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# Muscarinic Cholinergic Binding in Rat Brain

**(quinuclidinyl benzilate/receptors)**

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**ABSTRACT Binding sites with high affinity and specificity for [$^3$H]quinuclidinyl benzilate (QNB) are present in homogenates of rat brain. The characteristics of the binding sites resemble those of muscarinic cholinergic receptors. Specific binding is saturable with respect to [$^3$H]QNB and tissue concentration and is time-, temperature-, and pH-dependent. The bimolecular rate of association ($2.0 \times 10^8$ $M^{-1}$ $min^{-1}$) and dissociation ($1.2 \times 10^{-2}$ $min^{-1}$) at 35° indicate a dissociation constant of 60 pM and a density of 65 pmol/g of brain. Muscarinic antagonists and agonists displace specific [$^3$H]QNB binding, while nicotinic and non-cholinergic drugs possess little affinity for [$^3$H]QNB-binding sites.**

Identification of neurotransmitter receptor sites by chemical measurements of direct binding has been reported for nicotinic cholinergic receptors of invertebrate electric organs (1), mammalian neuromuscular junction (2) and central nervous system (3), and for the glycine receptor in the mammalian central nervous system (4). Attempts to study the muscarinic cholinergic receptor biochemically have involved measuring the binding of atropine to the guinea pig intestine (5) and subcellular fractions of rat brain (6). Recently an alkylating agent derived from the muscarinic antagonist benzilylcholine has been employed in the guinea pig intestine (7) and rat brain (8). 3-Quinuclidinyl benzilate (QNB) has been reported to be a potent central muscarinic antagonist (9, 10). Moreover, in the periphery, QNB has been shown to antagonize the acetylcholine-induced contractile response of the guinea pig ileum 50% at 0.01 $\mu$M (11). Since QNB possesses potency, specificity, and persistence of action, it appears to be a suitable agent for receptor labeling.

We now report a simple and sensitive assay for specific muscarinic cholinergic binding in the rat central nervous system using QNB, and describe kinetic properties of the binding, its regional and subcellular localization, and the relative affinity of cholinergic and noncholinergic drugs.

## MATERIALS AND METHODS

QNB was labeled by catalytic tritium exchange at New England Nuclear Corp., Boston, Mass. Fifty milligrams of QNB dissolved in 0.3 ml of glacial acetic acid were mixed with 25 mg of platinum catalyst and 10 Ci of $^3H_2O$. After stirring 18 hr at 80°, labile $^3$H was removed *in vacuo* with methanol as a solvent and, after filtration from the solvent, the product was dissolved in 10 ml of methanol. In our laboratory, the product was purified by thin-layer chromatography in Silica-Gel F-254 plates, 0.25-mm thickness (EM Laboratories, Inc., Elmsford, N.Y.) in 1-butanol:glacial acetic acid:water [4:1:1] and the purity determined in three solvent systems (1-butanol:glacial acetic acid:water [4:1:1]; chloroform:acetone:diethylamine [50:40:10] and chloroform:diethylamine [90:10]). Purified [$^3$H]QNB (90–95%) moved as a single peak with authentic QNB in all three systems. The specific activity of the [$^3$H]-QNB was 1.6–4.0 Ci/mmol as determined by comparison with the ultraviolet absorption of standard solutions at 258 nm.

Male Sprague–Dawley rats (100–150 g) were decapitated and their brains were rapidly removed. After excision of the cerebella, which were nearly devoid of receptor activity as determined in preliminary experiments, each brain was homogenized in 10 volumes of ice-cold 0.32 M sucrose in a Potter–Elvehjem glass homogenizer fitted with a Teflon pestle. The whole homogenate was centrifuged for 10 min at $1000 \times g$. The pellet (crude nuclear fraction) was discarded and the resultant supernatant fluid ($S_1$) was homogenized with a Polytron (setting no. 5, 60 sec) and used for [$^3$H]QNB-binding studies.

To assay specific binding of [$^3$H]QNB, 25–50 $\mu$l of this preparation were incubated at 25° with 2 ml of 0.05 M sodium-potassium (Na–K) phosphate buffer, pH 7.4, containing [$^3$H]QNB. After a 60-min incubation 3 ml of ice-cold Na–K phosphate buffer were added and the contents were passed through a glass filter (GF/B) positioned over a vacuum. The filters were washed three times under vacuum with 3 ml of ice-cold buffer. Every determination of binding was performed in triplicate, together with triplicate samples containing unlabeled QNB (0.01 $\mu$M) or oxotremorine (100 $\mu$M) to determine nonspecific [$^3$H]QNB binding. The filters were placed in vials containing 12 ml of Triton X-100:toluene-phosphor, maintained at 25° for 8–12 hr and the radioactivity then assayed by liquid scintillation spectrometry (Packard Tri-Carb models 3375, 3385), at a counting efficiency of 40%.

Choline acetyltransferase (EC 2.3.1.6) and cholinesterase (EC 3.1.1.7) activities were assayed by a modification (12) of radiometric assays (13). Protein was determined by the method of Lowry *et al.* (14) using bovine-serum albumin as a standard.

Compounds were obtained as follows: acetylcholine (Eastman Organic Chem, Rochester, N.Y.); acetyl-$\beta$-methylcholine, physostigmine, (Calbiochem, Los Angeles); isopropamide, *d*-chlorpheniramine (Smith, Kline & French, Phila., Pa.), atropine, neostigmine (Sigma Chem, St. Louis, Mo.); oxotremorine, scopolamine pilocarpine (Aldrich Chem. Co., Milwaukee, Wis.); *l*-chlorpheniramine, *l*-brompheniramine, (Schering Corp., Bloomfield, N.J.); *d*-brompheniramine (A. H. Robins Co., Richmond, Va.); carbamylcholine, (Merck, Sharp & Dohme, West Point, Pa.); 3-quinuclidinyl benzilate, and neurotoxin and corticotoxin from the cobra (*Naja naja*) were gifts from Edgewood Arsenal, Md.

Abbreviation: QNB, 3-quinuclidinyl benzilate.

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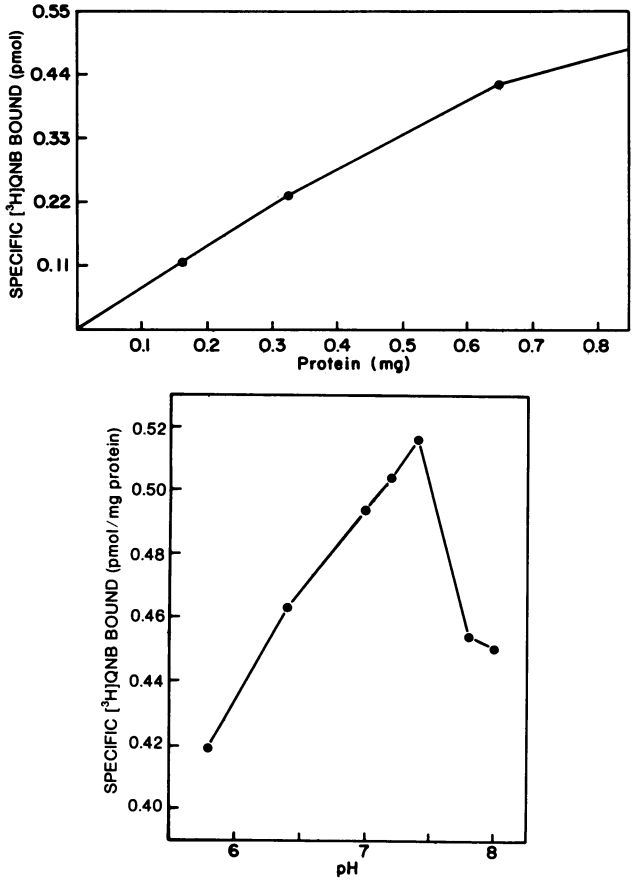


FIG. 1. Specific binding of [³H]QNB as a function of concentration of tissue and pH. *Bottom:* Rat brain homogenates were incubated with 0.05 M Na-K phosphate buffers (pH 5.0–8.0) and 0.6 nM [³H]QNB. After incubation at 25° for 60 min, specific binding was determined as described in the *text*. *Top:* Various amounts of tissue were incubated 60 min at 25° in 2.0 ml of 0.05 M Na–K phosphate buffer (pH 7.4) with 0.6 nM [³H]QNB.

## RESULTS

*Subcellular Localization of Specific QNB Binding.* In typical experiments in which 0.5 mg of whole brain protein is incubated with 2000 cpm of [³H]QNB, about 400 cpm total binding is observed. In the presence of 0.01 μM unlabeled QNB or 100 μM oxotremorine, binding is reduced to 50–70 cpm. Essentially no binding (<10 cpm) to the filters occurs when tissue is omitted from the incubation. Specific [³H]QNB binding is defined as the total binding minus the binding in the presence of 0.01 μM unlabeled QNB or 100 μM oxotremorine, both of which give the same values. Under these conditions the ratio of specific to nonspecific binding is 8–10:1. About 10–20% of the [³H]QNB in the medium is bound in a specific fashion.

To measure the relative amounts of specific [³H]QNB-binding activity in various subcellular fractions, homogenates of the whole brain without the cerebellum have been subjected to differential centrifugation (Table 1). Only about 11% of the total binding is recovered in the crude nuclear fraction. The crude mitochondrial fraction, which includes synaptosomes (pinched-off nerve endings) and mitochondria, contains about 57% of the total binding, while the crude microsomal fraction, which contains a variety of membranes and some myelin, displays about 35% of total binding. Although the crude mitochondrial fraction contains the largest total amount

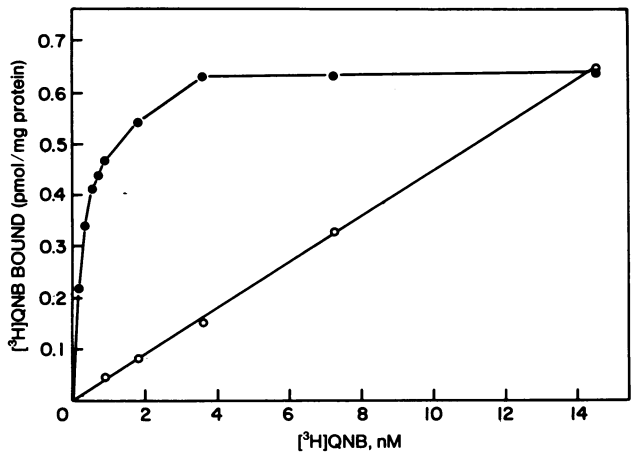


FIG. 2. Specific binding of [³H]QNB to rat brain homogenates as a function of the concentration of QNB. Six-tenths milligram of tissue was incubated at 25° for 60 min in 2.0 ml of 0.05 M Na–K phosphate buffer (pH 7.4) with various concentrations of [³H]QNB. Specific binding (●——●) and nonspecific binding (○——○) of QNB to tissue were determined as described in the *text*.

of binding, the specific activity of [³H]QNB binding is greatest in the crude microsomal fraction, whose specific activity is about 2.5 times that of the crude mitochondrial fraction. Virtually all the binding of the whole homogenate is recovered in the three particulate subcellular fractions. No direct studies have been performed to rule out QNB binding in the soluble supernatant fraction. This pattern of subcellular localization, with the greatest total amount of binding in the crude mitochondrial fraction and the highest specific activity in the microsomal fraction, is similar to results obtained in this laboratory for specific opiate receptor (15) and glycine receptor binding in the rat central nervous system (4).

Because the crude nuclear fraction displays very little spe-

TABLE 1. *Subcellular distribution of specific [³H]QNB binding in rat brain homogenates*

| Fraction | Specifically bound [³H]QNB (pmol/g of protein) | Total [³H]QNB bound/ fraction (nmol/ fraction) | Percent of total specific [³H]QNB binding of whole homogenates |
|---|---|---|---|
| Whole homogenate | 363 ± 74 | 218 ± 44 | — |
| Crude nuclear pellet (1000 × *g* for 10 min) | 107 ± 72 | 25.7 ± 1.7 | 11 |
| Crude mitochondrial pellet (17,500 × *g* for 20 min) | 525 ± 6 | 12.6 ± 0.2 | 57 |
| Crude microsomal pellet (100,000 × *g* for 60 min) | 1284 ± 98 | 7.7 ± 0.6 | 35 |

Tissues were homogenized and subjected to differential centrifugation. The various pellets were resuspended in 24 ml of 0.05 M sodium-potassium phosphate buffer, pH 7.4, and an aliquot assayed as described in *Methods*. The data represent the mean of three experiments ± SEM.

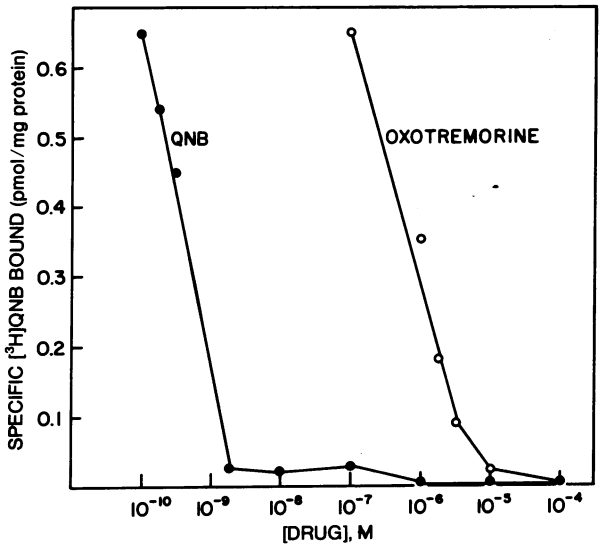


FIG. 3. Displacement of specific [$^3$H]QNB binding by various concentrations of QNB and oxotremorine. Six-tenths milligram of tissue in 20 ml of Na–K phosphate buffer (pH 7.4) was incubated with 0.06 nM [$^3$H]QNB and the indicated amounts of QNB or oxotremorine for 60 min at 25°. Specific QNB binding was determined as described in the *text*.

cific [$^3$H]QNB binding, we have employed the supernatant fluid from the crude nuclear pellet for routine assays.

Specific [$^3$H]QNB binding is linear between 0.1 and 0.6 mg of tissue protein and displays a fairly broad pH optimum between pH 6 and 8, but with a distinct maximum at pH 7.4 (Fig. 1).

Ionic manipulations do not markedly affect specific [$^3$H]-QNB binding. Sodium and potassium concentrations as high as 500 mM and 100 mM, respectively, do not alter specific [$^3$H]QNB binding. Calcium and magnesium have no effect at 1 mM but lower binding 20% at 10 mM.

*Saturability of [$^3$H]QNB Binding to Rat Brain Homogenates.* Specific [$^3$H]QNB binding is saturable with increasing concentrations (Fig. 2), with half-maximal binding at about 0.4 nM. By contrast, nonspecific binding, in the presence of 0.01 $\mu$M unlabeled QNB or 100 $\mu$M oxotremorine, is not saturable and increases linearly with increasing [$^3$H]QNB. [$^3$H]QNB binding is displaced by nonradioactive QNB with half-maximal displacement at about 0.4–0.5 nM nonradioactive QNB and maximal displacement at 0.01 $\mu$M (Fig. 3). The fact that half-maximal saturation occurs at similar concentrations of nonradioactive and radiolabeled QNB indicates that [$^3$H]QNB is biologically equivalent to the nonradioactive drug in terms of receptor binding and confirms the validity of the determined specific activity of [$^3$H]QNB.

The same maximal displacement of [$^3$H]QNB binding is obtained with oxotremorine as with QNB (Fig. 3). Half-maximal displacement occurs with 0.5–0.8 $\mu$M oxotremorine, while maximal displacement requires 100 $\mu$M oxotremorine.

*Association and Dissociation of Specific [$^3$H]QNB Binding.* At 35°, specific [$^3$H]QNB binding to whole rat brain homogenates is half maximal at about 4 min and plateaus by 40 min (Fig. 4). The bimolecular rate constant for the [$^3$H]QNB–receptor association, $k_1$, is $2.0 \pm 0.2 \times 10^8$ $M^{-1}$ $min^{-1}$. By contrast, nonspecific QNB binding is not time-dependent and is only about 12% of the total specific [$^3$H]QNB binding.

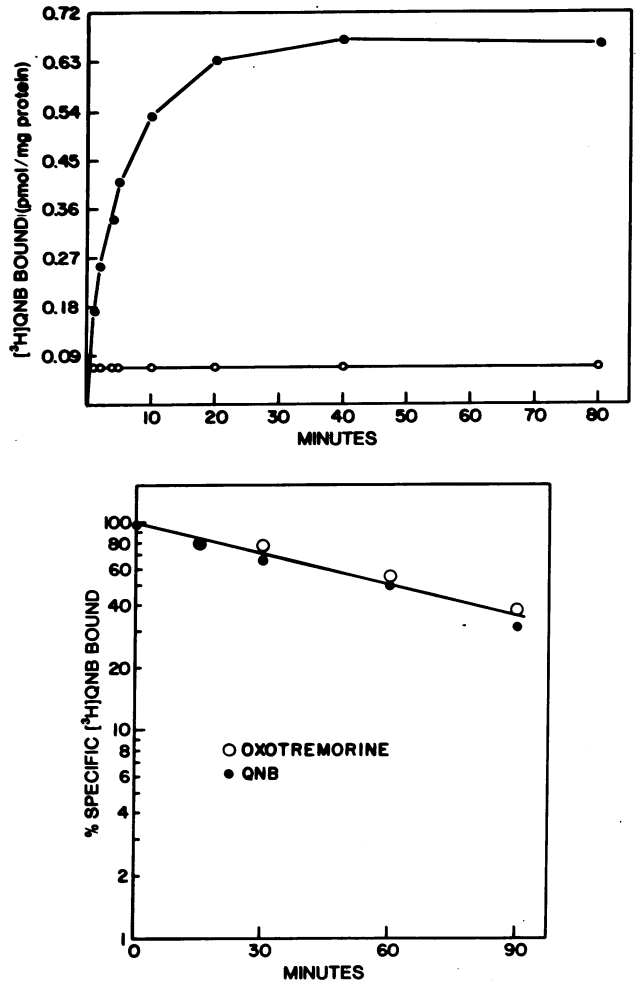


FIG. 4. Time dependence of specific (●——●) and nonspecific (○——○) binding of [$^3$H]QNB and dissociation of the [$^3$H]QNB complex. *Top:* The incubation medium contained 0.6 mg of tissue in 2.0 ml of Na–K phosphate buffer (pH 7.4) and 0.6 nM [$^3$H]QNB. Specific and nonspecific binding were determined at various times at 35° as described in the *text*. *Bottom:* The incubation medium contained 0.6 mg of rat brain tissue in 2.0 ml of Na–K phosphate buffer (pH 7.4) with 0.6 nM [$^3$H]QNB. After incubation for 60 min at 25°, nonradioactive QNB (0.01 $\mu$M) or oxotremorine (100 $\mu$M) were rapidly added and samples were either filtered immediately (time 0) or maintained for various intervals at 35° before rapid cooling and filtration.

The rate of dissociation of the QNB–receptor complex has been studied by labeling brain preparations with [$^3$H]QNB at 25° and then measuring the decline of bound [$^3$H]QNB after further incubation at 35° with either 0.01 $\mu$M nonradioactive QNB or 100 $\mu$M oxotremorine. (Fig. 4). The rate of dissociation was measured at 35°, because only negligible dissociation occurs at 25° for up to 40 min. When plotted semilogarithmically, the half-life of the QNB–receptor complex at 35° is about 60 min. The rate constant for dissociation at 35°, $k_{-1}$, is $1.2 \times 10^{-2}$ $min^{-1}$. The dissociation constant $k_{-1}/k_1$ is 0.06 nM.

*Effects of Cholinergic and Noncholinergic Drugs on Specific [$^3$H]QNB Binding.* If specific QNB binding reflects an interaction with the muscarinic receptor, muscarinic cholinergic drugs should have significant affinity for the binding site, while nicotinic and noncholinergic drugs should not. As shown in Table 2, the muscarinic antagonists, scopolamine, isoprop-

amide, and atropine are the most potent agents of the drugs examined. All three agents inhibit specific [$^3$H]QNB binding 50% at 0.4–2 nM. By contrast, the nicotinic antagonists, *d*-tubocurarine, pempidine, hexamethonium, mecamylamine, and a neurotoxin from the cobra (*Naja naja*), fail to alter binding at 10 $\mu$M concentration.

Muscarinic agonists have considerably less affinity than their antagonists. Acetylcholine and acetyl-$\beta$-methylcholine incubated together with 5 $\mu$M physostigimine to inhibit acetylcholinesterase have $ED_{50}$ values (the dosage effective in displacing 50% of the specific binding) of about 2–4 $\mu$M and 3–5 $\mu$M, respectively. Oxotremorine, whose central muscarinic cholinergic potency is greater than that of acetylcholine, displays 5 times more affinity for QNB binding sites than acetylcholine. The muscarinic agonists arecoline, pilocarpine, and carbamylcholine have $ED_{50}$ values of 10 $\mu$M, 7 $\mu$M, and 20–30 $\mu$M, respectively. Nicotinic agonists, nicotine and dimethylphenylpiperazinium (DMPP) inhibit specific [$^3$H]QNB binding only minimally (10–20%) at 10 $\mu$M. Both (+) and (−) isomers of the antihistamines, chlorpheniramine and brompheniramine, which display mild muscarinic anticholinergic pharmacologic effects, inhibit specific [$^3$H]QNB binding about 50% at 30–50 $\mu$M concentrations. A variety of drugs without known anticholinergic actions, which fail to alter specific [$^3$H]QNB binding at 10 $\mu$M levels includes: $\Delta^9$-tetrahydrocannabinol, corticotoxin, glycine, $\gamma$-aminobutyric acid (GABA), glutamic acid, aspartic acid, proline, naloxone, levorphanol, dextrorphan, diazepam, and chlordiazepoxide.

*Regional Localization of Specific [$^3$H]QNB Binding.* If specific [$^3$H]QNB binding involves the brain's muscarinic cholinergic receptor, its regional distribution should parallel that of muscarinic synapses. We have measured specific [$^3$H]-QNB binding, choline acetyltransferase and cholinesterase activities in nine discrete regions of the rat brain (Table 3). Specific [$^3$H]QNB binding and both enzyme activities are highest in the corpus striatum, the area of the brain which is richest in acetylcholine (16). The cerebral cortex, which contains almost as much specific [$^3$H]QNB binding as the corpus striatum, is one of the least enriched brain regions for choline acetyltransferase and cholinesterase. The least specific [$^3$H]-QNB binding is detected in the cerebellar cortex, which also contains the least choline acetyltransferase and cholinesterase activities of the brain regions. QNB binding in other areas tends to parallel choline acetyltransferase better than cholinesterase.

TABLE 2. *Relative potencies of drugs in reducing [$^3$H]QNB binding to rat brain homogenates*

| Drug | $ED_{50}$* ($\mu$M) |
|---|---|
| Quinuclidinyl benzilate (QNB) | 0.0004–0.0005 |
| Scopolamine | 0.0008–0.0009 |
| Isopropamide | 0.001–0.002 |
| Atropine | 0.001–0.002 |
| Oxotremorine | 0.5–0.8 |
| Pilocarpine | 7 |
| *d*-Chlorpheniramine | 20–30 |
| *l*-Chlorpheniramine | 40–50 |
| *d*-Brompheniramine | 20–30 |
| *l*-Brompheniramine | 40–50 |
| Arecoline | 1–3 |
| Acetylcholine† | 2–4 |
| Carbamylcholine | 20–30 |
| Acetyl $\beta$-methylcholine† | 3–5 |
| *No effect at 10 $\mu$M* | |

Aspartic acid, $\gamma$-aminobutyric acid, glutamic acid, proline, naloxone, methylphenidate, glycine, pempidine, levorphanol, dextrorphan, purified corticotoxin and neurotoxin from the cobra (*Naja naja*), hexamethonium, *d*-turbocurarine, mecamylamine, $\Delta^9$-tetrahydrocannabinol, neostigmine, diazepam, and chlordiazepoxide.

Values are the mean of data from three separate experiments performed in triplicate whose results varied less than 15%.

* Concentration of drug which displaced specific [$^3$H]QNB binding by 50%.

† Corrections for spontaneous hydrolysis were not possible; however physostigmine (5 $\mu$M) was added to the incubation medium to prevent enzymatic (cholinesterase) hydrolysis.

## DISCUSSION

The specific binding of [$^3$H]QNB to homogenates of rat brain has many characteristics which might be expected of interactions with muscarinic cholinergic receptors in the brain. Displacement of [$^3$H]QNB binding is greatest with muscarinic antagonists and the relative affinity of muscarinic cholinergic agonists tends to parallel their pharmacological potency. By contrast nicotinic and noncholinergic drugs have much less affinity for these binding sites. The extremely high affinity in the nanomolar range of muscarinic antagonists for QNB-binding sites is quite similar to their molar affinity for muscarinic receptors as demonstrated in smooth muscle. Atropine can interact with the specific choline uptake system of cholinergic neurons in the brain but only at concentrations several orders of magnitude greater than its affinity for QNB-binding sites (18). Muscarinic antagonists have negligible affinity for choline acetyltransferase, cholinesterase, or acetylcholine storage sites (19). Thus the selectively high affinity of muscarinic anticholinergic drugs for muscarinic receptors and for QNB-binding sites strongly favors the identity of the binding sites with muscarinic cholinergic receptors in the brain.

Subcellular fractionation experiments involving disruption of synaptosomes indicate an association of QNB binding with synaptic membrane fractions (20). Recently we have identified specific QNB binding to membrane fractions of the guinea pig ileum with kinetic properties and affinities for muscarinic antagonists and agonists very similar to values in the brain (22). In the ileum molar pharmacologic potencies (5) and binding affinities can be directly compared, providing additional evidence that QNB-binding sites involve muscarinic cholinergic receptors and suggesting that the receptor responds to drugs similarly in the peripheral and central nervous system. After septal lesions interrupting the septal-hippocampal cholinergic tract, QNB binding in the hippocampus is unaltered even though hippocampal choline acetyltransferase activity is depleted by 80% (21). This indicates that QNB binding sites are not located on cholinergic pre-synaptic terminals.

In some respects the regional distribution of specific [$^3$H]-QNB binding parallels that of choline acetyltransferase and cholinesterase. All three are highest in the corpus striatum and lowest in the cerebellar cortex. The lesser correlation of some other brain areas may relate to a variety of factors such as nicotinic (19), in addition to muscarinic (16), synapses and localization of enzymes in cell bodies and noncholinergic structures as well as at synapses. Very similar regional differences in [$^3$H]QNB binding, choline acetyltransferase and cholinesterase activities have been observed in 30 areas of the rhesus monkey brain (23).

TABLE 3. *Regional distribution of specific [$^3$H]QNB binding, choline acetyltransferase (C.A.T.), and cholinesterase (ChE) activities in rat brain homogenates*

| | [$^3$H]QNB specifically bound (pmol/g of protein) | C.A.T. (nmol of acetylcholine/mg of protein per hr) | ChE (nmol of acetylcholine/mg of protein per min) |
|---|---|---|---|
| Corpus striatum | 478 ± 82 | 36.0 | 153 |
| Hypothalamus | 131 ± 38 | 7.9 | 41.1 |
| Midbrain and pons | 150 ± 61 | 32.8 | 55.5 |
| Thalmus | 137 ± 35 | 15.3 | 49.0 |
| Medulla oblongata–cervical spinal cord | 51 ± 5 | 24.3 | 37.6 |
| Superior and inferior colliculi | 230 ± 83 | 11.9 | 35.4 |
| Cerebral cortex | 390 ± 23 | 8.6 | 28.2 |
| Cerebellar cortex | 34 ± 3 | 2.3 | 23.9 |
| Hippocampus | 243 ± 11 | 10.8 | 23.8 |

Specific [$^3$H]QNB binding was assayed as described in the *text*. Binding values represent the mean ± SEM of three determinations. Choline acetyltransferase (C.A.T.) and cholinesterase (ChE) activities are the average of two determinations, each performed in duplicate.

Muscarinic antagonists have 3 to 5 orders of magnitude more affinity for QNB binding sites than do cholinergic agonists. Similarly, strychnine, a glycine receptor antagonist, has about 1000 times more affinity for the glycine receptor than does glycine itself (4). The affinity of acetylcholine for QNB-binding sites is about the same as that of glycine for the glycine receptor and similar to that of acetylcholine for nicotinic receptor binding sites (19) and norepinephrine (24) and dopamine (25) for receptor-associated adenylate cyclase preparations.

It can be calculated from the direct measurement of specific [$^3$H]QNB binding at saturation that 1 g of rat brain without cerebellum has sufficient receptors to bind 65 pmol of [$^3$H]-QNB. Assuming that each [$^3$H]QNB molecule interacts with one receptor site, we calculate the number of receptor units in one rat brain (1.6 g) to be about $4 \times 10^{13}$. This value is close to the calculated density of nicotinic cholinergic receptors in the electric organ of the electric eel but about $^1/_{10}$th the number of receptors in the electric organ of *Torpedo* (1, 19). The density of apparent muscarinic receptor sites in rat brain is similar to the number of opiate receptors (15), and to the number of glycine receptors in the rat spinal cord (26). The number of receptor sites in rat brain we have calculated is the same as that estimated by Hiley *et al.* (8) for muscarinic sites in rat cerebral cortex by measuring the binding of a radiolabeled homologue of benzilylcholine mustard and similar to that reported for high affinity binding of [$^3$H]atropine to whole rat brain homogenates (6).

The binding of [$^3$H]QNB appears closely similar to the binding of a benzilylcholine mustard homologue (8), and may resemble [$^3$H]atropine binding to rat brain homogenates (6).

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