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Cholinergic Muscarinic Receptor: Biochemical and Light Autoradiographic Localization in the Brain

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The muscarinic cholinergic antagonist 3-quinuclidinyl benzilate (QNB) binds avidly but reversibly to the muscarinic cholinergic receptor of mammalian brain and peripheral tissues. [^{3}H]-QNB binding provides a simple, sensitive and specific assay for the muscarinic cholinergic receptor binding. Inhibition of [^{3}H]-QNB binding to homogenates of brain and guinea pig ileum by muscarinic drugs correlates with their pharmacologic potencies, while nicotinic agents and noncholinergic drugs have negligible affinity. The regional distribution of [^{3}H]-QNB binding throughout rat and monkey brain parallels to a major extent other cholinergic markers, suggesting that the majority of cholinergic synapses in the brain are muscarinic. [^{3}H]-QNB accumulation in various brain regions after intravenous injection provides a means of labelling the muscarinic receptor *in vivo*. By labelling the receptor *in vivo*, autoradiographic studies under the light microscope have been performed to visualize the muscarinic receptor.

Neurotransmitter receptor sites may be operationally defined as the »recognition« sites for the transmitter. A great deal of research effort has been directed in the past few years to studies of the nicotinic cholinergic receptor, especially in the electric organ of certain invertebrates[1–3]. Most of these studies have made use of a specific and tightly binding neurotoxin derived from snake venom, whose interactions with the receptor are almost irreversible. Some studies have employed equilibrium dialysis of purified receptor or tissue fragments against radiolabelled cholinergic agonists. With techniques from investigations of opiate receptor binding in animal brain[4], we identified the synaptic receptor for the neurotransmitter actions of glycine in mammalian brain by measuring the reversible binding of the glycine antagonist strychnine to synaptic membrane fractions[5]. Because most cholinergic synapses in the brain are thought to be muscarinic rather than nicotinic, we were interested in investigating muscarinic cholinergic receptor binding in the brain. Using [^{3}H]-atropine, Paton and Rang[6] first studied muscarinic receptor binding in the guinea pig ileum. Based on our experience with the glycine receptor, we have examined musca-

rinic receptor binding both in the brain and the guinea pig ileum by measuring interactions of tissue membrane fractions with a reversible muscarinic cholinergic antagonist, 3-quinuclidinyl benzilate (QNB)[7,8]. Other groups have studied the interaction of brain preparations with cholinergic agonists or alkylating agents derived from muscarinic agonists as well as atropine-like agents[6,9–16].

The binding of [^{3}H]-QNB to membrane preparations of brain and guinea pig ileum has provided a rapid, sensitive, simple and specific assay for the muscarinic cholinergic receptor and has enabled us to compare the properties of this receptor in the brain and in peripheral tissues[17–19]. By regional comparisons of the distribution of the muscarinic receptor with other cholinergic markers, we have been able to assess the extent to which muscarinic synapses account for cholinergic function in the brain. The avid but reversible binding of [^{3}H]-QNB has enabled us to label muscarinic receptors in the brain *in vivo*. By labelling muscarinic receptors *in vivo* with [^{3}H]-QNB, it has been possible to perform autoradiographic studies to localize receptor binding in a detailed fashion and, hopefully, ultimately to map muscarinic cholinergic pathways in mammalian brain.

Pharmacologic Specificity of the Muscarinic Receptor

In initial studies, we were able to show that low concentrations of [^{3}H]-QNB bind to brain homogenates in a fashion that can be almost completely inhibited by unlabelled QNB or cholinergic muscarinic agonists such as oxotremorine. Under the standard binding conditions only about 50—70 c.p.m. of [^{3}H]-QNB are entrapped by tissue in the presence of excess amounts of QNB or oxotremorine so that with low concentrations of [^{3}H]-QNB (0.1—0.6 nM) specific receptor binding is about 10 times nonspecific binding[17]. In various subcellular fractions, specific QNB binding is most abundant in crude mitochondrial fractions which are enriched in synaptosomes or pinched-off nerve endings[17].

Using whole rat brain homogenates, [^{3}H]-QNB binding is saturable with half-maximal binding at about 0.4 nM. By contrast, nonspecific binding, in the presence of 0.01 μM unlabelled QNB or 100 μM oxotremorine, is not saturable and increases linearly with increasing [^{3}H]-QNB. [^{3}H]-QNB binding is inhibited by nonradioactive QNB with half-maximal displacement at about 0.4—0.5 nM nonradioactive QNB, essentially the same value for half-saturation obtained with [^{3}H]-QNB. By contrast, the agonist oxotremorine has much less affinity for binding sites so that half-maximal inhibition requires about 0.7 μM oxotremorine. These characteristics of [^{3}H]-QNB binding, especially saturation and displacement by QNB and oxotremorine are closely similar in homogenates of the guinea pig ileum and in the brain[19].

The fact that QNB binding is saturable at low concentrations and can be inhibited by a muscarinic agonist is by no means sufficient evidence to ensure that the binding involves the pharmacologically relevant muscarinic receptor. To ensure specificity, one must evaluate a wide range of muscarinic drugs as well as numerous drugs which are not thought to interact with the muscarinic receptor. Ideally, one would like to correlate the molar affinity for binding sites with pharmacologic potency. Such correlations cannot be obtained in the brain. However, in the guinea pig ileum, one can measure in the same intestinal

strip the ability of drugs to inhibit [^{3}H]-QNB binding and to alter the contractile properties of the muscle. Accordingly, we compared the ability of a wide range of muscarinic agonists and antagonists to inhibit [^{3}H]-QNB binding in the brain and in the guinea pig ileum and examined the relationship of these values to those obtained for the influence of these drugs upon contraction of the guinea pig ileum (Table I).

TABLE I.

Relative Potencies of Drugs in Reducing [^{3}H]QNB *Binding to Rat Brain Homogenates and to the Longitudinal Muscle of the Guinea Pig*

Drugs	Rat Brain ID_{50}/M[a]	Guinea Pig Ileum ID_{50}/M[a]	Biological Values ED_{50}/M[b]	Ref. (19)
Antagonists				
3-Quinuclidinyl benzilate	$4—5\times10^{-10}$	$2—3\times10^{-10}$	5×10^{-10}	
Scopolamine	$7—9\times10^{-10}$	$2—3\times10^{-10}$	3×10^{-10}	(6)
Atropine	$1—2\times10^{-9}$	$2—4\times10^{-9}$	1×10^{-9}	(6)
Isopropamide	$1—2\times10^{-9}$	$6—7\times10^{-9}$	—	(57)
Diphenyldioxolane	$1—2\times10^{-8}$	3×10^{-8}	2×10^{-8}	
Mixed agonists-antagonists				
Pilocarpine	7×10^{-6}	$7—9\times10^{-7}$	1×10^{-6}	(58, 59)
DL-Diisopropyl-dioxolane	4×10^{-5}	7×10^{-5}	3×10^{-5}	(60)
D-Diisopropyl-dioxolane	$2—3\times10^{-5}$	3×10^{-5}	4×10^{-5}	(60)
L-Diisopropyl-dioxolane	7×10^{-5}	8×10^{-5}	9×10^{-5}	(60)
Agonists				
Oxotremorine	$5—8\times10^{-7}$	$5—8\times10^{-7}$	—	
Arecoline	$1—3\times10^{-6}$	$3—4\times10^{-6}$	—	
Acetylcholine[c]	$2—4\times10^{-6}$	$2—4\times10^{-6}$	$4—6\times10^{-8}$	(58,61)
Acetyl-β-methylcholine	$3—5\times10^{-6}$	$2—3\times10^{-6}$	$5—6\times10^{-8}$	(58, 60)
DL-Cis-methyl-dioxolane	$7—9\times10^{-6}$	$6—8\times10^{-6}$	2×10^{-8}	(60)
D-Cis-metyl-dioxolane	$2—3\times10^{-5}$	$3—4\times10^{-5}$	2×10^{-7}	(60)
L-Cis metyl-dioxolane	$7—9\times10^{-6}$	—	2×10^{-8}	(60)

No Effect at 10 μM:

D-Acetyl-β-methylcholine, 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-tubocurarine, mecamylamine, Δ^9-tetrahydrocannabinol, diazepam, napthyl--vinylpyridinium (NVP), chlordiazepoxide and angiotensin.

[a] Concentration of drug which inhibited specific [^{3}H]QNB binding by 50%.

[b] Values listed are the ED_{50} (concentrations for 50% of maximal response) obtained in pharmacological procedures using intact smooth muscle preparations. The smooth muscle used in references 57, 60, 61 and 59 was the longitudinal muscle of guinea pig ileum, while that used in reference 58 was the circular muscle of the rabbit stomach fundus.

[c] Physostigmine (1 μM) was added to the incubation to prevent enzymatic (AChE) hydrolysis.

QNB itself is one of the most potent muscarinic antagonists, causing 50% inhibition of [^{3}H]-QNB binding at concentrations under 0.5 nM both in the brain and the guinea pig ileum. Other muscarinic antagonists such as scopolamine, atropine, diphenyldioxolane and isopropamide are also very potent inhibitors of [^{3}H]-QNB binding in brain and intestine and are potent antagonists of acetylcholine induced contractions of the muscle. The pharmacologic potencies of the antagonists are similar to the concentrations of the agents which inhibit QNB binding 50%. Mixed agonist-antagonists, such as pilocarpine and di-isopropyl-dioxolane, also have similar biological and binding potencies.

There are some marked discrepancies between biological potencies of agonists and their potencies as inhibitors of QNB binding. Agonists for which we have both biological and binding data are two to four orders of magnitude more potent in contracting intestinal strips than in reducing [^{3}H]-QNB binding. For instance, acetylcholine elicits 50% of maximal contraction at about 0.05 μM concentration (ED_{50} value) but requires about 3 μM concentration to lower [^{3}H]-QNB binding by 50%. If the ED_{50} values correspond to the concentration of drug for half maximal receptor occupation, then there are major differences between biological and binding data.

Some workers have postulated that the majority, as much as 90% or more, of muscarinic receptors in the guinea pig intestine are »spare« receptors so that occupation by a pure agonist such as acetylcholine of only a small proportion will elicit a maximal biologic response[20]. In this situation one may determine the extent of receptor occupation biologically by measuring the ED_{50} of the agonist after »removing« all the spare receptors with an alkylating drug. In these experiments 50% receptor occupation appears to occur at 2—3 μM concentrations of acetylcholine and acetyl β-methylcholine, similar to the concentrations for 50% inhibition of QNB binding. However, it is possible that the ability of alkylating drugs to reduce the potency of agonists without altering the maximal response does not result from destruction of spare receptors for the agonist. According to this latter formulation, there are no spare muscarinic receptors in the guinea pig intestine. Instead, the biological ED_{50} values for an agonist correspond closely to the concentration required for 50% receptor occupation. In this case, the major discrepancies between binding and biological data require explanation.

An allosteric model of receptor function is consistent with the close correspondence between biologic and binding data for antagonists but not for agonists[21,22]. Neurotransmitter receptors are postulated to exist in interconvertible »agonist« and »antagonist« conformations. Agonists have high and low affinities respectively for the agonist and antagonist receptor states while the reverse situation occurs for antagonists. Neurotransmission occurs only when an agent binds to the agonist conformation of the receptor and is associated with a change in binding to the receptor of a relevant ion, triggering a change in the ion's membrane conductance. We have obtained direct evidence for the existence of interconvertible agonist and antagonist states of the opiate and glycine receptors[23–28]. Low concentrations of sodium selectively transform the opiate receptor into the antagonist form[24,29]. Chloride and other anions inhibit receptor binding of strychnine, the glycine antagonist, in proportion to their ability to mimic the synaptic actions of chloride at glycine synapses[5].

Conceivably such a model of receptor dynamics may apply to the muscarinic receptor. According to this formulation, QNB and other antagonists bind to

the antagonist conformation of the receptors, explaining the good agreement of biologic and binding data. Because pure agonists have much less affinity for the antagonist than for the agonist conformation of the receptor, they are much less potent in reducing QNB binding than in contracting intestinal strips, the latter reflecting their high affinity for the agonist form of the receptor. Mixed agonist-antagonists, with similar affinities for both agonist and antagonist receptor states, should inhibit QNB binding and contract intestinal strips with similar potencies, as is observed experimentally.

As a check on specificity of QNB binding to muscarinic receptor, a wide range of nicotinic cholinergic agents as well as noncholinergic drugs have been examined for their ability to inhibit QNB binding. All of those agents have little effect at concentrations of 10 μM (Table I).

Regional Distribution of the Muscarinic Receptor

Although neurophysiologic studies in general suggest that muscarinic cholinergic receptors account for a major portion of cholinergic synapses in mammalian brain, the identification of specific synapses as muscarinic or nicotinic has never been altogether clear. Muscarinic receptors seem to predominate in the caudate nucleus, putamen and cerebral cortex[30–32], while in the spinal cord, acetylcholine receptors of the Renshaw cells are nicotinic[33–35]. In several regions both nicotinic and muscarinic receptors appear to be present, according to neurophysiologic studies. In an effort to examine this question, we evaluated the density of [^{3}H]-QNB binding sites in numerous regions of the monkey brain and compared QNB binding with high affinity choline uptake, thought to label cholinergic nerve terminals selectively[36–38], the activity of choline acetyltransferase, the acetylcholine synthesizing enzyme, and the activity of cholinesterase[39] (Table II).

There are marked regional variations in QNB binding with at least a 29-fold variation between the putamen, containing the highest density of binding sites and the optic chiasm, the lowest. The head and body of the caudate contain about the same amount of binding as the putamen, which in turn contains 6 times as much binding as the closely juxtaposed globus pallidus. The six areas of the cerebral cortex examined contain similar amounts of QNB binding, about half the levels of the caudate and putamen. The amygdala and hippocampus possess about the same amount of binding as the cerebral cortex. In all three regions of the thalamus examined, binding is similar and levels are somewhat higher than in the hypothalamus. Within the midbrain, highest binding occurs in the superior colliculi, almost 1.5 times that of the inferior colliculi and 3 times binding in raphe area. Binding of QNB in the pons is only about half that of the superior colliculi but double that of the medulla oblongata. The lowest QNB binding in the brain stem occurs in the inferior olivary nucleus, less than half that of the medulla oblongata.

QNB binding in the cervical spinal cord is the same as in the inferior olivary nucleus and similar to values in the optic chiasm, the lowest area of the brain. Interestingly, although QNB binding is low in areas containing exclusively white matter such as the corpus callosum, corona radiata and cerebral peduncles, levels are definitely demonstrable in these regions and are comparable to binding in the cerebellar cortex. Conceivably, in white matter areas which contain axons but no cells or nerve terminals, QNB

TABLE II.

Regional Distribution of Specific [^{3}H]QNB *Binding,* [^{3}H]*Choline Uptake, Choline Acetyltransferase* (CAT) *and Cholinesterase* (ChE) *Activities in Monkey Brain Regions*

	[^{3}H]QNB Binding	[^{3}H]Choline Uptake	CAT	ChE
Cerebral Hemispheres				
Frontal Pole	439 ± 88	1.72	4.4 ± 0.6	15.7
Occipital Pole	578 ± 80	1.16	3.28 ± 0.6	14.8
Precentral Gyrus	483 ± 42	1.73	6.47 ± 1.1	18.6
Postcentral Gyrus	516 ± 29	2.46	7.95 ± 1.3	30.5
Cingulate Gyrus	546 ± 90	2.48	6.35 ± 0.5	18.5
Pyriform Cortex	474 ± 83	5.97	18.6 ± 5.2	29.3
White Matter Areas				
Corpus Callosum	107 ± 28	0.94	1.4 ± 0.5	11.7
Corona Radiata	87.4	0.55	3.49	18.4
Optic Chiasm	34.4 ± 25	—	3.60 ± 0.3	32.9
Cerebral Peduncles	140 ± 45	1.2	3.2 ± 1.7	44.2
Limbic Cortex				
Amygdala	496	—	26.2	122.0
Hippocampus	502 ± 58	6.25	13.2 ± 1.5	45.2
	241 ± 46	9.67	13.9 ± 1.4	57.4
Hypothalamus				
Thalamus				
Anterior	285 ± 85	7.29	14.6 ± 2.0	59.1
Medial	369 ± 66	7.20	23.9 ± 3.6	73.1
Lateral	360 ± 54	3.96	12.7 ± 1.7	59.8
Extra-Pyramidal Areas				
Caudate Nucleus (head)	976 ± 110	28.1	72.8 ± 14.3	281.0
Caudate Nucleus (body)	1061 ± 114	56.1	90.0 ± 10.8	172.0
Putamen	1126 ± 55	35.0	111.0 ± 13.9	354.0
Globus Pallidus	168 ± 47	2.4	9.3 ± 2.0	72.0
Midbrain				
Superior Colliculi	381 ± 83	10.5	35.2 ± 1.4	121.0
Inferior Colliculi	279 ± 39	4.58	10.5 ± 0.6	46.1
Raphe Area	149 ± 32	4.54	36.0 ± 2.4	61.1
Cerebellum-Lower Brain Stem				
Pons	212 ± 112	2.70	9.2 ± 1.7	28.5
Cerebellar Cortex	125 ± 65	1.45	0.8 ± 0.5	54.3
Floor of Fourth Ventricles	96.5 ± 35	5.05	21.4 ± 3.2	45.3
Medulla Oblongata	114 ± 31	4.29	10.2 ± 0.8	36.5
Inferior Olivary Nucleus	47.6 ± 31	3.85	2.4 ± 1.0	68.8
Pontine Tegmentum	146 ± 27	—	12.6 ± 0.1	63.8
Spinal Cord				
Cervical Cord	47.6 ± 3	3.19	8.1 ± 2.0	22.2

Specific [^{3}H]QNB binding values are given in pmol/g protein and are the mean of 4 samples ± S.E.M. See Table III for description of binding assay. Choline uptake was performed in duplicate and is the mean of 2 samples. Velocities of choline uptake are given in nmol/g protein/4 min. [^{3}H]choline (1 μM) was incubated in 1.9 ml of Krebs PO_4 buffer, pH=7.4 at 30° C for 4 min. Accumulation of [^{3}H]choline occurring at 0 °C was subtracted from total uptake values. Choline acetyltransferase (CAT) activities are given in nmol ACh synthesized/(mg protein)h and are the mean ± S.E.M. of 4 samples. Cholinesterase (ChE) activities are given in nmoles ACh hydrolyzed/(mg protein) min and are the mean of 2 samples. Data are adapted from Yamamura *et al.*[39]. Value for one monkey.

binding reflects the presence of muscarinic cholinergic receptors on axons[14]. This would accord with recent evidence for the existence of presynaptic receptors for a variety of neurotransmitters in nerve endings and cell bodies of the neurons which synthesize the neurotransmitter[40–49]. However lesioning studies indicate that QNB binding is almost wholly associated with postsynaptic muscarinic receptors[18]. The septal-hippocampal cholinergic pathway in the brain can be destroyed by selective septal lesions[36]. These lesions deplete hippocampal acetylcholine and choline acetyltransferase but fail to alter QNB binding, which therefore is predominantly post-synaptic[18].

[^{3}H]-Choline uptake and choline acetyltransferase activity closely parallel each other throughout the brain and correlate well with QNB binding in some areas. Thus the highest choline uptake and choline acetyltransferase activity occur in the head and body of the caudate and in the putamen with values several times that of the globus pallidus, just as occurs with QNB binding. Similarly the cerebellar cortex and white matter areas contain very low amounts of choline acetyltransferase, choline uptake and QNB binding.

However, there are some striking discrepancies in these correlations. Thus within the cerebral hemispheres, the pyriform cortex contains the most choline acetyltransferase activity and [^{3}H]-choline uptake, while its QNB binding is about the same as for other cerebral regions. Perhaps these data have bearing on the relative distribution of muscarinic and nicotinic cholinergic receptors throughout the cerebral cortex. One might speculate that the pyriform cortex contains a substantial density of nicotinic receptors not present in other cerebral cortical regions.

Except for the caudate and putamen, the superior colliculi and raphe area possess the highest choline acetyltransferase activity in the brain, while QNB binding is quite low in the raphe area and moderate in the superior colliculi. By contrast, the floor of the fourth ventricle, with one of the lowest QNB binding levels, contains substantial choline acetyltransferase activity. Similar regional variations in muscarinic receptor binding have been observed for dog brain[14].

As already mentioned, some of the discrepancies between QNB binding and other cholinergic markers may relate to relative amounts of nicotinic and muscarinic receptors in the brain. However, interpretations must be made cautiously. Transmitter synthesis takes place both in cell bodies and nerve terminals, while receptor binding presumably is largely associated with synaptic areas as is also the case with high affinity choline uptake. Even if choline uptake were to label the nerve terminals of a given synapse and QNB binding to involve the associated postsynaptic membrane, the relative surface area of nerve ending and postsynaptic element might vary in different brain regions.

Identification of Muscarinic Receptor Binding in vivo

Although the labelling of the muscarinic receptor with QNB *in vitro* enables one to evaluate many characteristics of the receptor site, it is possible that important functional characteristics may be obscured. Thus, changes in the number of muscarinic receptor sites under varying physiological and pharmacologic conditions may be evanescent and »wash out« during the time that one kills the animal and prepares brain homogenates for receptor assay. For this reason, the ability to detect cholinergic receptor binding *in vivo* would be valuable. Perhaps even more important, to perform autoradiographic studies

of the receptor under conditions in which morphological relationship of neuronal elements are preserved, one would like to label the receptor *in vivo*.

Several workers measured the accumulation of cholinergic drugs in mammalian brain, but most of the accumulation was rather evenly distributed throughout the brain and did not appear to be associated with the muscarinic receptor. Because QNB has such a high affinity for the muscarinic receptor, we examined its accumulation by various regions and have been able to demonstrate that QNB labels selectively the muscarinic cholinergic receptor *in vivo*[50].

The experimental approach has been rather straightforward. Rats anesthetized with ether receive tail vein injections of [^{3}H]-QNB, their brains are rapidly dissected and the entire brain region or membrane preparations from it analyzed for accumulated radioactivity. In all experiments there is no evidence for any metabolism of QNB and all accumulated radioactivity in various regions of the brain can be accounted for completely by authentic [^{3}H]-QNB.

The principal evidence that QNB labels muscarinic receptors *in vivo* is that the regional distribution of the label after *in vivo* administration of QNB is essentially the same as the regional variation of QNB assayed *in vitro* (Table III). Highest binding occurs in the corpus striatum, with somewhat less binding in the cerebral cortex and hippocampus. The hypothalamus and medulla-pons contain only about a third of the binding of the corpus striatum

TABLE III.

Regional Distribution (in vitro) *and Accumulation* (in vivo) *of* [^{3}H]QNB *in Rat Brain*

Brain Regions	*In vitro* [^{3}H]QNB Specifically Bound[a]	*In vivo* Atropine-Displaceable [^{3}H]QNB[b]
	pmol/g of protein	min^{-1}/mg tissue
Corpus Striatum	478 ± 82	426
Cerebral Cortex	390 ± 23	348
Hippocampus	243 ± 11	278
Superior and Inferior Colliculi	230 ± 83	—
Midbrain and Pons	150 ± 61	—
Thalamus	137 ± 35	—
Hypothalamus	131 ± 38	59
Medulla Oblongata-Cervical Spinal Cord	51 ± 5	15
Cerebellar Cortex	34 ± 3	0

To assay specific binding of [^{3}H]QNB, an aliquot of each brain region was incubated at 25 °C with 2 ml of 0.05 M sodium-potassium phosphate buffer (pH=7.4), containing radiolabelled QNB. After a 60-min incubation 3 ml of ice-cold buffer was added and the contents were passed through a glass filter (GF/B) positioned over a vacuum. The filters were washed 3 times under vacuum with 3 ml of ice-cold buffer. Every determination of binding was performed in triplicate, together with triplicate samples containing unlabelled QNB (0.01 μM) or oxotremorine (100 μM) 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 °C for 8—12 h and the radioactivity then assayed by liquid scintillation spectrometry. Values are the mean ± S.E.M.

while QNB accumulation in the cerebellum is the lowest in the brain, only 1/6th levels in the corpus striatum.

Another reason to feel confident that [^{3}H]-QNB *in vivo* labels the muscarinic receptor is that pretreatment of animals with atropine inhibits QNB binding markedly in all areas with the exception of the cerebellum and to a certain degree of the medulla-spinal cord (Table 3). This effect of atropine in preventing QNB binding is a very specific one. The nicotinic cholinergic antagonist, mecamylamine (5 mg/kg), phenobarbital (50 mg/kg) or L-dihydroxyphenylalanine (50 mg/kg) fail to prevent [^{3}H]-QNB binding in any areas. Interestingly, QNB accumulation in various brain regions is saturable with increasing doses of intravenously injected [^{3}H]-QNB. Half-maximal accumulation of [^{3}H]-QNB in the corpus striatum is fairly similar to values obtained for half-maximal saturation *in vitro*.

The time-course relationships of QNB accumulation in various brain regions are striking (Figure 1). Peak levels occur in all brain regions at 2.5 min, the first time point measured. In the cerebellum, QNB levels then fall so rapidly

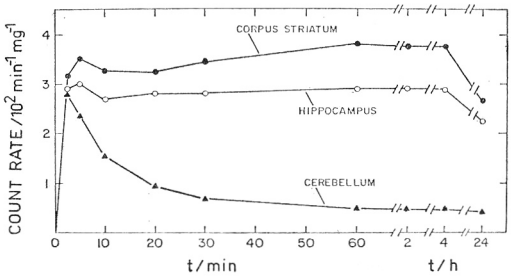

Fig. 1. Time course of [^{3}H]QNB accumulation into various regions of rat brain. Rats were given 60 µCi of [^{3}H]QNB (4 Ci/nmol) intravenously via the tail vein in 0.3 ml of saline and sacrified after various time intervals. Brain regions were placed in scintillation vials containing 1 ml of NEN solubilizer (Protosol) and incubated at 37 °C until solubilized. Each point represents the mean of at least 3 animals which did not vary more than 10—15%. Adapted from Yamamura *et al.*[50].

that by 10 min they are only half of peak values. Thereafter cerebellar QNB content declines to value about 1/5th of peak values of 60 min with little change for the next 24 hours. In dramatic contrast, the corpus striatum and hippocampus evince no reduction in [^{3}H]-QNB levels between 2.5 min and 4 hours; between 4 and 24 hours levels decline only about 25%. The fact that cerebellar QNB accumulation cannot be prevented by atropine and declines much more rapidly than in other regions suggests that QNB accumulation in the cerebellum does not involve the cholinergic receptor. The extremely slow decline of QNB levels in the corpus striatum and hippocampus indicates that QNB binds tightly to the muscarinic receptor.

Autoradiographic Studies

One of the aims of obtaining muscarinic receptor binding *in vivo* is to identify the receptor by autoradiographic techniques in the light microscope[51].

Animals were injected with [^{3}H]-QNB under conditions similar to those used for *in vivo* binding studies and brains frozen by partial immersion in liquid nitrogen, sectioned in a cryostat, »thaw mounted«, developed, fixed, and examined using autoradiographic procedures essentially similar to those of

Anderson and Greenwald[52] and Gerlach and McEwen[53]. After 30 days of exposure, a number of interesting features are observed. Areas of the brain containing high densities of autoradiographic grains are the corpus striatum, the hippocampus and the cerebral cortex, which also display high levels of QNB binding both *in vivo* and *in vitro* (Table IV). As expected, the density of

TABLE IV.

Relative density of autoradiographic grains in representative brain regions

Region	Relative Grain Density
Hippocampus	
Stratum oriens	++++
Stratum radiatum	++++
Stratum moleculare	++++
Alveus	+
Striatum	++++
Cerebral Cortex	
Cingulate Cortex	++++
Pyriform Cortex	+++
Intermediate depth of cortex	++
Thalamus	
Lateral thalamic nuclei	++
Nucleus parafascicularis	++
Medial habenular nucleus	++
Hypothalamus	
Arcuate Nucleus	++
Dorsomedial nucleus pars ventralis	++
Midbrain	
Raphe nucleus	+
Interpeduncular nucleus	+
Cerebellar Cortex	0 to +

Relative densities were determined by examining the brain region after a 30-d exposure. Densities refer to specific binding as they are corrected for blank values obtained by examining similar regions from atropine-pretreated animals. Relative grain densities for cerebral cortex and striatum were measured on coronal sections of rat brain showing Brodal's area 6 of the cerebral cortex. Data adapted from Kuhar and Yamamura[51].

grains in all brain regions in animals pretreated with atropine is greatly lowered. Within the corpus striatum, a high density of grains occurs relatively evenly across the entire nucleus and is several times more dense than the distribution over the adjoining globus pallidus and about 20 times greater than background grains.

Hippocampus has been chosen for more detailed investigation, because its cholinergic properties have been characterized extensively biochemically[18,36], neurophysiologically and histochemically[31,54,55]. About 50% of hippocampal neurons are excited by acetylcholine, an effect which is blocked by atropine[54,56]. Nerve terminals which histochemically strain for acetylcholinesterase in the electron microscope are distributed throughout regions containing the dendritic shafts of the pyramidal cells. Thus it is of interest that within the hippocampus a high density of autoradiographic grains is observed over the stratum oriens and stratum radiatum, regions containing the apical and basal dendrites of the pyramidal cells. Grain density then falls abruptly in the region of the alveus, a fiber tract (Figure 2). The stratum moleculare of the dentate gyrus, a region containing dendritic shafts of the granule cells, also possesses a high grain density which falls abruptly at the ventral boundary of the dentate gyrus (Figure 2). The high grain densities around cells in regions described

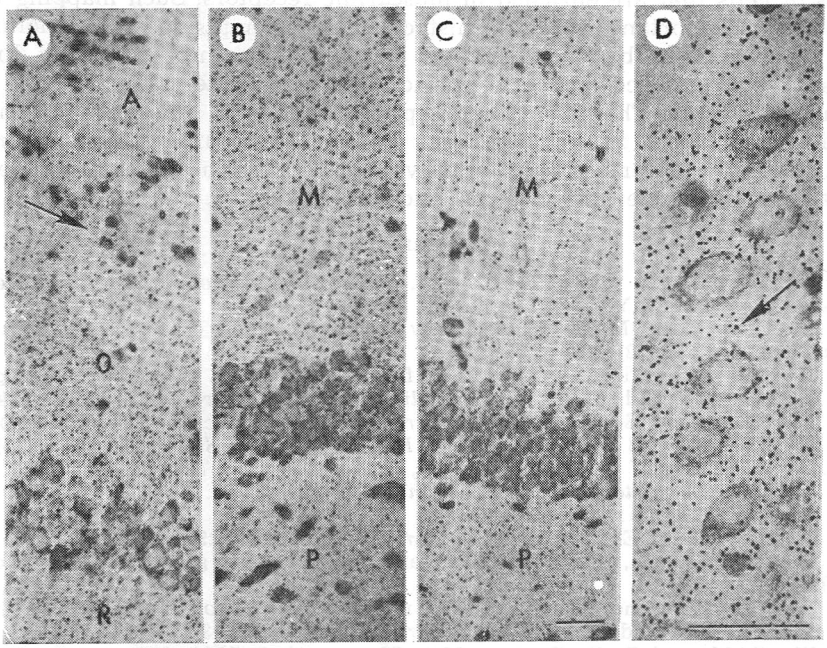

Fig. 2. Light autoradiographs of regions of the brain. The grains are shown most clearly in (*D*) (arrow), a higher power micrograph of the striatum. Lower power micrographs are shown in (*A*), (*B*) and (*C*) to make the pattern of distribution more observable. Bars represent 25 μm. *A*, section of hippocampus proper showing pyramidal cells and adjacent areas in CA1. Note the high density of grain over regions on either side of the pyramidal cells, the stratum oriens (O) and the stratum radiatum (R), which contain the basal and apical dendrites of the pyramidal cells. The density decreases sharply at the boundary of (see arrow pointing along the boundary) and over the alveus (*A*), a fibre tract. *B*, section of the dentate gyrus showing the granule cell layer and the adjacent areas, stratum moleculare (M) and stratum polymorphe (P). The stratum moleculare contains the dendritic processes of the granule cells, while the stratum polymorphe (this picture shows an area away from the pyramidal cells in CA4), is a heterogenous zone containing the axons of the granule cells. Note the much higher density of grains in the regions containing the dendritic processes. *C*, same region as in (*B*) except it is from an atropine-pretreated animal. Note the great reduction in density of grain in the stratum moleculare, the dendrite-containing region while there is little or no change in the striatum polymorphe. *D*, higher magnification of a section of the striatum, a region with a similar density of grains as that of the dendrite-containing regions of the hippocampus. The density was relatively uniform across the entire nucleus caudatus-putamen. Data from Kuhar and Yamamura[51].

above contrast with much lower densities around cells in other nuclei such as the dorsal midbrain raphe nucleus, medial habenular nucleus, lateral thalamic nucleus and periventricular nucleus of the hypothalamus (Table IV).

Throughout the brain, there is a tendency for autoradiographic grains to appear more frequently between rather than over cells, a feature which has been verified by grain counts showing more than 4 times as many grains per unit area in noncellular as in cellular areas. This suggests that most of the [^{3}H]-QNB binds to dendritic and postsynaptic regions, which are localized to areas devoid of cell bodies. In these regions which do not contain cells, one presumes that the grains are localized over dendrites containing cholinergic receptors. However it is not possible to visualize such dendrites in the light microscope so that one cannot precisely determine which cells have the binding sites.

These preliminary studies demonstrate the feasibility of localizing discrete groups of cells that are enriched in muscarinic receptors. Such mapping endeavors are of importance, because histochemical attempts to study cholinergic tracts in the brain by cholinesterase staining have met with only limited success due to the occurrence of considerable cholinesterase activity in non-cholinergic systems. The much greater specificity of QNB binding may facilitate significant advances in understanding cholinergic neuronal systems in the brain. Studies thus far are only tentative and detailed localization will require autoradiography under the electron microscope.

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SAŽETAK

Kolinergični muskarinski receptor: Biokemijska i svjetlosna autoradiografska lokalizacija u mozgu

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Muskarinski kolinergični antagonist 3-kinuklidinil-benzilat (KNB) veže se brzo ali reversibilno na muskarinski kolinergični receptor mozga sisavaca i perifernog tkiva. Vezanje [^{3}H]-KNB omogućava jednostavnu, osjetljivu i specifičnu metodu istraživanja načina vezivanja na muskarinski kolinergični receptor. Inhibicija vezanja [^{3}H]-KNB u homogenatima mozga i ileuma zamorca muskarinskim spojevima u korelaciji je s njihovim farmakološkim učinkom, dok je afinitet nikotinskih agensa i nekolinergičnih spojeva zanemarljiv. Regionalna raspodjela mjesta vezivanja [^{3}H]-KNB u mozgu štakora i majmuna uglavnom se slaže s mjestima vezivanja ostalih markiranih kolinergičnih spojeva te ukazuje na to, da je većina kolinergičnih sinapsi u mozgu muskarinske prirode. Nagomilavanje [^{3}H]-KNB u različitim dijelo-

vima mozga nakon intravenozne injekcije pretstavlja način markiranja muskarinskih receptora *in vivo*. Da bi se dobila predodžba o muskarinskom receptoru, načinjene su autoradiografske studije pod mikroskopom pomoću markiranja receptora *in vivo*.

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