QUANTITATIVE *IN VIVO* RECEPTOR BINDING

I. Theory and Application to the Muscarinic Cholinergic Receptor¹

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Abstract

A novel approach to *in vivo* receptor binding experiments is presented which allows direct quantitation of binding site densities. The method is based on an equilibrium model of tracer uptake and is designed to produce a static distribution proportional to receptor density and to minimize possible confounding influences of regional blood flow, blood-brain barrier permeability, and nonspecific binding. This technique was applied to the measurement of regional muscarinic cholinergic receptor densities in rat brain using [³H]scopolamine. Specific *in vivo* binding of scopolamine demonstrated saturability, a pharmacologic profile, and regional densities which are consistent with interaction of the tracer with the muscarinic receptor. Estimates of receptor density obtained with the *in vivo* method and *in vitro* measurements in homogenates were highly correlated. Furthermore, reduction in striatal muscarinic receptors following ibotenic acid lesions resulted in a significant decrease in tracer uptake *in vivo*, indicating that the correlation between scopolamine distribution and receptor density may be used to demonstrate pathologic conditions. We propose that the general method presented here is directly applicable to investigation of high affinity binding sites for a variety of radioligands.

Extensive attempts in the past to demonstrate the *in vivo* distribution of radiolabeled drugs within the central nervous system encompassed a variety of approaches. The goals of initial studies were to demonstrate the regional distributions of labeled drugs and neurotoxins at their presumed sites of action in the brain (Roth and Barlow, 1961). With the development of receptor binding techniques, a second application of *in vivo* tracer distribution emerged: tracer distribution experiments were employed to reveal the anatomic localization of high affinity drug-binding sites previously detected in tissue homogenates (Yamamura et al., 1974; Holtt et al., 1977; Chang and Snyder, 1978; Kuhar et al., 1978). Autoradiographic localization of drug binding revealed anatomic detail at the light (Kuhar and Yamamura, 1976; Murrin et al., 1977; Murrin and Kuhar, 1979) and electron microscopic (Kuhar et al., 1981) levels, beyond that available by tissue punch and homogenate binding techniques.

Recently, there has been renewed interest in *in vivo* ligand distribution experiments with the ultimate goal of imaging

neurotransmitter receptors and other high affinity binding sites in human brain utilizing positron and single-photon emission tomography (Eckelman et al., 1979, 1984; Raichle, 1979; Wagner et al., 1983). The approach holds the promise of directly testing hypotheses implicating receptor alterations in a number of neurologic and psychiatric diseases. Design strategies for such clinical applications differ from previous *in vivo* studies in that accurate quantitation of receptor numbers and/or affinities in discrete brain regions is necessary. Thus, requirements for these *in vivo* tracer experiments exceed those of earlier localization studies in that they impose additional constraints associated with modeling of tracer distribution and binding.

Correlation between muscarinic receptor distribution in the brain and the *in vivo* distribution of [³H]quinuclidinyl benzilate was originally reported by Yamamura et al. (1974) following bolus injection of the tracer into the tail vein of the rat. Attempts in this laboratory as well as in those of others (Drayer et al., 1982; Vora et al., 1983) to extend these findings to lower drug doses compatible with eventual clinical imaging protocols have demonstrated poorer correlation between receptor density and tissue radioactivity and have prompted a re-examination of the design of *in vivo* receptor binding experiments. In this report we describe a quantitative approach to *in vivo* measurement of regional receptor densities based on the equilibrium distribution of radiolabeled tracers. The feasibility of this approach has been examined using [³H]scopolamine to label the muscarinic receptor in the rat brain. The ultimate purpose of these experiments is to identify a suitable positron-labeled imaging agent for human studies. Additional experiments indicating the suitability of scopolamine for this purpose are in

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progress (K. A. Frey, R. L. E. Ehrenkauf, R. D. Hichwa, and B. W. Agranoff, manuscript in preparation).

Theory

Tracer distribution in samples of brain tissue involves two anatomic compartments: the intravascular and extravascular spaces. The total extravascular tracer concentration, C_E , may be determined from total tissue tracer concentration, C_T , and blood tracer levels, C_B , when the tissue blood volume, V_B (expressed as milliliters of blood per milliliter of tissue), is known:

$$C_E = (C_T - C_B V_B) / (1 - V_B) \quad (1)$$

This correction for intravascular tracer is frequently ignored, since brain blood volumes are estimated to be 1 to 5% (Kuhl et al., 1980; Cremer and Seville, 1983). It has been omitted from analysis of the data in the present study since tissue tracer levels were found to be greater than or equal to blood tracer levels at all times examined.

The extravascular tracer consists of three components: L_E , NS_E and S_E , representing free, nonspecific, and specifically bound tracer, respectively.

$$C_E = L_E + NS_E + S_E \quad (2)$$

If it is assumed that free and nonspecifically bound tracer concentrations at equilibrium are proportional to the plasma ligand concentration, an apparent tissue-to-blood partition coefficient, λ , may be defined:

$$\lambda = (L_E + NS_E) / C_B \quad (3)$$

and, by substitution into equation 2 and rearrangement of terms:

$$S_E = C_E - \lambda C_B \quad (4)$$

Equation 4 can be used to determine the amount of tracer in tissue specifically bound to the receptor site at equilibrium when C_E and C_B are measured and the local value of λ is known. At saturating ligand concentrations, the local tissue density of binding sites may be estimated by this method directly. The regional values of λ may be determined from animals in which specific binding has been eliminated either by pretreatment with a saturating dose of a long-acting competitor or by reducing the specific activity of a saturating dose of the radioactive ligand so that S_E is small compared to C_E , in which cases:

$$\lambda = C_E / C_B \quad (5)$$

Alternatively, under conditions where C_E is much greater than λC_B in equation 4, total tissue ligand uptake is directly proportional to regional receptor density. Thus, autoradiographic imaging in animals or tomographic scanning in humans of this tracer distribution will yield a graphic representation of receptor distribution in tissue. Successful implementation of this static, single-picture approach to receptor quantitation requires true equilibrium of tracer between all of the compartments in the proposed model. This is most readily achieved by utilizing tracer injection protocols which produce constant arterial concentrations of tracer. Since ligand delivery to the brain and subsequent receptor binding have first-order dependence on C_B , use of saturating ligand concentrations should shorten equilibration time, but at the expense of increased nonspecific ligand uptake.

Alternative approaches utilizing bolus systemic administration of tracers generally do not satisfy requirements for equilibrium and, thus, do not lead to a static tracer distribution that is directly proportional to receptor density. However, it is possible that kinetic analysis may be employed to extract

binding parameters from the time courses of regional brain and blood tracer levels under non-equilibrium conditions.

Materials and Methods

Scopolamine, methylscopolamine, atropine, and di-isopropylfluorophosphate (DFP) were purchased from Sigma Chemical Co. (St. Louis, MO). Ibotenic acid was obtained from Regis Chemical Co. (Morton Grove, IL). Unlabeled ($\pm$)-quinuclidinyl benzilate (QNB) was provided by Dr. Charlotte Otto, University of Michigan (Dearborn, MI). Tritiated CH_3I (specific activity, 10 to 13 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Tritiated ($-$)-QNB (specific activity, 33.1 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Male Sprague-Dawley rats weighing between 180 and 200 gm were purchased from Harlan Sprague-Dawley Inc. (Indianapolis, IN).

Synthesis of [3H]scopolamine. Scopolamine was tritiated by methylation of ($-$)-norscopolamine (Werner and Schickflus, 1969) with [3H]CH $_3I$ using a modification of the method of Schmidt et al. (1965). The product was purified by thin layer chromatography on silica gel using the solvent system tetrahydrofuran-di-isopropylethylamine, 95:5. Radiochemical purity of the product was routinely greater than 97% and it remained stable for several months when stored at $-20^\circ C$ in ethanol solution. The specific activity of the product was determined by comparison of its *in vitro* receptor-binding capacity compared to that obtained with [3H]QNB of known specific activity. The specific activity of [3H]scopolamine determined in this manner agreed to within 10% with the stated specific activity of the precursor [3H]CH $_3I$.

In vitro binding. *In vitro* scopolamine binding to homogenates of rat brain was measured with a filtration assay (Kloog and Sokolovsky, 1978). Rat forebrains were homogenized in 25 vol of phosphate-buffered saline (NaCl, 124 mM; KCl, 2.7 mM; Na $_2$ HPO $_4$, 7.7 mM; KH $_2$ PO $_4$, 1.5 mM, pH 7.4). A crude particulate fraction was obtained by centrifugation at $20,000 \times g$ for 10 min. The pellet was resuspended and the centrifugation was repeated before final reconstitution in buffer and subsequent storage at $-20^\circ C$. In regional QNB binding capacity experiments, centrifugation was omitted and determinations were carried out on the total homogenate.

Binding of [3H]scopolamine was determined by incubating aliquots of the homogenates (80 to 160 μg of protein) with various ligand concentrations (0.1 to 10 nM) in 1 ml of buffer for 30 min at $25^\circ C$. Incubations were terminated by rapid vacuum filtration through glass fiber filters (Whatman GF/C), followed by three washes with 2-ml portions of ice-cold buffer. Nonspecific binding was determined in the presence of 1 μM atropine. Filters were placed in scintillation vials and dispersed in 10 ml of ACS scintillation cocktail (Amersham) with 1 ml of H $_2$ O, and radioactivity was determined using a Beckman LS9000 scintillation counter with external quench correction.

Binding of [3H]QNB to brain homogenates was determined as described previously (Yamamura and Snyder, 1974), with minor modifications. Tissue was incubated with a saturating (1 nM) concentration of [3H]QNB in 2 ml of PBS at $25^\circ C$ for 60 min. The incubation was terminated by filtration over Whatman GF/C filters, followed by washing and determination of radioactivity as described above. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as reference.

In vivo scopolamine distribution. Preliminary studies following bolus intravenous injection of [3H]scopolamine into rats indicated that the plasma decay curve was dominated by a single exponential component with a half-life of 17 min. An infusion protocol was designed to yield an approximately constant arterial tracer concentration by following the bolus with a constant rate infusion of tracer calculated to replace that lost in the observed first-order decay (Patlak and Pettigrew, 1976). The resulting infusion protocol consisted of a bolus of unit magnitude, followed by infusion of tracer at a rate of 0.04 units/min. This injection protocol was used throughout the *in vivo* experiments to be described.

Cannulations of a femoral artery and vein were performed in rats under light diethyl ether anesthesia. Animals were allowed to recover for 3 to 4 hr before initiation of binding experiments. [3H]Scopolamine was administered intravenously according to the protocol described above at specific activities ranging from 0.25 to 250 Ci/mol. Additional nonradioactive competing ligands were administered intravenously using the injection protocol described for [3H]scopolamine, except that the bolus of competing drug (14% of total dose) was administered 5 min before initiating the [3H]scopolamine injection, and additional competitor (86%) was incorporated into the constant infusion. DFP

was suspended in olive oil at a concentration of 20 mg/ml and administered by intramuscular injection.

Striatal lesions. Unilateral striatal lesions were produced by stereotaxic injection of the excitatory neurotoxin ibotenic acid. Animals were anesthetized with diethyl ether and mounted in a stereotaxic frame with reference planes according to the atlas of König and Klippel (1963). Twenty micrograms of ibotenic acid were injected in 1 μ l over a period of 8 min through a 30 gauge steel cannula positioned at the following coordinates: 1.0 mm anterior to the bregma, 2.6 mm lateral to the midline, and 5.5 mm below the dura. The cannula was left in place for an additional 2 min following the injection to minimize reflux of ibotenate up the cannula track.

Tissue dissection. Animals were killed by decapitation at various intervals following initiation of tracer infusion. Brains were dissected, and a coronal slice approximately 3 mm thick extending from the optic chiasm rostrally to the midpoint of the olfactory tubercles was removed. This slice served as a source for striatal as well as cortical tissue samples. Additional samples of cortex were obtained from the rostral poles of the frontal lobes. The hippocampal formation was removed by blunt dissection of the overlying parietal and temporal cortex and subsequent separation from the underlying diencephalon. The cerebellum was separated from the brainstem by severing the cerebellar peduncles. The samples from left and right hemispheres of each animal were pooled and resulted in the following tissue yields: striatum, 40 to 60 mg; frontoparietal cortex (areas 2, 3, 4, 6, 8, and 10 of Brodmann), 100 to 150 mg; hippocampus (including dentate gyrus), 100 to 140 mg; and cerebellum (including white matter and deep nuclei), 200 to 250 mg. Samples were wrapped in foil and stored at -70°C in sealed plastic bags to minimize dessication. Plasma was obtained by centrifugation of heparinized blood taken from the arterial catheter at the end of the infusion period.

Determination of [^3H]scopolamine and metabolites. Brain samples were weighed, homogenized in 10 vol of 80% ethanol, and centrifuged at $15,000 \times g$ for 10 min. Plasma samples were mixed with 3 vol of 95% ethanol and centrifuged. Aliquots of the ethanol supernatants were analyzed for total radioactivity and for nonvolatile activity following drying at 80°C . Scopolamine content of the nonvolatile fraction was determined by thin layer chromatography. Aliquots of the ethanol supernatants were concentrated by vacuum dessication and chromatographed with carrier scopolamine on silica gel plates with fluorescent indicator using the solvent system described above. Scopolamine was located by fluorescence quench and quantitated by scintillation counting. Extraction of activity from tissue and plasma into the ethanol supernatant was essentially complete. Recovery of [^3H]scopolamine standards following addition to tissue blanks was greater than 80%.

Results

***In vitro* scopolamine binding.** In agreement with previous studies (Kloog and Sokolovsky, 1978), equilibrium binding of scopolamine to homogenates of rat forebrain was consistent with ligand interaction with a single population of binding sites ($K_D = 0.38 \pm 0.01$ nM; $B_{\text{max}} = 0.81 \pm 0.04$ fmol/ μg of protein, 65 ± 1 fmol/mg of tissue; $n = 3$; see Fig. 1). Specific binding of [^3H]scopolamine was completely displaced by addition of 1 μM nonradioactive scopolamine or 100 nM QNB. In similar fashion, specific binding of [^3H]QNB was displaced by unlabeled scopolamine, suggesting identity of the high affinity binding sites for the two ligands.

***In vivo* scopolamine distribution.** The time course of accumulation of scopolamine and metabolites in brain was examined following administration of 150 $\mu\text{Ci/kg}$ of [^3H]scopolamine at specific activities of 250 and 0.25 Ci/mol (6×10^{-7} and 6×10^{-4} mol/kg, respectively) over a period of 240 min. Plasma scopolamine concentrations produced by these doses were 23 ± 4 nM and 20 ± 2 μM , respectively.

Volatile radioactivity increased linearly with time in all tissues studied (Fig. 2). Nonvolatile metabolites contributed significantly to plasma radioactivity but accounted for only 10% of the activity in the cerebellum following the high specific activity tracer infusion, and even less in other brain regions. In low specific activity experiments, volatile radioactivity and labeled metabolites accounted for a greater portion of total recoverable brain radioactivity, whereas the distribution of counts in plasma was qualitatively similar to that observed at 250 Ci/mol. No regional differences were observed in the distribution or time course of accumulation of labeled metabolites of [^3H]scopolamine.

[^3H]Scopolamine itself showed wide regional differences in its distribution following infusion at 250 Ci/mol (Fig. 3A). In contrast, its distribution following infusion at 0.25 Ci/mol was relatively homogeneous and appears to reflect primarily non-specific tracer uptake (Fig. 3B). Blood and regional brain tracer concentrations determined from this latter group of animals were used to determine regional values of λ as described previously (see "Theory," equation 5) and resulted in the following estimates: striatum, 1.5; cortex, 1.7; hippocampus, 1.6; and

Figure 1. Binding of [^3H]scopolamine to homogenate of rat forebrain. Values for total binding (upper line) and nonspecific binding (lower line) represent means from triplicate determinations in a typical experiment. Inset, Scatchard plot of specific binding.

Figure 2. Metabolism of [^3H]scopolamine during *in vivo* infusion. Radioactivity was separated into three components: scopolamine (●), nonvolatile metabolites (■), and volatile metabolites (○). Rats received 30 μCi of [^3H]scopolamine at a specific activity of 250 Ci/mol over 240 min. Each point represents the mean $\pm$ SEM for three animals.

cerebellum, 1.1. Total scopolamine uptake in the group receiving the high specific activity tracer was corrected for nonspecific binding using plasma tracer levels and the regional values of λ according to equation 4 (Fig. 3C). Although this correction is not rigorously applicable in the absence of tracer equilibration, the resulting underestimation of specific binding at early times is likely to be small since nonspecific uptake (Fig. 3B) is rapid compared to the time course of total uptake (Fig. 3A). Specific binding of scopolamine reached equilibrium rapidly in the cerebellum, appeared to approach asymptotic values in hippocampus and cerebral cortex, and continued to increase in almost linear fashion in the striatum during the 240-min tracer infusion. Comparison of specific *in vivo* scopolamine binding at 240 min with the regional distribution of muscarinic receptors determined *in vitro* with [^3H]QNB demonstrates close correlation between the two parameters (Table I).

Distribution of scopolamine 240 min after establishment of constant plasma tracer levels was additionally examined at specific activities of 125 and 16 Ci/mol (1.2×10^{-6} and 9.4×10^{-6} mol/kg, respectively; Fig. 4). Specific [^3H]scopolamine binding was decreased by approximately 60% following the reduction in specific activity from 250 to 125 Ci/mol, suggesting that the former distribution (Table I) reflected equilibrium between the receptor sites and a saturating concentration of ligand. No effect of tracer specific activity on plasma levels of [^3H]scopolamine was observed.

Specific binding of [^3H]scopolamine was inhibited by competing doses of QNB and atropine (Table II). In addition, the effect of atropine was dose dependent and consistent with competitive inhibition at a single class of binding sites. In contrast, a relatively large dose of methylscopolamine was

ineffective in displacement of *in vivo* scopolamine binding. Inhibition of acetylcholinesterase (AChE) following DFP administration was also ineffective in reduction of specific [^3H]scopolamine accumulation. The variability associated with measurement of displacement of specific binding in cerebellum reflects the lower levels of total isotope uptake, as well as the lower ratio of specific to nonspecific binding in this region.

Three weeks after unilateral striatal ibotenic acid lesions, the accumulation of [^3H]scopolamine in the lesioned striatum was markedly reduced compared to the contralateral side (Table III).

Discussion

In the present study, we present a model for *in vivo* quantitation of high affinity binding sites in brain regions based on the equilibrium distribution of radiolabeled tracers. A major difference between this approach and previous work is recognition of the need to control the regional brain concentrations of free ligand available for subsequent binding. Thus, whereas receptor-mediated tracer distributions have been reported with several labeled ligands for binding sites within the central nervous system, the tracer distribution at a single point in time following bolus systemic injection is representative of receptor density only under select conditions. For example, if the chosen ligand dissociates slowly from the receptor site and the amount of drug injected produces saturating concentrations throughout the brain, the resulting distribution of bound tracer will be relatively unaffected by regional differences in free ligand concentration. As the time between injection and tissue sampling increases, nonspecific binding decreases as a result of declining plasma levels of the tracer, and high ratios of specific to nonspecific binding may be achieved. Thus, better correlation between receptor density and *in vivo* QNB distribution was observed in the original report by Yamamura et al. (1974) than by subsequent investigators, who used lower doses of [^3H]QNB (Gibson et al., 1984) or [^{123}I]- or [^{125}I]iodo-QNB (Drayer et al., 1982; Gibson et al., 1984). Furthermore, the higher degree of correlation between receptor density and tracer distribution observed with QNB (Yamamura et al., 1974) than was found in a recent study employing bolus administration of scopolamine (Vora et al., 1983) may be attributable to dissociation of the latter tracer from receptors during the time allowed for washout of nonspecific binding.

In the present study, an infusion protocol producing a constant arterial concentration of tracer was used to allow regional brain pools of free and bound tracer to equilibrate, thus eliminating the effects of cerebral blood flow and regional blood-brain barrier permeability on the free tissue ligand concentrations which serve as precursor to receptor binding. This experimental approach has resulted in the highest correlation between *in vivo* tracer accumulation and regional density of muscarinic receptors yet reported. Whereas the regional densities of *in vitro* muscarinic receptor binding reported here are in good agreement with previous reports (Yamamura and Snyder, 1974; Kobayashi et al., 1978; Birdsall et al., 1980), the estimated B_{max} values obtained *in vivo* are 1.5 times higher than the *in vitro* measurements. This unexplained systematic difference between the two measures may reflect the presence of soluble receptors or receptors on tissue fragments too small to be trapped on the filters used for the *in vitro* assays, degradation of receptors during tissue homogenization, or other postmortem alterations in the tissue samples.

The role of muscarinic receptors in the *in vivo* distribution of scopolamine is confirmed by the observed effects of removal of striatal binding sites following ibotenic acid lesions. Ibotenic acid or kainic acid lesions of the striatum are known to preferentially effect neurons with cell bodies near the injection site without damaging presynaptic elements from extrinsic neurons,

Figure 3. Time course of regional brain [³H]scopolamine uptake. A, Scopolamine uptake in striatum (Δ), cortex (●), hippocampus (□), and cerebellum (○) in rats receiving 30 μCi of [³H]scopolamine at a specific activity of 250 Ci/mol over 240 min. Plasma activity (■) remained constant during the 240-min infusion. B, Scopolamine uptake in rats receiving 30 μCi of [³H]scopolamine at a specific activity of 0.25 Ci/mol. C, Time course of specific scopolamine uptake based on correction of total uptake (A) for nonspecific uptake (B) using equation 4. Values shown represent the mean ± SEM for three animals at each time point.

TABLE I

Scopolamine uptake at 240 min: Comparison with *in vitro* binding

Tissue	In Vivo Scopolamine Distribution			In Vitro QNB Binding (Specific)
	Total	Nonspecific	Specific	
	dpm/mg of tissue		fmol/mg of tissue	fmol/mg of tissue
Striatum	151 ± 6 ^a	14 ± 1 ^a	239 ± 5 (15 ^b)	177 ± 4 ^c (18)
Cortex	120 ± 6	16 ± 1	178 ± 4 (11)	112 ± 4 (11)
Hippocampus	97 ± 5	16 ± 1	139 ± 2 (8.7)	88 ± 3 (8.8)
Cerebellum	22 ± 3	10 ± 1	16 ± 2 (1.0)	10 ± 1 (1.0)
Plasma	13 ± 2	11 ± 1		

^a Mean ± SEM, *n* = 3.^b Relative to cerebellum.^c Mean ± SEM, *n* = 6.

axons of passage, glia, or endothelial elements (Coyle et al., 1978; Murrin et al., 1979; Schwarcz et al., 1979). Lesions of this type result in a marked local reduction in muscarinic receptor density (Schwarcz and Coyle, 1977) in the presence of relatively

normal levels of cerebral blood flow (Frey and Agranoff, 1983). The reduction in scopolamine uptake observed in the lesioned striatum thus appears to reflect receptor loss rather than ischemia or other secondary processes.

Specific binding of scopolamine in all brain regions examined was saturable. The lowest concentration used, 0.6 μmol/kg, resulted in a saturating plasma concentration of ligand. Lower drug doses were not investigated since the equilibration time for these additional concentrations was expected to exceed the 240-min experimental period used. An upper limit for the *in vivo* affinity of scopolamine for the muscarinic receptor was estimated to be 2 nM. This result is consistent with *in vitro* estimates of the scopolamine affinity constant obtained in this study, as well as in previous reports (Burgermeister et al., 1978; Hulme et al., 1978; Kloog and Sokolovsky, 1978).

The specific binding of [³H]scopolamine additionally displayed a pharmacologic profile consistent with the central muscarinic receptor as previously defined in several bioassays. Scopolamine and QNB were more potent inhibitors of binding of the tracer than atropine, and all three were much more

Figure 4. Displacement of *in vivo* scopolamine uptake. Rats received 30 μ Ci of [3 H]scopolamine over 240 min. Additional unlabeled scopolamine was included in the infusions to produce total drug doses ranging from 0.6 μ mol to 0.6 mmol/kg. Values represent the mean $\pm$ SEM of three animals at each drug dose in the following tissues: striatum (Δ), cortex ($\bullet$), hippocampus ($\square$), cerebellum ($\circ$), and arterial plasma ($\blacksquare$).

TABLE II

Pharmacological profile of specific in vivo binding

All animals received 30 μ Ci of [3 H]scopolamine at a specific activity of 250 Ci/mol over 240 min. Nonradioactive competing ligands were included at the doses indicated. Absolute values for specific binding in striatum, cortex, hippocampus, and cerebellum are 132, 99, 77, and 9 dpm/mg of tissue, respectively. Values represent the mean $\pm$ SEM of three animals in each group.

Competing Drug	Dose mol/kg	Specific Binding (percentage of control)			
		Striatum	Cortex	Hippo- campus	Cere- bellum
None		100 $\pm$ 2	100 $\pm$ 2	100 $\pm$ 1	100 $\pm$ 10
QNB	9.3 $\times 10^{-6}$	-2 $\pm$ 1	-3 $\pm$ 1	-3 $\pm$ 1	-8 $\pm$ 5
Scopolamine	6.0 $\times 10^{-7}$	41 $\pm$ 1	39 $\pm$ 2	36 $\pm$ 1	16 $\pm$ 2
	9.3 $\times 10^{-6}$	-1 $\pm$ 1	-2 $\pm$ 2	-4 $\pm$ 3	-33 $\pm$ 10
Atropine	9.3 $\times 10^{-6}$	39 $\pm$ 1	36 $\pm$ 3	39 $\pm$ 2	48 $\pm$ 5
	9.3 $\times 10^{-5}$	3 $\pm$ 1	2 $\pm$ 1	2 $\pm$ 2	14 $\pm$ 5
Me-scopol- amine	1.4 $\times 10^{-5}$	82 $\pm$ 1	84 $\pm$ 2	76 $\pm$ 2	88 $\pm$ 8
DFP	- ^a	79 $\pm$ 1	84 $\pm$ 2	84 $\pm$ 1	77 $\pm$ 16

^a The dose was 2 mg/kg, i.m., 30 min before scopolamine infusion.

TABLE III

Effect of striatal ibotenic acid lesion on total in vivo scopolamine uptake

Three weeks after unilateral striatal injection of 20 μ g of ibotenic acid, rats were infused with 30 μ Ci of [3 H]scopolamine (250 Ci/mol) over 240 min. Lesioned and control (contralateral) striata were pooled from two animals for each measurement.

Experiment No.	dpm [3 H]Scopolamine/ mg of Tissue		Lesion/Control Ratio
	Control Striatum	Lesioned Striatum	
1	115	64	0.55
2	122	50	0.41
3	115	41	0.36
4	124	45	0.36
Mean $\pm$ SEM	119 $\pm$ 2	50 $\pm$ 5 ^a	0.42 $\pm$ 0.05

^a $p < 0.001$ versus control, Student's t test.

effective than methylscopolamine. This rank order is identical to that obtained for elicitation of behavioral changes in the rat (Herz et al., 1965; Parkes, 1965), mouse (Herz et al., 1965; Parkes, 1965; Rahavi et al., 1978), dog (Albanus, 1970), and human (Ketchum et al., 1973), as well as EEG alterations in the rabbit (Herz et al., 1965). These potency differences are

presumed to be due to differential permeability of the blood-brain barrier to the agents (Herz et al., 1965; Weinstein et al., 1977), which is largely a function of lipid solubility at physiologic pH (Brodie et al., 1960; Rapaport et al., 1979). Thus, scopolamine and methylscopolamine have similar potencies in assays of peripheral anticholinergic activity, yet they differ in their central efficacies by several orders of magnitude. For this reason, the lack of effect of methylscopolamine on the *in vivo* binding of [3 H]scopolamine suggests that central muscarinic receptors are located predominantly on the parenchymal side of the blood-brain barrier and that the contribution of binding sites on blood elements or the luminal surfaces of cerebral endothelial cells is minor.

A frequently overlooked aspect of *in vivo* binding experiments concerns the possible effects of endogenous competitors and modulators of tracer binding. It has been well documented, for example, that removal of endogenous cortisol following adrenalectomy significantly enhances detection of steroid binding to hippocampal receptors (McEwen et al., 1969). With respect to quantitation of synaptic neurotransmitter receptors, the problem of endogenous competitors becomes considerably more complex, since neurotransmitters and neuromodulators are intrinsic to the tissue of interest. In addition, their intrasynaptic concentrations may vary regionally and as a function of either physiologic or pathologic alterations in neuronal activity. In this regard, a recent study by van der Werf et al. (1983) demonstrated that removal of endogenous dopamine, either by lesions of the substantia nigra or by reserpine treatment, resulted in significant increases in *in vivo* binding of *N*-propyl-norapomorphine to striatal dopamine receptors.

In order to examine possible effects of endogenous acetylcholine (ACh) on *in vivo* scopolamine binding, we studied a group of animals which had been pretreated with a dose of DFP shown previously to increase ACh in brain (Fonnum and Gutormsen, 1969) and to eliminate histochemical detection of AChE (Fibiger, 1982). This treatment proved relatively ineffective in altering tracer binding and did not reveal the regional specificity anticipated based on the known heterogeneity of ACh levels and CAT activity (Cheney et al., 1975; Hoover et al., 1978) in the brain regions studied. Although a selective effect on a minor population of binding sites cannot be ruled out, a more parsimonious interpretation is that the saturating tracer concentrations used in the present work competed effectively with synaptic ACh for available receptor sites.

The protracted time course of *in vivo* scopolamine binding observed at plasma ligand concentrations near 20 nM was unexpected. Tissue uptake of scopolamine studied at 20 μ M levels indicates that nonspecifically bound and free ligand

equilibrates rapidly with plasma. Thus, neither blood-brain barrier permeability nor local cerebral blood flow limitations are adequate explanations for the slow equilibration of bound tracer with free tissue and plasma pools. Furthermore, the *in vitro* kinetics of scopolamine binding indicate rapid association of tracer with the receptor in homogenates (Kloog and Sokolovsky, 1978).

The observed time courses of *in vivo* scopolamine binding are compatible with several interpretations. First, the kinetics of receptor-ligand interaction under *in vivo* conditions may differ substantially from the *in vitro* situation. Modifying effects of substances such as guanine nucleotides and divalent cations or of receptor phosphorylation may differ between intact preparations and tissue homogenates. Reports of antagonist binding to muscarinic receptors on intact cells in culture, however, do not support this speculation (Burgermeister et al., 1978; Frick et al., 1983; Nathanson, 1983). Second, tracer access to the receptor sites may be restricted *in vivo* at a point subsequent to extraction from the plasma. If a significant number of receptors are present within a relatively small tissue compartment which is separated from the extracellular space by a diffusion barrier, detection of this compartment would be difficult in the low specific activity infusion experiments, yet its presence could explain the slow time course observed under conditions of high specific activity. This explanation has been proposed to account for observations on the kinetics of *in vivo* distribution of opiate antagonists, with the speculation that the synaptic cleft may be the anatomic site of the isolated micro-compartment (Perry et al., 1980). An additional possibility is that the presence of endogenous ACh, bound to receptors and in competition with the tracer for free receptors, retards the rate of tracer binding. The reversal in the pattern of tracer distribution in cortex and striatum observed between 60 and 180 min in the present study may reflect the greater levels of ACh in the striatum competing with [³H]scopolamine. Present evidence does not permit distinction between these possibilities.

The equilibrium model presented here may be applied to a variety of ligands and receptors *in vivo*. It was validated with the use of radiolabeled scopolamine to measure muscarinic binding. The calculated *in vivo* equilibrium distribution of [³H]scopolamine appears to accurately reflect the regional binding capacity for muscarinic antagonists determined by *in vitro* methods. As predicted by the model, at appropriately chosen ligand concentrations, total tissue tracer levels are proportional to receptor numbers. It is anticipated that this *in vivo* experimental approach will permit the quantitative autoradiographic localization of receptors in experimental animals, as well as receptor imaging in humans using positron or single-photon emission tomography.

References

- Albanus, L. (1970) Central and peripheral effects of anticholinergic compounds. *Acta Pharmacol. Toxicol.* 28: 305-326.
- Birdsall, N. J. M., E. C. Hulme, and A. Burgen (1980) The character of the muscarinic receptors in different regions of the rat brain. *Proc. R. Soc. Lond. (Biol.)* 207: 1-12.
- Brodie, B. B., H. Kurz, and L. S. Schanker (1960) The importance of dissociation constant and lipid-solubility in influencing the passage of drugs into the cerebrospinal fluid. *J. Pharmacol.* 130: 20-25.
- Burgermeister, W., W. L. Klein, M. Nirenberg, and B. Witkop (1978) Comparative binding studies with cholinergic ligands and histrionicotoxin at muscarinic receptors of neural cell lines. *Mol. Pharmacol.* 14: 751-767.
- Chang, R. S. L., and S. H. Snyder (1978) Benzodiazepine receptors: labeling in intact animals with [³H]flunitrazepam. *Eur. J. Pharmacol.* 48: 213-218.
- Cheney, D. L., H. F. LeFevre, and G. Racagni (1975) Choline acetyltransferase activity and mass fragmentographic measurement of acetylcholine in specific nuclei and tracts of rat brain. *Neuropharmacology* 14: 801-809.
- Coyle, J. T., M. E. Molliver, and M. J. Kuhar (1978) *In situ* injection of kainic acid: A new method for selectively lesioning neuronal cell bodies while sparing axons of passage. *J. Comp. Neurol.* 180: 301-324.
- Cremer, J. E., and M. P. Seville (1983) Regional brain blood flow, blood volume, and haematocrit values in the adult rat. *J. Cerebr. Blood Flow Metab.* 3: 254-256.
- Drayer, B., R. Jaszczak, E. Coleman, A. Storni, K. Greer, N. Petry, M. Lischko, and S. Flanagan (1982) Muscarinic cholinergic receptor binding: *In vivo* depiction using single photon emission computed tomography and radioiodinated quinuclidinyl benzilate. *J. Comput. Assist. Tomogr.* 6: 536-543.
- Eckelman, W. C., R. C. Reba, R. E. Gibson, W. J. Rzeszutarski, F. Vieras, J. K. Mazaitis, and B. Francis (1979) Receptor-binding radiotracers: A class of potential radiopharmaceuticals. *J. Nucl. Med.* 20: 350-357.
- Eckelman, W. C., R. C. Reba, W. J. Rzeszutarski, R. E. Gibson, T. Hill, B. L. Holman, T. Budinger, J. J. Conklin, R. Eng, and M. P. Grissom (1984) External imaging of cerebral muscarinic acetylcholine receptors. *Science* 223: 291-292.
- Fibiger, H. C. (1982) The organization and some projections of cholinergic neurons of the mammalian forebrain. *Brain Res. Rev.* 4: 327-388.
- Fonnum, F., and D. M. Guttormsen (1969) Changes in acetylcholine content of rat brain by toxic doses of di-isopropylphosphofluoridate. *Experientia* 25: 505-506.
- Frey, K. A., and B. W. Agranoff (1983) Barbiturate-enhanced detection of brain lesions by carbon-14-labeled 2-deoxyglucose autoradiography. *Science* 219: 879-881.
- Frick, W., F. Hefti, K. Citherlet, A. Dravid, and G. Gmelin (1983) Muscarinic receptors on intact, cultured neurons. Characterization by [³H]quinuclidinyl-benzilate binding. *Neurosci. Lett.* 40: 45-50.
- Gibson, R. E., D. J. Weckstein, E. M. Jagoda, W. J. Rzeszutarski, R. C. Reba, and W. C. Eckelman (1984) The characteristics of I-125 4-IQNB and H-3 QNB *in vivo* and *in vitro*. *J. Nucl. Med.* 25: 214-222.
- Herz, A., H. Teschemacher, A. Hofstetter, and H. Kurz (1965) The importance of lipid-solubility for the central action of cholinolytic drugs. *Int. J. Neuropharmacol.* 4: 207-218.
- Holt, V., A. Czlonkowski, and A. Herz (1977) The demonstration *in vivo* of specific binding sites for neuroleptic drugs in mouse brain. *Brain Res.* 130: 176-183.
- Hoover, D. B., E. A. Muth, and D. M. Jacobowitz (1978) A mapping of the distribution of acetylcholine, choline acetyltransferase and acetylcholinesterase in discrete areas of rat brain. *Brain Res.* 153: 295-306.
- Hulme, E. C., N. J. M. Birdsall, A. S. V. Burgen, and P. Mehta (1978) The binding of antagonists to brain muscarinic receptors. *Mol. Pharmacol.* 14: 737-750.
- Ketchum, J. S., F. R. Sidell, E. B. Crowell, Jr., G. K. Aghajanian, and A. H. Hayes, Jr. (1973) Atropine, scopolamine, and ditran: Comparative pharmacology and antagonists in man. *Psychopharmacologia (Berlin)* 28: 121-145.
- Kloog, Y., and M. Sokolovsky (1978) Studies on muscarinic acetylcholine receptors from mouse brain: Characterization of the interaction with antagonists. *Brain Res.* 144: 31-48.
- Kobayashi, R. M., M. Palkovits, R. E. Hruska, R. Rothschild, and H. I. Yamamura (1978) Regional distribution of muscarinic cholinergic receptors in rat brain. *Brain Res.* 154: 13-23.
- König, J. F. R., and R. A. Klippel (1963) *The Rat Brain: A Stereotaxic Atlas of the Forebrain and Lower Parts of the Brain Stem*, Robert E. Krieger, New York.
- Kuhar, M. J., and H. I. Yamamura (1976) Localization of cholinergic muscarinic receptors in rat brain by light microscopic radioautography. *Brain Res.* 110: 229-243.
- Kuhar, M. J., L. C. Murrin, A. T. Malouf, and N. Klemm (1978) Dopamine receptor binding *in vivo*: The feasibility of autoradiographic studies. *Life Sci.* 22: 203-210.
- Kuhar, M. J., N. Taylor, J. K. Wamsley, E. C. Hulme, and N. J. M. Birdsall (1981) Muscarinic cholinergic receptor localization in brain by electron microscopic autoradiography. *Brain Res.* 216: 1-9.
- Kuhl, D. E., A. Alavi, E. J. Hoffman, M. E. Phelps, R. A. Zimmerman, W. D. Obrist, D. A. Bruce, J. H. Greenberg, and B. Uzzell (1980) Local cerebral blood volume in head-injured patients. Determination by emission computed tomography of ^{99m}Tc-labeled red cells. *J. Neurosurg.* 52: 309-320.

- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265-275.
- McEwen, B. S., J. M. Weiss, and L. S. Schwartz (1969) Uptake of corticosterone by rat brain and its concentration by certain limbic structures. *Brain Res.* 16: 227-241.
- Murrin, L. C., and M. J. Kuhar (1979) Dopamine receptors in rat frontal cortex: An autoradiographic study. *Brain Res.* 177: 279-285.
- Murrin, L. C., S. J. Enna, and M. J. Kuhar (1977) Autoradiographic localization of [³H]reserpine binding sites in rat brain. *J. Pharmacol. Exp. Ther.* 203: 564-574.
- Murrin, L. C., K. Gale, and M. J. Kuhar (1979) Autoradiographic localization of neuroleptic and dopamine receptors in the caudate-putamen and substantia nigra: Effects of lesions. *Eur. J. Pharmacol.* 60: 229-235.
- Nathanson, N. M. (1983) Binding of agonists and antagonists to muscarinic acetylcholine receptors on intact cultured heart cells. *J. Neurochem.* 41: 1545-1549.
- Parkes, M. W. (1965) An examination of central actions characteristic of scopolamine: Comparison of central peripheral activity of scopolamine, atropine and some synthetic basic esters. *Psychopharmacologia* 7: 1-19.
- Patlak, C. S., and K. D. Pettigrew (1976) A method to obtain infusion schedules for prescribed blood concentration time courses. *J. Appl. Physiol.* 40: 458-463.
- Perry, D. C., K. B. Mullis, S. Øie, and W. Sadeé (1980) Opiate antagonist receptor binding *in vivo*: Evidence for a new receptor binding model. *Brain Res.* 199: 49-61.
- Raichle, M. E. (1979) Quantitative *in vivo* autoradiography with positron emission tomography. *Brain Res. Rev.* 1: 47-68.
- Rapoport, S. I., K. Ohno, and K. D. Pettigrew (1979) Drug entry into the brain. *Brain Res.* 172: 354-359.
- Rehavi, M., B. Yaavetz, Y. Kloog, S. Maayani, and M. Sokolovsky (1978) *In vivo* and *in vitro* studies on the antimuscarinic activity of some amino esters of benzoic acid. *Biochem. Pharmacol.* 27: 1117-1124.
- Roth, L. J., and C. F. Barlow (1961) Drugs in the brain. Autoradiography and radioassay techniques permit analysis of penetration by labeled drugs. *Science* 134: 22-31.
- Schmidt, A. L., G. Werner, and G. Kumpe (1965) Radioaktive Markierung von Tropan-Alkaloiden. IV. Synthetischer Einbau von ¹⁴C in (-)-Scopolamin, Scopin and Scopolin. *J. Liebigs Ann. Chem.* 688: 228-232.
- Schwarcz, R., and J. T. Coyle (1977) Striatal lesions with kainic acid: Neurochemical characteristics. *Brain Res.* 127: 235-249.
- Schwarcz, R., T. Hökfelt, K. Fuxe, G. Jonsson, M. Goldstein, and L. Terenius (1979) Ibotenic acid-induced neuronal degeneration: A morphological and neurochemical study. *Exp. Brain Res.* 37: 199-216.
- Van Der Werf, J. F., J. B. Sebens, W. Vaalburg, and J. Korf (1983) *In vivo* binding of *N*-*n*-propylnorapomorphine in the rat brain: Regional localization, quantification in striatum and lack of correlation with dopamine metabolism. *Eur. J. Pharmacol.* 87: 259-270.
- Vora, M. M., R. D. Finn, T. E. Boothe, D. R. Liskowsky, and L. T. Potter (1983) [*N*-methyl-¹⁴C]-Scopolamine: Synthesis and distribution in rat brain. *J. Labelled Compd. Radiopharm.* 20: 1229-1236.
- Wagner, H. N., Jr., H. D. Burns, R. F. Dannals, D. F. Wong, B. Langstrom, T. Duelfer, J. J. Frost, H. T. Ravert, J. M. Links, S. B. Rosenbloom, S. E. Lukas, A. V. Kramer, and M. J. Kuhar (1983) Imaging dopamine receptors in the human brain by positron tomography. *Science* 221: 1264-1266.
- Weinstein, H., S. Srebrenik, S. Maayani, and M. Sokolovsky (1977) A theoretical model study of the comparative effectiveness of atropine and scopolamine action in the central nervous system. *J. Theor. Biol.* 64: 295-309.
- Werner, G., and R. Schickfluss (1969) Zur Chemie von Tropan-Derivaten. III. Darstellung von Nor-(-)-Scopolamin sowie einiger Nor-scopin- und Scopinester. *J. Liebigs Ann. Chem.* 729: 152-157.
- Yamamura, H. I., and S. H. Snyder (1974) Muscarinic cholinergic binding in rat brain. *Proc. Nat. Acad. Sci. U. S. A.* 71: 1725-1729.
- Yamamura, H. I., M. J. Kuhar, and S. H. Snyder (1974) *In vivo* identification of muscarinic cholinergic receptor binding in rat brain. *Brain Res.* 80: 170-176.