchard analysis; the 2.3-fold discrepancy may result from an underestimation of the k_{-1}, because its calculation was based on the assumption of a monophasic dissociation (see Fig. 3*B*).

Autoradiographic data are presented as representative autoradiograms (as in Figs. 4, 6, 7) and as means ± SEM of densitometric measurements of selected regions from a number of sections taken from several animals (Table 1). A preliminary determination of the time course of the binding of ^{125}I-NBT to brain sections confirmed that, as with membrane preparations, 1 hr was an appropriate incubation period to use in performing autoradiographic experiments (data not shown). It was also

demonstrated that optimal specific to nonspecific binding ratios in tissue sections could be obtained by washing the sections twice for 30 min in ice-cold buffer (data not shown).

The binding of 2.0 nM ^{125}I-NBT alone at the level of the caudal thalamus is shown in Figure 4A. Areas with particularly dense labeling at this level include the lateral geniculate nucleus, the olivary pretectal nucleus, the medial mammillary nucleus (lateral part), and the fasciculus retroflexus. Competition studies established that NBT bound to 3 pharmacologically distinct sites. One class of sites was recognized by both NBT and αBT, as shown by incubating adjacent sections with (Fig. 4B) and without (Fig. 4A) nonradioactive αBT (0.5 μM). Thus, as seen in Figure 4, αBT largely abolished NBT binding in the medial mammillary nucleus (lateral part) and reduced it substantially in the lateral geniculate. Figure 4B depicts those NBT sites not recognized by αBT. To determine the nicotinic nature of these sites, sections were incubated in the presence of 100 μM nicotine plus αBT (Fig. 4C). Note that nicotine competed for a significant component of the ^{125}I-NBT binding that remains in the presence of αBT, for example, in the lateral geniculate nucleus and the olivary pretectal nucleus (cf. Fig. 4B,C). Nonspecific binding, as defined by binding in the presence of 0.5 μM nonradioactive NBT (in addition to nonradioactive αBT and nicotine), is illustrated in Figure 4D. Note that nicotine plus nonradioactive αBT did not compete for all of the saturable ^{125}I-NBT binding (cf. Fig. 4C,D), suggesting that this final component of binding was not nicotinic in nature. This failure of nicotine to recognize all specific ^{125}I-NBT sites was observed at all levels of the brain. However, the density of the portion of specific binding not recognized by nicotine or αBT was found to be approximately the same in all brain regions examined. The indication that some regions are actually more densely labeled in the presence of nicotine (Fig. 4C) than in its absence (Fig. 4B) appears to be due to experimental variability and was not reproducible (see Table 1 for quantitative analysis from several animals).

Quantitative measurement of these pharmacologically distinct components of ^{125}I-NBT binding is illustrated in Figure 5 from experiments on the superficial gray layer of the superior colliculus. Optical density values determined from the photographic film for the binding of 2.0 nM ^{125}I-NBT were converted to receptor density values (units in amol/mm²). Of the total saturable ^{125}I-NBT binding in this brain region, about 40% was competed for by αBT and about 75% by nicotine plus αBT. Note that, when αBT (0.5 μM) and nicotine (100 μM) were both included in the incubation medium, the density of labeled sites was no less than if only nicotine were added. This suggests that there are no sites recognized by αBT that are not also recognized by a high concentration of nonradioactive nicotine (100 μM).

Figure 6, A–C, presents typical autoradiograms depicting the binding of 2.0 nM ^{125}I-NBT at 3 other levels of the brain under 3 experimental conditions. Autoradiograms in the left column represent binding in the absence of competing ligand; those in the middle column, binding in the presence of 0.5 μM αBT; and those in the right column, binding in the presence of αBT and 100 μM nicotine. Again, αBT competes for only a part of ^{125}I-NBT binding, while a portion of the remaining αBT-insensitive sites are recognized by nicotine. At the level of the caudate-putamen, αBT competes for binding in the endopiriform nucleus and islands of Calleja (Fig. 6A), while there are no areas at this brain level with notably high densities of αBT-insensitive, but nicotine-sensitive sites. More caudally (Fig. 6B), it can be seen that the high levels of binding in the subthalamic nucleus are
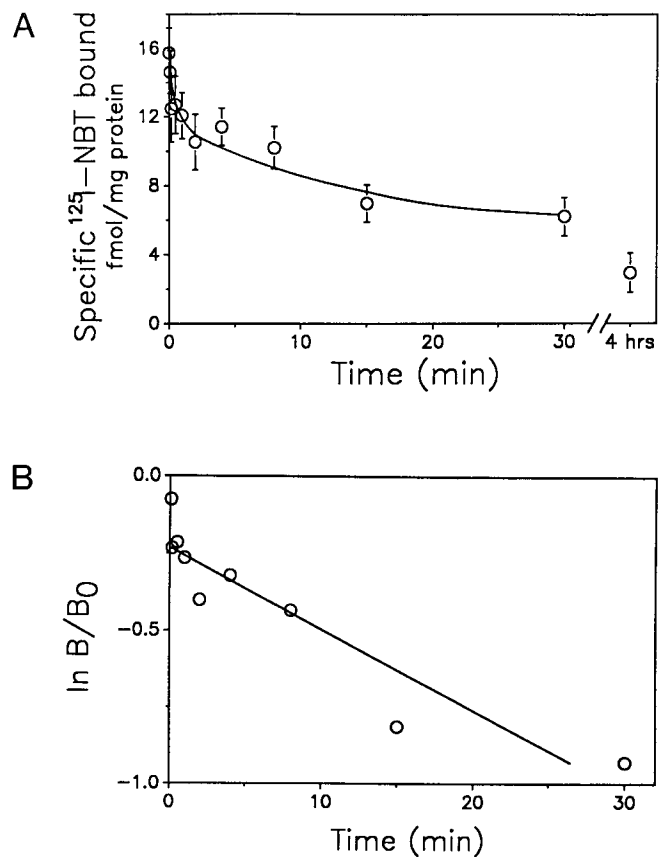

A

B

Figure 3. Dissociation of ^{125}I-NBT binding from striatal membranes. A, Washed membranes from rat striatum were incubated in 2.0 nM ^{125}I-NBT for 90 min at 37°C. In this experiment, equilibrium was interrupted by addition of unlabeled NBT (0.5 μM). Dissociation kinetics did not differ if equilibrium was interrupted instead by dilution with buffer. Each *point* represents mean ± SEM for specific binding resulting from quadruplicate determinations. B, Linear transformation of the data from A, yielding a k_{-1} value of 0.025 min^{-1}.

no longer detectable when αBT is present as a competing ligand (middle autoradiogram). However, in the same figure, there is substantial ^{125}I-NBT binding in the medial habenula in the presence of αBT. Similarly, αBT competes for only a portion of the dense labeling observed in the superficial gray layer of the superior colliculus and in the dorsal and medial terminal nuclei of the accessory optic tract (Fig. 6C, middle autoradiogram). In the presence of nicotine, no selective labeling of these sites is seen (Fig. 6B,C, right autoradiograms). In addition, labeling is seen in an area of the hippocampal formation that seems to correspond to the hilus of the dentate gyrus (Fig. 6C), and this labeling is competed for completely by αBT (Table 1).

As seen in Figure 7, in sections taken from the level of the caudal superior colliculus, the nicotinic antagonists dihydro-β-erythroidine (Fig. 7B) and d-tubocurare (Fig. 7C) compete for αBT-resistant binding (Fig. 7A) in a manner that is similar to competition by the nicotinic agonist nicotine.

The density of ^{125}I-NBT binding was compared for various regions of the brain after incubation with a single concentration (2.0 nM) of ^{125}I-NBT (Table 1). This concentration is slightly less than the K_d for binding to striatal membranes. Thus, regional densities of total ^{125}I-NBT binding sites cannot be directly determined from the table, because a subsaturating concentration
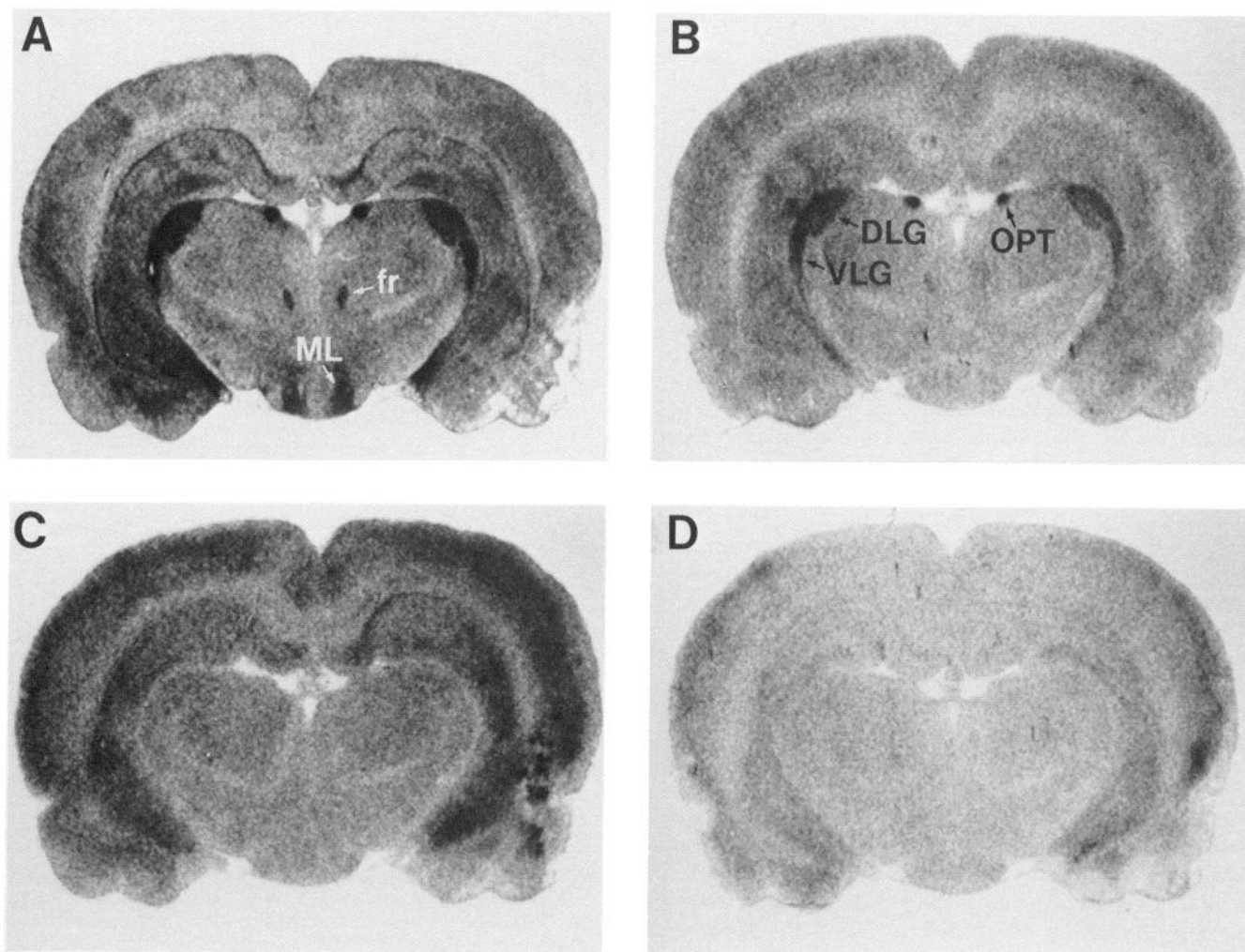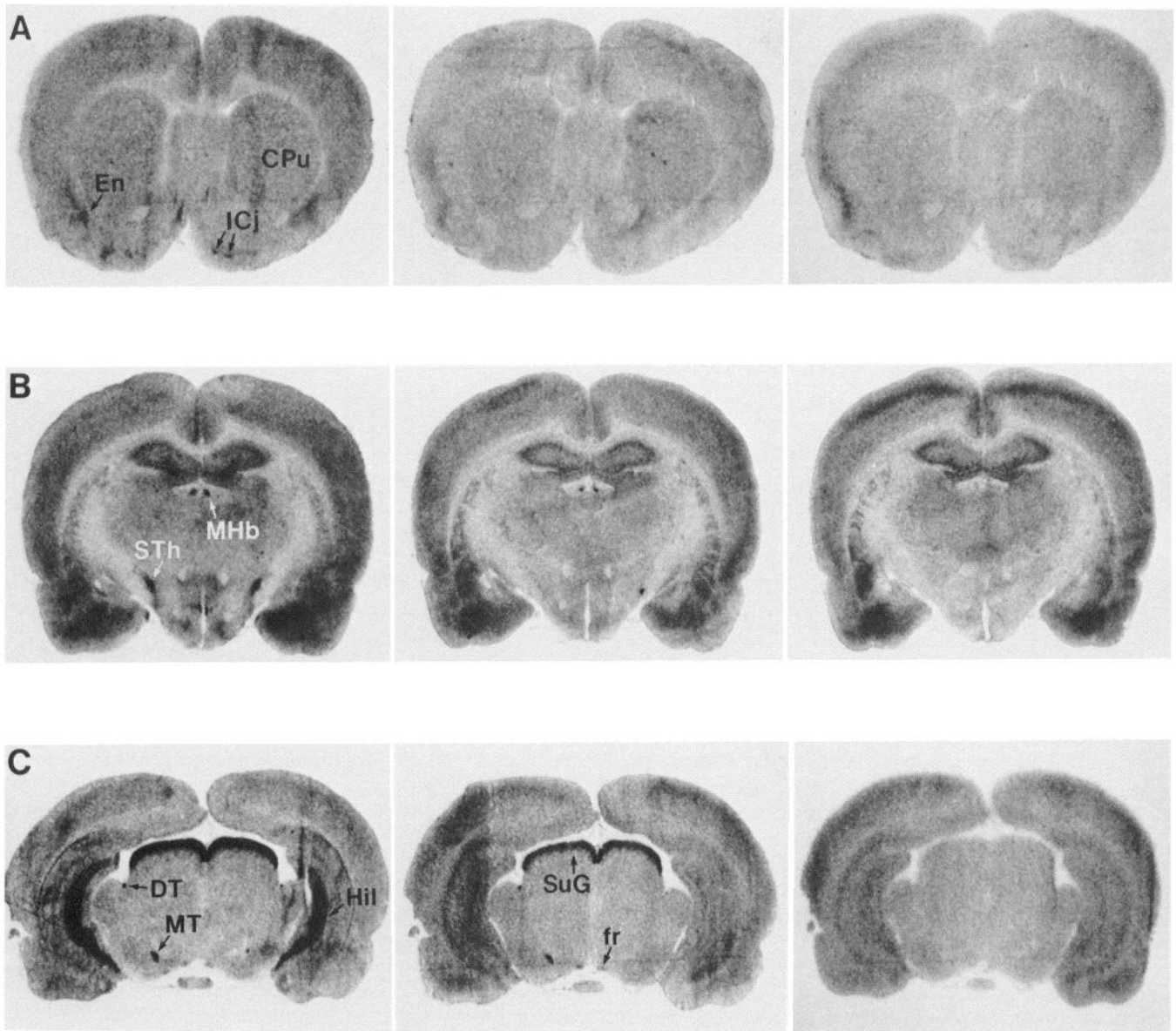
Figure 4. Binding of ^{125}I-NBT (2.0 nM) to frozen rat brain sections at level of thalamus. Binding was examined under 4 conditions: *A*, Total binding. *B*, Binding in the presence of 0.5 μM unlabeled αBT. *C*, Binding in the presence of 0.5 μM αBT plus 100 μM nicotine. *D*, Binding in the presence of 0.5 μM αBT, 100 μM nicotine, and 0.5 μM unlabeled NBT. *fr*, fasciculus retroflexus; *ML*, medial mammillary nucleus, lateral part; *DLG*, dorsal lateral geniculate nucleus; *VLG*, ventral lateral geniculate nucleus; *OPT*, olivary pretectal nucleus. The apparent increased density in parts of the cortex and hippocampus in *C*, as compared with *B*, probably reflects experimental variability rather than an actual difference in extent of NBT binding (see Results and Table 1).
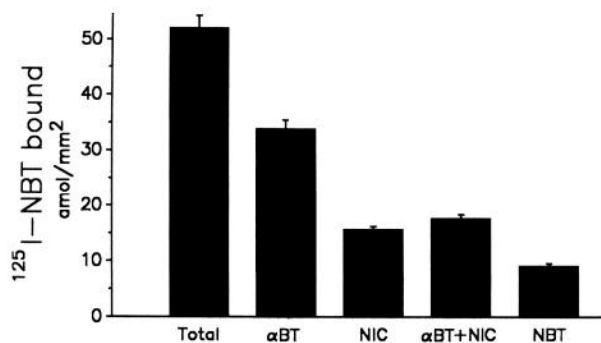
Figure 5. Quantitation of various pharmacological subtypes of ^{125}I-NBT binding sites. Optical density readings were taken from the superficial gray layer of the superior colliculus, and computer-assisted microdensitometry was used to determine receptor density values in amol/mm^2. ^{125}I-NBT at a concentration of 2.0 nM was present alone (*Total*) or in combination with the indicated competing ligands [*αBT* and *NBT* at 0.5 μM; nicotine (*Nic*) at 100 μM].

of ^{125}I-NBT was used. This list includes most regions where ^{125}I-NBT binding is particularly high. (Preliminary studies revealed that, while there were areas with high density of ^{125}I-NBT binding in the pons and medulla, these sites were blocked totally by addition of nonradioactive αBT alone. These αBT-sensitive sites were not analyzed quantitatively.) Also included are areas where high densities of nicotinic receptors have been found by other methods (see introductory remarks), as well as representative nuclei from various regions commonly included in anatomical distribution studies (e.g., amygdala, cortex, hippocampus, hypothalamus, and thalamus). Nonspecific binding (i.e., binding in the presence of 0.5 μM nonradioactive NBT) has been subtracted, so that the 3 columns listed in Table 1 are total specific binding, specific binding in the presence of αBT (0.5 μM), and specific binding in the presence of αBT and nicotine (100 μM). Binding to sites shared by the 2 toxins (i.e., sites recognized by both NBT and αBT) may be determined by subtracting the values on the second column from those in the first. Areas with high densities of these sites include the dorsal tegmental nucleus,

Figure 6. A–C, Binding of ^{125}I-NBT (2.0 nM) to frozen rat brain sections at 3 different levels of the rat brain, under the first 3 conditions described for Figure 4. *Left autoradiograms,* Total binding, no competing ligand; *middle autoradiograms,* 0.5 μM αBT; *right autoradiograms,* 0.5 μM αBT plus 100 mM nicotine. *CPu,* caudate-putamen; *En,* endopiriform nucleus; *ICj,* islands of Calleja; *MHb,* medial habenular nucleus; *STh,* subthalamic nucleus; *DT* and *MT,* dorsal and medial terminal nuclei of the accessory optic tract; *Hil,* hilus dentate gyrus; *SuG,* superficial gray layer of the superior colliculus; *fr,* fasciculus retroflexus.

the mediolateral nucleus of the hypothalamus, the hilus of the dentate gyrus, the ventral lateral geniculate nucleus, the subthalamic nucleus, and the superficial gray layer of the superior colliculus. The density of NBT-selective sites recognized by nicotine is obtained by subtracting values in the third column from those in the second. The areas with the greatest densities of these sites are the medial terminal nucleus of the accessory optic system, the superficial gray layer of the superior colliculus, the olivary pretectal nucleus, the dorsal lateral geniculate nucleus, the medial habenula, and the fasciculus retroflexus. The third category of saturable ^{125}I-NBT binding sites, those that are not recognized by nicotine or αBT, are represented by the third column of Table 1. Note that there is little regional heterogeneity with respect to the density of these sites.

Discussion

NBT is a neurotoxin that blocks nicotinic receptors in a variety of neuronal preparations, including several regions of the CNS. ^{125}I-NBT has been extremely useful in characterizing and localizing nicotinic receptors in autonomic ganglia, and we have therefore examined its binding in the brain. The binding of ^{125}I-NBT to membranes from the rat caudate-putamen was found to be saturable, reversible, and of high affinity. Values for the dissociation constant of the binding, whether determined by Scatchard or kinetic analyses, were similar (approximately 2–5 nM). It is likely that some of the striatal sites labeled by ^{125}I-NBT correspond to nicotinic receptors that are localized on dopaminergic nerve terminals, because nicotine-stimulated do-
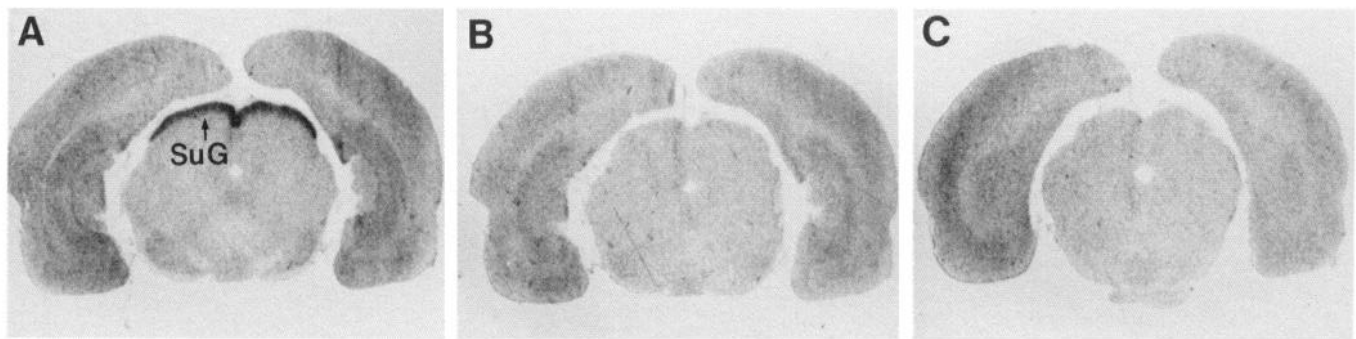
Figure 7. Binding of ¹²⁵I-NBT (2.0 nM) to frozen rat brain sections at level of brain stem. The following competing compounds were present: *left panel*, 0.5 μM αBT; *middle panel*, 0.5 μM αBT + 100 μM dihydro-β-erythroidine; *right panel*, 0.5 μM αBT + 100 μM d-tubocurare.

pamine release is potently blocked by NBT in striatal slices (Schulz and Zigmond, 1989). We have estimated that the K_i of NBT for this inhibitory effect is approximately 5 nM (Schulz and Zigmond, 1989; D. W. Schulz and R. E. Zigmond, unpublished observations). The closeness between this value and the K_d for striatal ¹²⁵I-NBT binding provides indirect evidence that the ¹²⁵I-NBT binding sites in the striatum are functional nicotinic receptors.

The autoradiographic data presented here demonstrate that the density of NBT binding sites is regionally very heterogeneous. Our studies also indicate that 3 pharmacologically distinct NBT binding sites exist in the brain, and that these can be distinguished by their affinity for αBT and for nicotine. Previous workers have demonstrated that there is a very poor correlation between the central distributions of ¹²⁵I-αBT binding and ³H-nicotine binding (Marks and Collins, 1982; Schwartz et al., 1982; Clarke et al., 1985b). Thus, it was of particular interest to compare the distribution of binding sites for NBT, a compound that blocks functional nicotinic activity, with those for these other 2 ligands.

In the avian autonomic ganglia and retina, αBT competes for some but not all ¹²⁵I-NBT binding sites (Chiappinelli, 1983; Loring et al., 1984). The data presented here indicate that, in the rat brain as well, there exist at least 2 sites that bind NBT, only one of which recognizes αBT. αBT has generally been found to be ineffective as an antagonist of neuronal nicotinic receptors. For example, αBT fails to block the nicotine-mediated facilitation of dopamine release in the striatum, while, as noted above, NBT does block this effect (Schulz and Zigmond, 1989). Therefore, it is likely that, in the brain as in the periphery, functional blockade of nicotinic activity by NBT occurs at "NBT-selective" sites (i.e., sites not recognized by αBT). Thus, in addition to examining total and nonspecific ¹²⁵I-NBT binding, we have localized NBT-selective sites in the brain by incubating anatomically adjacent sections with 0.5 μM nonradioactive αBT in order to mask sites that are also recognized by αBT. Note, when comparing the sections shown in Figure 6, that certain densely labeled areas (e.g., parts of the hippocampus and hypothalamus) are no longer prominently labeled when αBT is present. In addition, αBT partially blocks binding of NBT in other areas (e.g., the lateral geniculate). These areas have previously been found to be densely labeled when sections are incubated with nanomolar concentrations of ¹²⁵I-αBT (Segal et al., 1978; Marchand et al., 1979; Clarke et al., 1985b). However, the distribution of ¹²⁵I-αBT binding, for example, within the hippocampal and hy-

pothalamic regions, is much more widespread than is the distribution of the "shared" ¹²⁵I-NBT binding site, suggesting the possibility of multiple, pharmacologically distinct αBT binding sites in the brain. In addition, it should be noted that, while αBT has failed to block neuronal nicotinic receptors in vertebrates in most studies, there are some exceptions, raising the possibility that some of the αBT binding sites in the brain may be functional nicotinic receptors.

Many of the NBT binding sites were not blocked by the presence of a high concentration of αBT. We used the classical nicotinic ligand nicotine to characterize further those αBT-insensitive sites. Areas with high densities of NBT sites recognized by nicotine but not recognized by αBT include the medial habenula, the dorsal lateral geniculate nucleus, the olivary pretectal nucleus, the superficial gray layer of the superior colliculus, and the terminal nuclei of the accessory optic tract. All of these areas have been reported to contain high densities of nicotinic receptors, based on studies using ³H-nicotine autoradiography (Clarke et al., 1985b), immunohistochemical methods with monoclonal antibody 270 (Swanson et al., 1987), and measurement of ¹⁴C-2-deoxyglucose utilization following intravenous administration of nicotine (London et al., 1988). However, ¹²⁵I-NBT binding that is recognized by nicotine is far less widely distributed in the brain than is the labeling resulting from these other methodologies. For example, by comparing the second and third columns of Table 1, it can be seen that this component of ¹²⁵I-NBT binding is negligible in the substantia nigra, the ventral tegmental area, the medial geniculate nucleus, and most of the thalamus. In contrast, the studies cited above using other approaches all report high densities of nicotinic receptors in these regions.

By comparing the autoradiograms depicted in Figure 4, *C* and *D*, it can be seen that a substantial portion of saturable ¹²⁵I-NBT binding sites is not recognized by nicotine. Binding sites with similar properties have also been found in the chick retina (Loring et al., 1989), though the percentage of saturable ¹²⁵I-NBT binding sites that fall into this category is lower in this tissue (approximately 10–15%). These sites are fairly uniformly distributed in different regions of the rat brain. Furthermore, none of 15 different compounds that are pharmacologically active at various types of neurotransmitter receptors was found to recognize this site (e.g., atropine, fluphenazine, GABA, glutamate, glycine, naloxone; data not shown). Taken together, these observations raise questions concerning the functional significance of this category of ¹²⁵I-NBT binding sites.

As noted above, sites that are selectively labeled by ^{125}I-NBT and recognized by nicotine are less widely distributed than either ^{125}I-αBT binding sites or high-affinity ^{3}H-nicotine binding sites. Interestingly, these NBT binding sites appear to be restricted primarily to areas where primary visual afferent fibers are known to terminate. It has long been known that cholinergic neurotransmission exists in the mammalian retina (Masland and Ames, 1976; Ariel and Daw, 1982). At least one type of nicotinic receptor in retinal ganglion cells is recognized by NBT, because patch-clamp recordings from rat retinal ganglion cells in culture have demonstrated that responses to nicotinic agonists are potently blocked by NBT and not by αBT (Lipton et al., 1987; Aizenman et al., 1990). Furthermore, in the chick, there is a close correspondence between those areas of the retina exhibiting high ^{125}I-NBT binding and those having intense ChAT immunoreactivity (Loring et al., 1989). As with the nigrostriatal system, it has been postulated that some nicotinic receptors synthesized by retinal ganglion cells may be transported down the optic nerve and optic tract, and ultimately reside on the axon terminals of these neurons (Francis and Schechter, 1980; Swanson et al., 1987). Because nicotinic receptors have been shown to affect neurotransmitter release in other systems (reviewed by Starke, 1981), it is possible that endogenously released ACh may modulate release of the transmitter utilized by primary visual afferent fibers via a direct action on presynaptically localized nicotinic receptors. The possibility that nicotinic transmission occurs in areas such as the superior colliculus and lateral geniculate nucleus is supported by anatomical studies demonstrating that these areas receive cholinergic afferent fibers that originate in the pontomesencephalic tegmentum (Mesulam et al., 1983; Woolf and Butcher, 1986). Answers to questions regarding the cellular localization of ^{125}I-NBT binding sites in the brain, however, must await further study.

The relatively limited distribution of nicotinic ^{125}I-NBT binding sites, compared to that of ^{3}H-nicotine, raises the possibility that ^{125}I-NBT may label a particular subtype of neuronal nicotinic receptors. The use of a cDNA clone coding for an ACh receptor α subunit from mouse muscle and low-stringency hybridization conditions has led to the isolation of several clones coding for putative neuronal nicotinic receptor α subunits (α_2, α_3, and α_4; Boulter et al., 1986, 1987; Goldman et al., 1986, 1987). There is now direct evidence that there is a family of at least 3 genes, each coding for a different neuronal α subunit (Nef et al., 1988). All of these subunits have structural homology to the muscle-type α subunit (α_1). In addition, several clones coding for a second type of subunit, a β subunit, have been isolated, and it has been proposed that the complete neuronal nicotinic receptor consists of 1 or more α and 1 or more β subunits (Boulter et al., 1987).

The central distributions of mRNAs coding for the α_2, α_3, and α_4 subunits have now been described (Goldman et al., 1986, 1987; Wada et al., 1989), making it possible to compare the localizations of the expression of these subunits with the localization of ^{125}I-NBT binding sites. Of course, it is impossible to make direct comparisons between results obtained using these 2 methodologies. *In situ* hybridization identifies cell bodies that contain the mRNA that encodes a particular protein; however, the protein itself may be concentrated in another part of the neuron (e.g., the nerve terminal). This issue is of particular importance if one considers the possibility that some nicotinic receptors may be axonally transported over distances of several millimeters in the brain. Nevertheless, it is still informative to compare the results from *in situ* hybridization with the present findings concerning the localization of ^{125}I-NBT binding sites in the rat brain.

Overall, there is no obvious correspondence between the distribution of ^{125}I-NBT binding sites and the presence of mRNA coding for any of the 3 α subunits that have been characterized thus far. Expression of the α_2 gene in the rat brain appears to be restricted to the interpeduncular nucleus, an area that does not have appreciable ^{125}I-NBT binding (Boulter et al., 1986). The distributions of expression of α_3 and α_4 genes are much more extensive than that of α_2, but include areas where little ^{125}I-NBT binding is detected (Wada et al., 1989). However, in light of the high densities of ^{125}I-NBT binding sites found in areas receiving projections from the retina, it is interesting to note that both the α_3 and α_4 genes are expressed in retinal ganglion cells (Wada et al., 1989).

When the α_2 subunit was expressed in frog oocytes together with the β_2 subunit, a functional nicotinic receptor was formed that was not blocked by NBT or by αBT (Wada et al., 1988). However, when either the α_3 or the α_4 subunit mRNA was injected with the β_2 subunit mRNA, the nicotinic receptor that was formed was blocked by NBT, though not by αBT (Boulter et al., 1987). Interestingly, the potency of NBT is considerably higher for blocking the combination $\alpha_3\beta_2$ than for the combination $\alpha_4\beta_2$ (Luetje and Patrick, 1989). The issue of functional blockade is especially important when one considers that ^{125}I-NBT binding is relatively low in the cortex and the caudate-putamen, 2 regions where NBT has been shown to block nicotinic activity in brain slices *in vitro* (Schulz and Zigmond, 1989; Vidal and Changeux, 1989). Thus, it is unlikely that NBT blockade of nicotinic receptor-mediated effects is restricted only to those areas prominently labeled by ^{125}I-NBT in the present study. Finally, it has recently been found that injection of oocytes with mRNA for the α_3 subunit and for the newly discovered β_4 subunit leads to the formation of a nicotinic receptor that is insensitive to NBT (Duvoisin et al., 1989), indicating that the relationship between NBT sensitivity and, probably, binding and the presence of a particular α subunit in a neuron may be complex. This complexity is illustrated by the recent report of Mulle and Changeux (1990) that a nicotinic response recorded from enzymatically dissociated neurons from the habenula of 10–20-d-old rats is blocked neither by αBT nor by NBT. *In situ* hybridization studies indicate that the medial habenula contains mRNA for the α_3, α_4, β_2 (Wada et al., 1989), and β_4 (Duvoisin et al., 1989) subunits, and furthermore, that these subunits are not uniformly distributed throughout the nucleus (Wada et al., 1989).

In conclusion, we have demonstrated that NBT, a neurotoxin that selectively blocks nicotinic responses in a variety of neuronal preparations, may be used as a high-affinity ligand to study nicotinic receptors in the rat brain. Specific binding sites for ^{125}I-NBT may be divided into 3 categories: (1) those that are recognized by both NBT and αBT, (2) those that are *not* recognized by αBT but are recognized by nicotine, and (3) those that are recognized only by nonradioactive NBT and not by other nicotinic ligands. Binding sites included in the first 2 categories are heterogeneously distributed in the brain. However, because NBT, but not αBT, blocks nicotinic responses in most neuronal preparations studied, it is proposed that the second category probably represents the "physiologically relevant" component of ^{125}I-NBT binding. Areas with significantly high densities of these sites are the medial habenula and various

regions that receive primary visual input from retinal ganglion cells. The relatively limited distribution of these sites suggests that ^{125}I-NBT may bind to a particular subclass of nicotinic receptors in the brain. The regional localization of these sites, as described here, should aid future studies on the functional importance of nicotinic synaptic transmission in the brain.

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