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# Nicotinic Binding in Rat Brain: Autoradiographic Comparison of [$^3$H]Acetylcholine, [$^3$H]Nicotine, and [$^{125}$I]-$\alpha$-Bungarotoxin[1]

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## Abstract

**Three radioligands have been commonly used to label putative nicotinic cholinoceptors in the mammalian central nervous system: the agonists [$^3$H]nicotine and [$^3$H]acetylcholine ([$^3$H]ACh—in the presence of atropine to block muscarinic receptors), and the snake venom extract, [$^{125}$I]-$\alpha$-bungarotoxin([$^{125}$I]BTX), which acts as a nicotinic antagonist at the neuromuscular junction. Binding studies employing brain homogenates indicate that the regional distributions of both [$^3$H]nicotine and [$^3$H]ACh differ from that of [$^{125}$I]BTX. The possible relationship between brain sites bound by [$^3$H]nicotine and [$^3$H]ACh has not been examined directly. We have used the technique of autoradiography to produce detailed maps of [$^3$H]nicotine, [$^3$H]ACh, and [$^{125}$I]BTX labeling; near-adjacent tissue sections were compared at many levels of the rat brain. The maps of high affinity agonist labeling are strikingly concordant, with highest densities in the interpeduncular nucleus, most thalamic nuclei, superior colliculus, medial habenula, presubiculum, cerebral cortex (layers I and III/IV), and the substantia nigra pars compacta/ventral tegmental area. The pattern of [$^{125}$I]BTX binding is strikingly different, the only notable overlap with agonist binding being the cerebral cortex (layer I) and superior colliculus. [$^{125}$I]BTX binding is also dense in the inferior colliculus, cerebral cortex (layer VI), hypothalamus, and hippocampus, but is virtually absent in thalamus. Various lines of evidence suggest that the high affinity agonist-binding sites in brain correspond to nicotinic cholinergic receptors similar to those found at autonomic ganglia; BTX-binding sites may also serve as receptors for nicotine and are possibly related to neuromuscular nicotinic cholinoceptors.**

The concept of receptors for nicotine is an old one (Langley, 1905). In the peripheral nervous system, two types of receptor, namely, ganglionic (C-6) and neuromuscular (C-10), have been distinguished on the basis of antagonist selectivity (Paton and Zaimis, 1951). The existence of functional nicotine receptors in the central nervous system (CNS) is strongly suggested by various experimental approaches—electrophysiological (Krnjevic, 1975), biochemical (Giorguieff et al., 1979), physiological (Armitage and Hall, 1967), and behavioral (Clarke and Kumar, 1983).

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Putative central nicotinic receptors have been labeled using various ligands with known peripheral actions. *d*-Tubocurarine, $\alpha$-bungarotoxin (BTX), and *Naja-naja siamensis* $\alpha$-toxin all block nicotinic-cholinergic transmission at the neuromuscular junction and bind to rat brain membranes with high affinity (Speth et al., 1977; Nordberg and Larsson, 1980; Schmidt et al., 1980). Binding of [$^{125}$I]BTX, the most widely used radioligand, is saturable and reversible, and is displaced preferentially by nicotinic agents (Schmidt et al., 1980); hence, the BTX-binding site has been termed "nicotinic," and the displacement potency of nicotine *in vitro* suggests that nicotine could act at BTX sites *in vivo*.

More recently, the high affinity binding of agonists to putative nicotinic cholinoceptors in rodent brain membranes has also been demonstrated. Studies have employed either [$^3$H]nicotine (Yoshida and Imura, 1979; Romano and Goldstein, 1980; Marks and Collins, 1982; Costa and Murphy, 1983) or [$^3$H]acetylcholine ([$^3$H]ACh) in the presence of excess atropine to block muscarinic receptors (Schwartz et al., 1982). Binding of either ligand was potently displaced by nicotinic agonists, and both ligands had an affinity constant in the nanomolar range. There is no clear consensus as to the regional distribution of [$^3$H]nicotine-binding sites in brain (Yoshida and Imura, 1979; Marks and Collins, 1982; Costa and Murphy, 1983). Marks and Collins (1982) found a lack of correlation between the regional distributions of [$^3$H]nicotine and [$^{125}$I]BTX binding in mouse brain. A comparison of nicotinic [$^3$H]ACh binding and [$^{125}$I] BTX binding in rat brain reached the same conclusion (Schwartz et al., 1982). Clearly, nicotinic agonists and BTX do not always label the same molecule in brain.

Recently, we have used the autoradiographic method of Herkenham and Pert (1982) to visualize [$^3$H]-DL-nicotine binding to unfixed rat brain sections (Clarke et al., 1984). The binding was displaced by the unlabeled isomers of nicotine in a stereoselective manner; natural L-nicotine was approximately 17 times more potent than D-nicotine. The pharmacological displacement profile and binding affinity constant for [$^3$H]nicotine were similar to those previously found in membrane studies. The pattern of labeling was highly discrete and differed from that previously reported for BTX (Hunt and Schmidt, 1978; Segal et al., 1978). In addition, quantitative autoradiography recently has been used to identify nicotinic binding sites labeled by [$^3$H]ACh in rat brain slices (Rainbow et al., 1984). The kinetic constants and pharmacologic profile were similar to those obtained using brain homogenates (Schwartz et al., 1982). Again, the pattern of labeling was unlike that reported for BTX (Hunt and Schmidt, 1978; Segal et al., 1978). In the study described below, autoradiographic maps of [$^3$H]ACh, [$^3$H]nicotine, and [$^{125}$I]BTX were obtained from near-adjacent sections of rat brain; the resulting comparisons reveal that [$^3$H]ACh and [$^3$H]nicotine bind with a strikingly similar pattern which shows remarkably little overlap with [$^{125}$I] BTX labeling.

## Materials and Methods

Slide-mounted rat brain sections were prepared as previously described (Herkenham and Pert, 1982). Three male Sprague-Dawley rats (250 gm,

Taconic Farms, NY) were used. Two brains provided coronal sections; the third provided sagittal sections. Following decapitation, the brains were rapidly removed, immersed in isopentane at −35°C, and mounted on cryostat chucks. Cutting was performed at −16°C. At intervals through each brain, groups of three adjacent or near-adjacent sections were taken. Such a group never spanned more than 150 μm in the anterior-posterior direction. Each section in a group of three was transferred to a different subbed microscope slide and eventually was incubated with a different ligand. Sections allocated to [$^3$H]nicotine or [$^3$H]ACh were 24 μm thick. For incubation with [$^{125}$I]BTX, coronal sections from one rat were cut at 24 μm to permit direct comparisons with the autoradiographs of tritiated ligand binding; the remaining coronal and sagittal sections were cut at 12 μm in order to improve image definition obtained with the iodinated ligand. Sections were thaw-mounted on pre-cleaned gelatin-coated slides, dried overnight under partial vacuum at 4°C, and stored with dessicant at −70°C for at least 24 hr.

For autoradiography, the following general method was employed: slides were preincubated, incubated with radioligand (with or without the appropriate displacing drug to define nonspecific binding), rinsed in several changes of buffer, and rapidly dried under a stream of compressed air. Slides were left at room temperature and pressure in a dessicating jar for 24 hr and then juxtaposed tightly against tritium-sensitive film (Ultrofilm; LKB Instruments, Gaithersburg, MD) and stored at room temperature for several weeks in x-ray cassettes (Wolf: CGR Scientific, Columbia, MD). Films were processed in Kodak D19 developer at 22°C for 5 min and fixed for 4 min with Kodak Rapid Fixer (without hardener). The tissue sections were then demyelinated (defatted) through aqueous ethanol and xylene, before staining with thionin. Structures were identified by comparing stained sections with enlarged photographs of corresponding autoradiographic images.

The exact conditions employed were optimized for each ligand. For [$^3$H] nicotine autoradiography, there was no preincubation step, and sections were incubated at room temperature for 20 min in 50 mM Tris-HCl (pH 7.4; Sigma Chemical Co., St. Louis, MO), 8 mM $CaCl_2$, and 3.5 nM [$^3$H]DL-nicotine (*N-methyl*-$^3$H, 71.2 Ci/mmol, New England Nuclear, Boston, MA). Nonspecific binding was assessed in the presence of 10 μM L-nicotine bitartrate (BDH Chemicals, Ltd., Poole, England). Following incubation, the sections were immediately given four rinses of 30 sec each in buffer as described above, pH 7.4, at 4°C. Storage in cassettes lasted 12 weeks.

For [$^3$H]ACh autoradiography a modification of the procedure recently reported by Rainbow et al. (1984) was used. Sections were preincubated for 15 min at room temperature in 50 mM Tris-HCl buffer (pH 7.4) containing 1.5 μM atropine sulfate, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$. A second preincubation of 10 min duration followed in buffer of the same composition but with 100 μM di-isopropyl fluorophosphate added. Sections were then incubated for 1 hr at 4°C in buffer containing 10 nM [$^3$H]ACh (80 Ci/mmol, synthesized as described by Schwartz et al., 1982); nonspecific binding was assessed in the presence of carbachol (100 μM). Following incubation, the sections were given two rinses, each of 2 min, in 500 ml of buffer at 4°C. Finally, the sections were briefly rinsed in distilled water (2 sec) and dried. Storage in cassettes was for 5 weeks.

For [$^{125}$I]BTX binding, the sections were preincubated for 30 min at room temperature in 50 mM Tris-HCl (Sigma; pH 7.4) and 1 mg/ml of bovine serum albumin (Sigma). Incubation was 2 hr duration, carried out at room temperature, using the same buffer composition but including [$^{125}$I]BTX (50 to 61 Ci/mmol, New England Nuclear, used within 1.5 half-lives of synthesis). Prior to [$^{125}$I]BTX autoradiography, Scatchard and structure-activity analyses were performed using tissue sections which were processed as for autoradiography but which were scraped into tubes for gamma counting (Beckman 4000 counter; 60% efficiency) after drying. In these biochemical experiments, coronal sections (24 μm thick) were taken from a forebrain region with a minimal longitudinal gradient of binding (interaural 10.5 to 8.5, according to Paxinos and Watson, 1982). The structure-activity study served to verify that the displacement profile was nicotinic in tissue sections, as in homogenates; this was considered particularly important, since the pharmacological properties of commercially supplied BTX can vary according to the source (Costa et al., 1983). In the Scatchard experiment, six concentrations of [$^{125}$I]BTX were used, ranging from 0.18 nM to 7.8 nM. The structure-activity and autoradiographic studies utilized a concentration of 1.4 nM. Nonspecific binding was assessed by the addition of L-nicotine bitartrate (1 mM) to the preincubation and incubation media. Sections were given six rinses of 30 min each in 500 ml of buffer, pH 7.4, and 4°C. Storage in cassettes was for 14 days.

## Results

*Biochemical study of [$^{125}$I]BTX binding.* Figure 1 shows a saturation curve and Scatchard analysis of [$^{125}$I]BTX binding which reveals a single class of binding sites with an apparent affinity constant ($K_D$) of about 1.2 nM and number of binding sites ($B_{max}$) of about 1150 dpm/24-μm section (4.4 fmol/mg of tissue). The ratio of

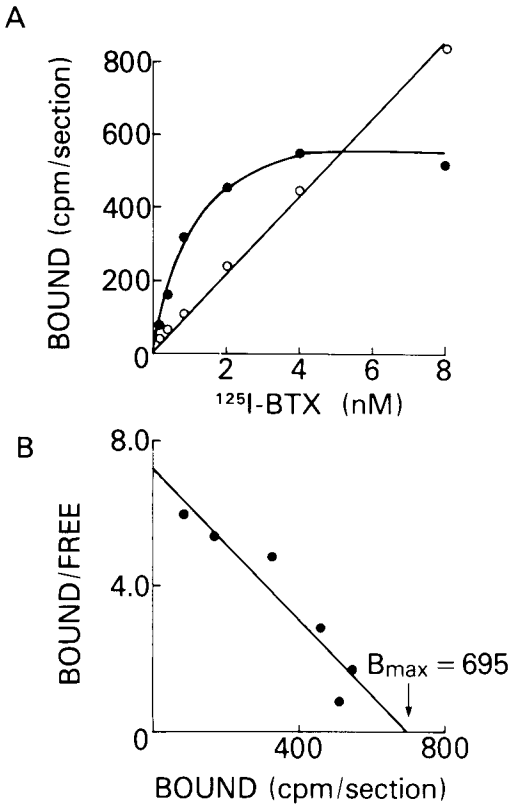



*Figure 1.* *A*, Saturation analysis of [$^{125}$I]BTX binding to unfixed rat brain sections. Frozen brain sections (24 μm thick) were thaw-mounted on subbed slides and preincubated for 30 min at 22°C in 50 mM Tris-HCl and 1 mg/ml of bovine serum albumin. Incubation was for 2 hr in the presence of [$^{125}$I] BTX (0.18 to 7.8 nM). Following incubation, sections were washed (3 hr), dried and scraped for gamma counting. Nonspecific binding (O) was determined in the presence of 1 mM L-nicotine bitartrate. Specific binding (●) is the difference between total binding (in the absence of displacing drug) and nonspecific binding. Each *data point* represents the mean counts from four brain sections. *B*, Scatchard analysis of the specific binding of [$^{125}$I]BTX. The $K_D$ and $B_{max}$ were determined by least squares regression.

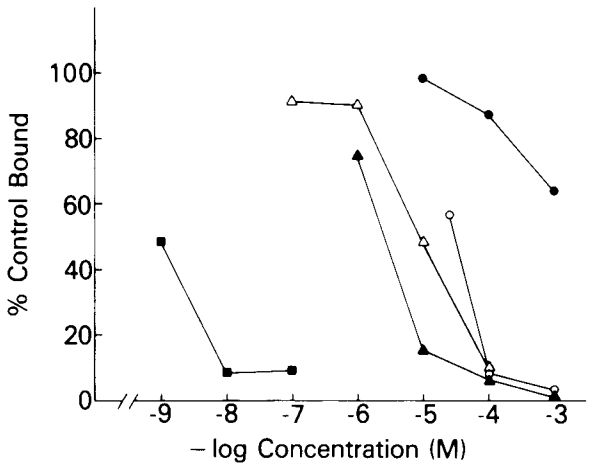



*Figure 2.* Displacement of [$^{125}$I]BTX binding in unfixed brain sections by various drugs. Slide-mounted brain sections were labeled with [$^{125}$I]BTX (1.4 nM) in the presence of various concentrations of displacing drugs (see the text): BTX, ■; L-nicotine, ▲; *d*-tubocurarine, △; acetylcholine, O; atropine, ●. Nonspecific binding was determined in the presence of 1 mM L-nicotine. Each *data point* represents the mean of six brain sections.

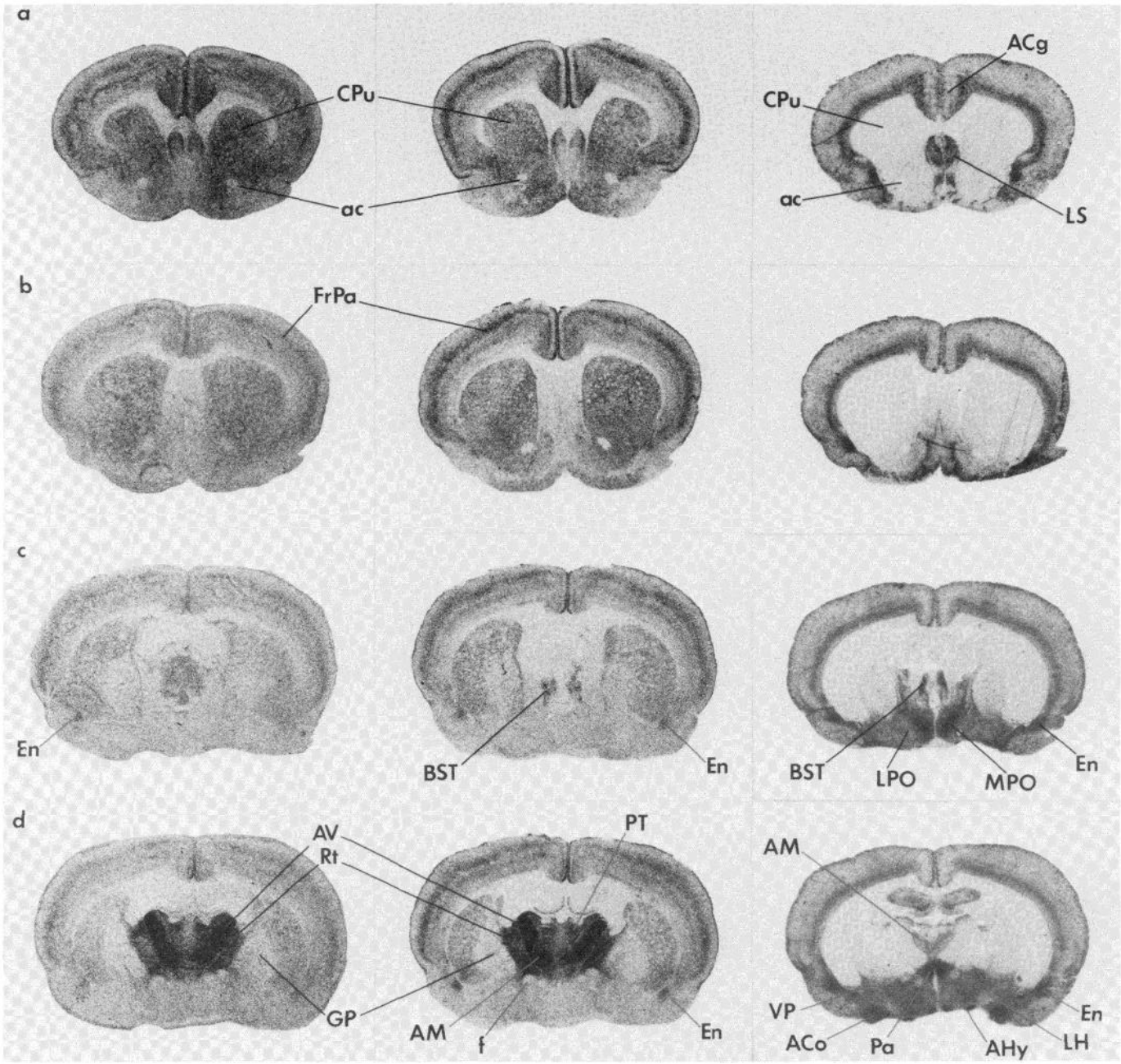
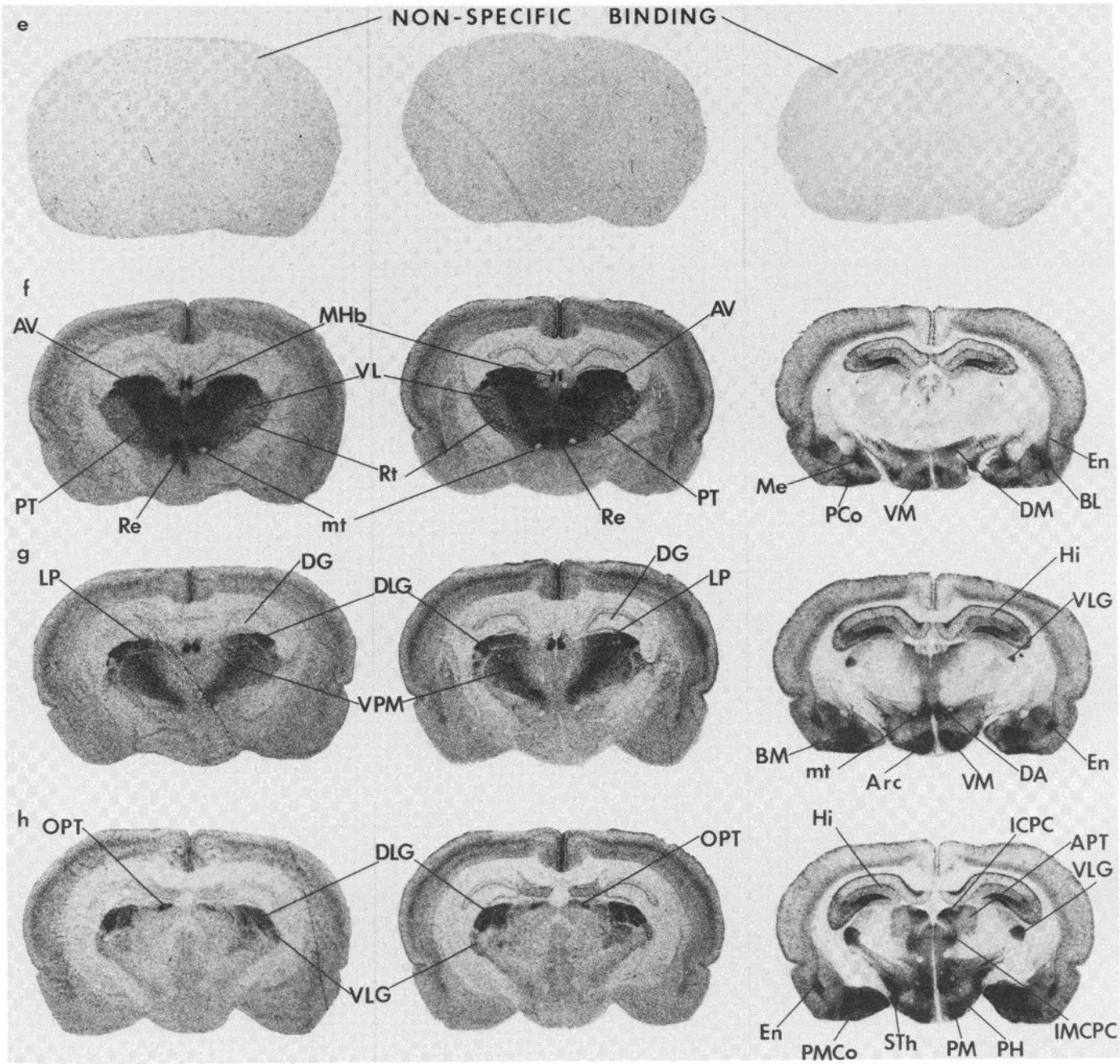



*Figure 3.* Autoradiographic images showing the distribution of [$^3$H]ACh (*left*), [$^3$H]nicotine (*middle*) and [$^{125}$I]BTX (*right*) labeling in rat brain. Near-adjacent brain sections were prepared unfixed and incubated with radioligand (see the text). Nonspecific binding was assessed in the presence of L-nicotine bitartrate (10 $\mu$M for [$^3$H]nicotine; 1 mM for [$^{125}$I]BTX) or carbachol (100 $\mu$M for [$^3$H]ACh). Images *a* to *n* represent coronal sections taken consecutively along a rostrocaudal axis. Images *o* and *p* represent parasagittal sections. Coronal and parasagittal sections were enlarged by constant but different factors. *ac,* anterior commissure; *Acb,* accumbens nucleus; *ACg,* anterior cingulate cortex; *ACo,* anterior cortical amygdaloid nucleus; *AHy,* anterior hypothalamic area; *AM,* anteromedial thalamic nucleus; *AO,* anterior olfactory nuclei; *APT,* anterior pretectal area; *Arc,* arcuate hypothalamic nucleus; *AV,* anteroventral thalamic nucleus; *BL,* basolateral amygdaloid nucleus; *BM,* basomedial amygdaloid nucleus; *BST,* bed nucleus of the stria terminalis; *CA4,* field CA4 of Ammon's horn, hippocampus; *Cb,* cerebellum; *CG,* central (periaqueductal) gray; *CPu,* caudate putamen; *DA,* dorsal hypothalamic area; *DCo,* dorsal cochlear nucleus; *DG,* dentate gyrus; *DLG,* dorsal lateral geniculate nucleus; *DM,* dorsomedial hypothalamic nucleus; *DPB,* dorsal (lateral) parabrachial nucleus; *DR,* dorsal raphe nucleus; *DTg,* dorsal tegmental nucleus; *En,* endopiriform nucleus; *f,* fornix; *FrPa,* Frontoparietal cortex; *Gi,* gigantocellular reticular nucleus; *GP,* globus pallidus; *Hi,* hippocampus; *IC,* inferior colliculus; *ICPC,* Intracommissural nucleus of the posterior commissure; *IMCPC,* interstitial magnocellular nucleus of the posterior commissure; *IO,* inferior olive; *IPN,* interpeduncular nucleus; *LH,* lateral hypothalamic area; *LM,* lateral mammillary nucleus; *LP,* lateral posterior thalamic nucleus (pulvinar); *LPO,* lateral preoptic area; *LS,* lateral septal nucleus; *LSO,* lateral superior olive; *Me,* medial amygdaloid nucleus; *MG,* medial geniculate nucleus; *MHb,* medial habenular nucleus; *ML,* medial mammillary nucleus, lateral part; *MPO,* medial preoptic area; *MSO,* medial superior olive; *mt,* mammillothalamic tract; *MVe,* medial vestibular nucleus; *OPT,* olivary pretectal nucleus; *Pa,* paraventricular hypothalamic nucleus; *PCo,* posterior cortical amygdaloid nucleus; *PGi,* paragigantocellular reticular nucleus; *PH,* posterior hypothalamic nucleus; *PM,* paramedian lobule; *PMCo,* Posteromedial cortical amygdaloid nucleus; *PT,* paratenial thalamic nucleus; *PVM,* premammillary nucleus, ventral part; *Re,* reuniens thalamic nucleus; *RLi,* rostral linear nucleus of the raphe; *RMg,* raphe magnus nucleus; *RPn,* raphe pontis nucleus; *RSpl,* retrosplenial cortex; *Rt,* reticular thalamic nucleus; *SNC,* substantia nigra, compact part; *Sp50,* nucleus of the spinal tract of the trigeminal nerve, oral part; *STh,* subthalamic nucleus; *SuG,* superficial gray layer of the superior colliculus; *SuM,* supramammillary nucleus; *VL,* ventrolateral thalamic nucleus; *VLG,* ventral lateral geniculate nucleus; *VM,* ventromedial hypothalamic nucleus; *VP,* ventral pallidum; *VPB,* ventral (medial) parabrachial nucleus; *VPM,* ventroposterior thalamic nucleus, medial part; *VTA,* ventral tegmental area; *ZI,* zona incerta.



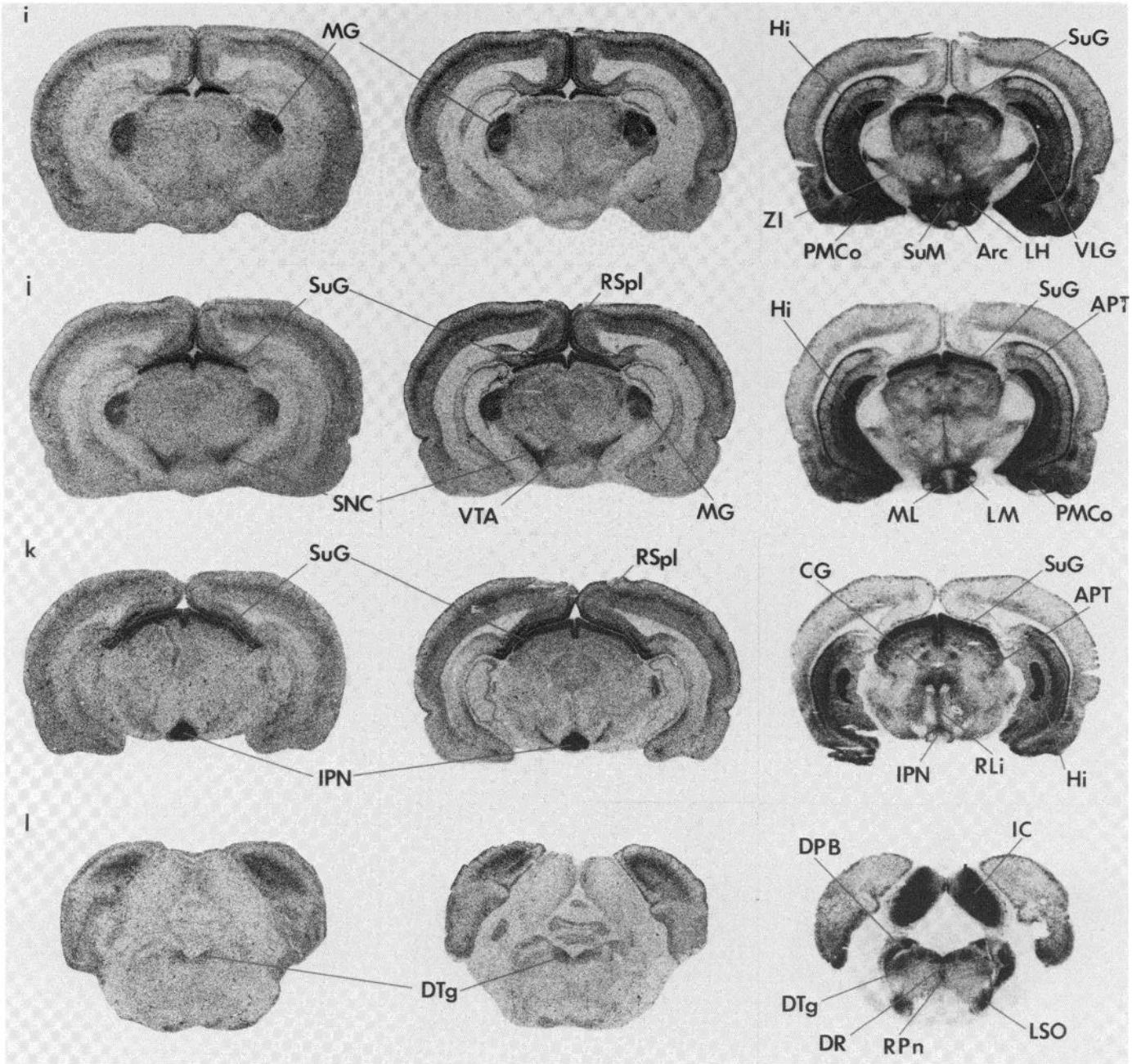

displaceable binding to total binding varied from 37% at 7.8 nM to 81% at 0.38 nM [$^{125}$I]BTX.

The structure-activity data, shown in Figure 2, confirm that [$^{125}$I] BTX is potently displaced by BTX itself ($IC_{50}$~1 nM) and is less potently displaced by L-nicotine bitartrate ($IC_{50}$~3 $\mu$M), *d*-tubocurarine ($IC_{50}$~10 $\mu$M), and ACh ($IC_{50}$~30 $\mu$M). All four nicotinic agents displaced at least 90% of specific binding. Atropine, a muscarinic antagonist, also displaced [$^{125}$I]BTX but was much less potent ($IC_{50}$~2 mM).

*Autoradiographic labeling.* No clear qualitative differences were found in the patterns of [$^3$H]ACh and [$^3$H]nicotine labeling, whereas [$^{125}$I]BTX bound with a completely different distribution (see Fig. 3). Possibly on account of the incubation conditions, the tissue quality of sections which had been incubated with [$^3$H]ACh was poorer than that of [$^3$H]nicotine-incubated sections; this difference is reflected in certain figures.

The autoradiographs of [$^3$H]ACh and [$^3$H]nicotine demonstrated high densities of labeling in the following structures: interpeduncular nucleus, all thalamic nuclei except posterior and intralaminar, superior colliculus, and medial (but not lateral) habenula. Binding was also prominent in the substantia nigra pars compacta and ventral tegmental area, molecular layer of the dentate gyrus (at least for [$^3$H] nicotine), presubiculum, and cerebral cortex. Cortical laminae III/IV were preferentially labeled; lamina I was labeled strongly by [$^3$H] nicotine and less clearly by [$^3$H]ACh. Moderate densities of labeling were found in neostriatum, ventral striatum, dorsal tegmental nucleus, and cerebellum.

[$^{125}$]BTX binding had an equally discrete distribution pattern. As expected, sections cut at 24 $\mu$m (not shown) gave darker autoradiographic imges, but the pattern of [$^{125}$I]BTX labeling was identical to that produced by 12-$\mu$m sections. In general, binding was highest in cerebral cortex, hypothalamus, hippocampus, inferior colliculus,




and in certain brainstem nuclei. In cerebral cortex, laminae I and VI were strongly labeled. Within the hypothalamus, binding was particularly dense in the mammillary body and in the suprachiasmatic, supraoptic, paraventricular, and posterior nuclei. The CA4 area of the hippocampus was also densely labeled. Regions of pons and medulla with appreciable [$^{125}$I]BTX binding included locus coeruleus, dorsal tegmental nucleus, dorsal and ventral parabrachial nuclei, inferior and lateral superior olive, medial vestibular nucleus, dorsal cochlear nucleus, gigantocellular reticular nucleus, and the nucleus of the spinal tract of the trigeminal nerve (oral part). The thalamus and striatum appeared devoid of binding, and there was little or no labeling in substantia nigra or ventral tegmental area. Other labeled areas included the medial septal nucleus, amygdala (basolateral, lateral, and medial nuclei), ventral lateral geniculate nucleus, and interpeduncular nucleus. The superior colliculus (superficial layers) appears unique in showing dense labeling by all three ligands.

## Discussion

A qualitative comparison of the labeling patterns produced by [$^{3}$H]nicotine and [$^{3}$H]ACh revealed a striking concordance. No clear differences were apparent. Binding of both tritiated agonists is displaced by unlabeled L-nicotine and ACh with very similar potency (Schwartz et al., 1982; Costa and Murphy, 1983; Clarke et al., 1984), and in the absence of conflicting evidence, it seems likely that [$^{3}$H]nicotine and [$^{3}$H]ACh label the same molecule. In contrast, use of iodinated BTX, which also has a nicotinic displacement profile, resulted in a very different distribution pattern. There was little overlap between tritiated agonist and iodinated BTX labeling. Significantly, neither distribution represented a subset of the other; hence, the marked difference of labeling patterns produced by agonists and by BTX cannot be explained either by differential quenching of tritium (Herkenham, 1983) or by the possible presence of contaminants in the sample of [$^{125}$I]BTX. The displacement study reported

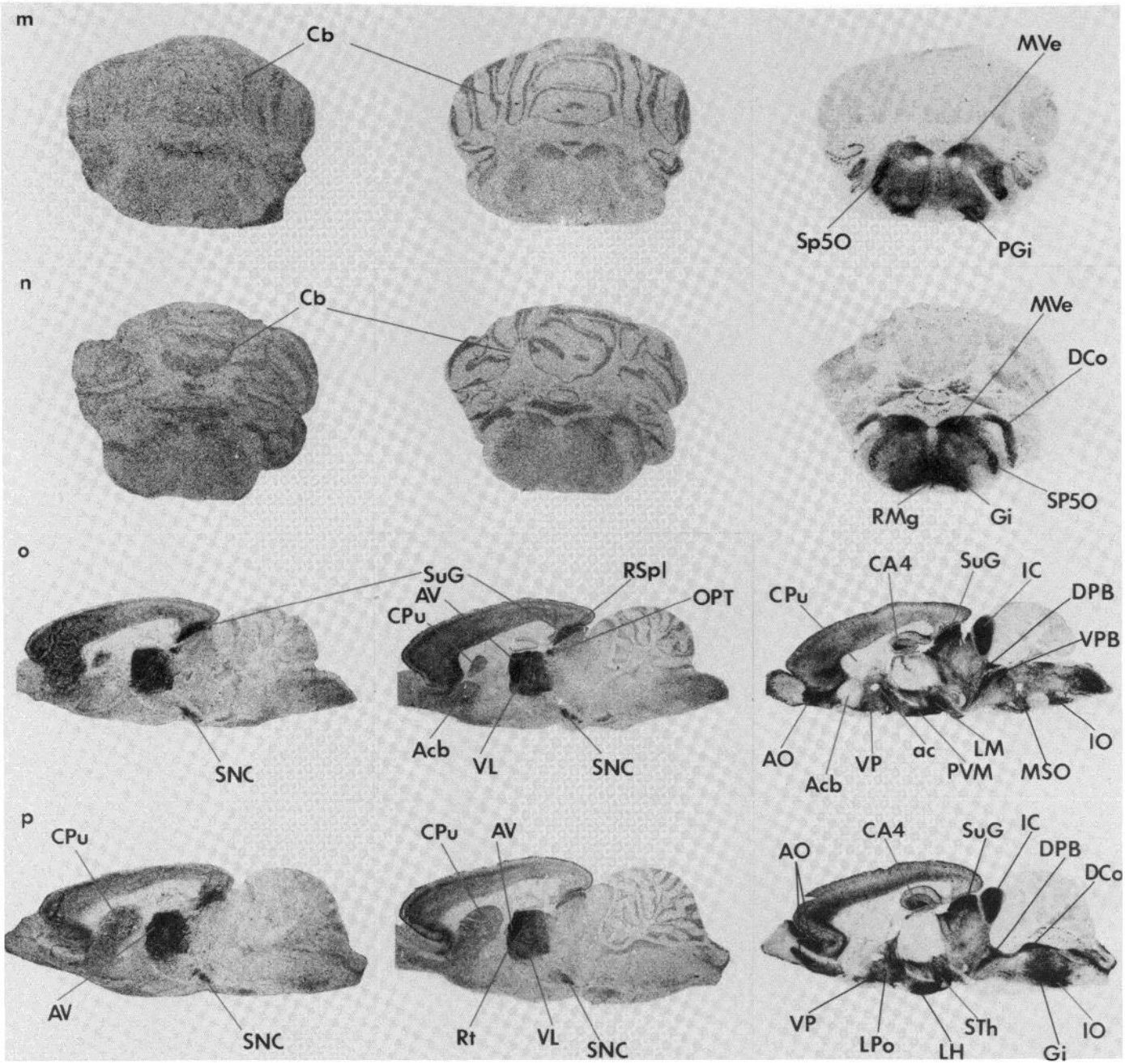



*Figure 3 M–P*

here confirms that nicotine and ACh displace [$^{125}$I]BTX binding only at micromolar concentrations, and it is not surprising that the two radiolabeled agonists failed to label BTX-binding sites at the nanomolar concentrations used. Conversely, high concentrations of BTX are required to displace the high affinity binding of tritiated nicotine or ACh to brain tissue (Romano and Goldstein, 1980; Marks and Collins, 1982; Schwartz et al., 1982; Hayashi et al., 1984).

The autoradiographs showed high densities of tritiated agonist labeling in the thalamus, whereas the hypothalamus and hippocampus appeared almost devoid of binding. Schwartz et al. (1982) reported a similar but less pronounced pattern of [$^{3}$H]ACh binding on the basis of regional dissection, and other studies have also revealed high binding densities of [$^{3}$H]nicotine in the thalamus (Yoshida and Imura, 1979; Abood et al., 1981; Marks and Collins, 1982). However, dense binding of [$^{3}$H]nicotine to hippocampus and/or hypothalamus has also been reported (Yoshida and Imura 1979; Abood et al., 1981; Costa and Murphy, 1983). At present, the source of these discrepancies is unclear. Conceivably, these two structures could contain binding sites of somewhat lower affinity which were not detected by our procedure.

The distribution of [$^{125}$I]BTX-binding sites in rodent brain has already been described (Morley et al., 1977; Hunt and Schmidt, 1978; Segal et al., 1978; Arimatsu et al., 1981). The autoradiographs shown here represent the only nonschematic atlas of [$^{125}$I]BTX labeling. Our results are generally in excellent agreement with previous work. Hunt and Schmidt (1978) found moderate [$^{125}$I]BTX binding localized in cerebral cortex layers, I, V, and VI, whereas Segal et al. (1978) reported low levels of labeling, scattered homogeneously throughout the neocortex; we concur with the former. Dense labeling was reported in medial habenula (Segal et al., 1978), but we found no evidence of this. The dense labeling of the interpeduncular nucleus reported previously in rat brain (Hunt and

Schmidt, 1978) was not seen, although in the rat, as in the mouse (Arimatsu et al., 1981), a subnuclear distribution is apparent (Rotter and Jacobowitz, 1984).

Clearly, there are at least two distinct populations of nicotinic binding sites in rat brain. Do either of these sites represent the functional receptors where nicotine and/or ACh act? As outlined below, present evidence suggests that the agonists ACh and nicotine label nicotinic-cholinergic receptors with pharmacological properties similar to those of ganglionic C-6-type receptors, whereas BTX may label another kind of nicotinic receptor, possibly similar to C-10-type cholinoceptors found at the neuromuscular junction.

Consistent with a cholinergic role, [$^3$H]nicotine and [$^3$H]ACh binding sites are found within previously described cholinergic systems (Hoover et al., 1978; Armstrong et al., 1983; Mesulam et al., 1983). Certain cell body areas, notably the dorsal tegmental nucleus and medial septum, were lightly labeled, but others such as the ventral pallidal region appeared to be devoid of binding. Terminal regions contained appreciable binding, including cingulate cortex (Sikes et al., 1983), layers I and III of retrosplenial cortex (Tenglesen and Robertson, 1982), anteroventral nucleus of thalamus (Rotter and Jacobowitz, 1981), and possibly the superior colliculus (Kvale et al., 1983). The pattern of labeling in the substantia nigra resembled that of acetylcholinesterase (Paxinos and Watson, 1982), and this structure appears to receive a cholinergic innervation (McGeer et al., 1984).

The existence of C-6-type nicotinic receptors in the CNS is suggested by pharmacological studies, in which a central action of nicotine is blocked by the administration of ganglion-specific antagonists which penetrate centrally (Yamamoto and Domino, 1965; Stitzer et al., 1970; Romano et al., 1981; Tripathi et al., 1982; Clarke and Kumar, 1983, 1984; Clarke, 1984). Two electrophysiological studies merit particular attention. Brown et al. (1983) recorded the responses of single units in the interpeduncular nucleus, a structure which has a high density of nicotinic agonist binding throughout, but a relatively low and localized density of BTX binding (see Fig. 3*k*). Many units were excited by ACh or carbachol, and excitation was greatly reduced by C-6-specific antagonists; BTX was ineffective. Lichtensteiger et al. (1982) reported that systemic or microiontophoresed nicotine or ACh excited dopaminergic cells in the substantia nigra pars compacta. This excitation can be prevented by administration of the centrally active, ganglion-specific blocker mecamylamine (P. B. S. Clarke, D. W. Hommer, A. Pert, and L. R. Skirboll, manuscript submitted for publication). Significantly, dopaminergic neurons of the substantia nigra possess [$^3$H]nicotine-binding sites (P. B. S. Clarke and A. Pert, manuscript submitted for publication), whereas this structure has a very low density of [$^{125}$I] BTX binding (Fig. 3*i*; see Hunt and Schmidt, 1978; Arimatsu et al., 1981).

Nicotine and ACh exert pharmacological actions when applied in the low micromolar range, both *in vitro* and *in vivo* (Schechter and Jellinek, 1975; Giorguieff et al., 1977, 1979; Yoshida et al., 1980). In contrast, both [$^3$H]nicotine and [$^3$H]ACh bind to brain membranes with nanomolar affinity. To account for this disparity, Romano and Goldstein (1980) and Schwartz and Kellar (1983a) suggested that, upon prolonged incubation with agonists, the nicotinic receptors may undergo a shift of affinity to an agonist-selective state, perhaps analogous to the phenomenon of *in vivo* desensitization. In the periphery, nicotine induces tachyphylaxis more readily at autonomic ganglia than at the neuromuscular junction (Paton and Savini, 1968). Tachyphylaxis at ganglia may outlast the initial period of depolarization blockade, and as discussed by Brown (1980), the residual lack of responsiveness is consistent with a change occurring at the nicotinic cholinoceptor and/or at associated ionic channels. Nicotine-induced tachyphylaxis (or "acute tolerance") is commonly encountered in the CNS (Domino, 1965; Yamamoto and Domino, 1965; Barrass et al., 1969; Schechter and Rosecrans, 1972; Stolerman et al., 1973; Tripathi et al., 1982). In several instances, central pharmacological actions of nicotine which are susceptible to tachyphylaxis have been prevented by a ganglion-specific antagonist (Domino, 1965; Yamamoto and Domino, 1965; Barrass et al., 1969; Romano et al., 1981; Tripathi et al., 1982; Clarke, 1984).

An alternative explanation of the disparity between high binding affinity and relatively low biological potency is suggested by observations in *Torpedo*, where the nicotinic-cholinergic macromolecule is proposed to possess two classes of binding sites: a low affinity site to which ACh binds, thus activating opening of the ion channel, and a higher affinity site which mediates desensitization (Conti-Tranconi et al., 1982). Since nicotinic antagonists need not occupy the agonist recognition site(s) in order to prevent channel opening (Conti-Tranconi and Raftery, 1982), it is not surprising that many nicotinic antagonists fail to inhibit the binding of tritiated agonists to brain tissue. Of possible significance, the ganglion-specific blocker surugatoxin potently inhibits tritiated nicotine binding to rat brain membranes, whereas antagonists which act selectively at the neuromuscular junction (e.g., BTX, decamethonium) have little or no effect (Schwartz et al., 1982; Hayashi et al., 1984).

Chronic drug studies *in vivo* support the suggestion that high affinity agonist-binding sites are the receptors where both nicotine and endogenous ACh act pharmacologically. In particular, chronic nicotine administration leads to an increase in the density of [$^3$H] nicotine- and [$^3$H]ACh-binding sites in several brain regions (Marks et al., 1983; Schwartz and Kellar, 1983b, 1985). Tolerance to certain acute effects of nicotine was found shortly after the termination of chronic nicotine infusion (Marks et al., 1983), consistent with the occurrence of tachyphylaxis. Of equal interest, studies employing acetylcholinesterase inhibitors suggest that ACh acts endogenously at tritiated agonist-binding sites (Costa and Murphy, 1983; Schwartz and Kellar, 1983b, 1985). Lesion studies reinforce the proposal that tritiated nicotine (P. B. S. Clarke and A. Pert, manuscript submitted for publication) and ACh (Schwartz et al., 1984) label functional nicotinic cholinoceptors.

The use of BTX as a general probe for nicotinic-cholinergic receptors in mammalian CNS has been widely questioned. For example, in the CNS, BTX can bind extrasynaptically (Ninkovic and Hunt, 1983). BTX fails to block nicotinic-cholinergic transmission at Renshaw cells (Duggan et al., 1976a), and it is unclear whether the toxin binds to nicotinic cholinoceptors associated with these neurones (Duggan et al., 1976b; Hunt and Schmidt, 1978). In the hippocampus, BTX binds to certain areas which lack cholinergic innervation (Hunt and Schmidt, 1979). In lower vertebrates, neurotransmission in the retinotectal pathway appears to include a nicotinic-cholinergic component which is blocked by BTX (Oswald and Freeman, 1980). In the rat, the homologous superior colliculus is densely labeled by BTX, but a cholinergic input from retina does not exist.

Although BTX-binding sites conceivably mediate the actions of a neuronal substance distinct from ACh (Quik, 1982) or some other function (see Oswald and Freeman, 1981), it is premature to rule out a role in nicotinic pharmacology, especially in certain brain areas with dense BTX labeling. (*1*) Certain of the effects of light on circadian rhythms appear to involve a BTX-sensitive cholinergic link in the suprachiasmatic nucleus of the hypothalamus (Zatz and Brownstein, 1981). (*2*) In the inferior colliculus, BTX blocks the effects of the agonists ACh and carbachol on single units (Farley et al., 1983). (*3*) Chronic nicotine administration in rats produces a small up-regulation of BTX binding, notably in the hippocampus (Marks et al., 1983); the binding density of BTX in the hippocampus is correlated with susceptibility to nicotine-induced seizures, across several strains of mouse (M. J. Marks, personal communication).

In summary, radiolabeled nicotinic agonists and BTX serve to distinguish two populations of binding sites in rat brain, which may both represent sites where nicotine exerts pharmacological actions. It seems likely that nicotinic agonists, which bind with high affinity *in vitro*, label receptors for endogenously released ACh. BTX-binding sites are somewhat enigmatic, but in certain regions they, too, may be cholinergic.

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