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REGIONAL DISTRIBUTION OF MUSCARINIC RECEPTORS PREFERRING GALLAMINE IN THE RAT BRAIN

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Abstract—The regional distribution of muscarinic receptors recognized by the antagonist gallamine was determined autoradiographically by the ability of gallamine to reduce the binding of [^{3}H]quinuclidinyl benzilate in rat brain slices. The inhibition data obtained from indirect binding assays on whole slices indicated that gallamine distinguished at least two sites with differing affinities. Analysis using a two-site model gave $K_h = 0.6\ \mu M$, $K_l = 10\ \mu M$. The regions of highest and lowest affinity for gallamine were apparent qualitatively by visual inspection of the autoradiograms. A number of regions in coronal sections at three different levels were compared by microdensitometry. Gallamine possessed greater overall affinity for the diencephalon and brainstem than for the forebrain. Within the forebrain, the septal nucleus was unique in that it displayed high affinity for gallamine. Within the brainstem, the superior colliculus had the greatest proportion of sites with high affinity for gallamine. In general, the binding profile of gallamine was opposite to that of the antagonist pirenzepine and similar to that of the agonist carbachol, suggesting that gallamine is selective for M2 muscarinic receptors.

The existence of at least three types of central muscarinic cholinergic receptors has been demonstrated in binding studies [1, 2]. It is apparent that agonists distinguish these subpopulations on the basis of affinity. Antagonists such as quinuclidinyl benzilate (QNB) and atropine, conversely, recognize only the presence of a homogeneous class of muscarinic receptors [3]; they do not differentiate brain regions on the basis of affinity. The binding characteristics of these classical antagonists are proposed to be competitive and mutually exclusive and to follow the law of mass-action [3]. Recently, however, the antagonists pirenzepine [4] and gallamine [5, 6] were identified as "nonclassical" antagonists; they discriminate subpopulations of central muscarinic receptors, yet do not possess any characteristics of an agonist or partial agonist. These results are supported by recent pharmacological studies which report that these antagonists differentially block central muscarinic receptors [7, 8].

In the present study, the anatomical distribution of the muscarinic receptors that possess different affinities for gallamine has been measured by the autoradiographic localization of [^{3}H]QNB binding in the presence and absence of various concentrations of gallamine.

MATERIALS AND METHODS

Materials. Tritiated *l*-quinuclidinyl benzilate, with a specific activity of 32 Ci/mmole, was purchased from the Amersham Corp. Atropine sulfate and gallamine triethiodide were purchased from the Aldrich Chemical Co. and K & K Laboratories respectively.

Section and slide preparation. Autoradiographic procedures were modifications of methods outlined by Young and Kuhar [9] and Wamsley *et al.* [10, 11]. Male Long–Evans rats were killed by cardiac perfusion with 40 mM sodium potassium phosphate buffer, pH 7.4, followed by 0.1% formaldehyde in the same buffer (200 ml). The brains were quickly removed and rapidly frozen with liquid nitrogen. A Hacker–Bright cryostat microtome was used to obtain 12 μm coronal sections from the level of the caudate nucleus and the anterior commissure, to the caudal end of the hippocampus at the level of the superior colliculus. The sections were mounted on acid-washed, subbed (coated with chromium potassium sulfate and gelatin) slides. There were four sections per slide. The slides were stored at −20° until they were used in the binding assays.

Binding assays. Direct and indirect binding assays were performed. The direct binding assay determined the B_{max} and K_d values of QNB. The value of the K_d, which was determined from Scatchard analysis, was 0.5 nM, similar to previously reported values [10]. Prepared slides were incubated in Coplin jars in the presence of two concentrations of [^{3}H]QNB (0.2 and 2 nM) for 1.5 hr. A concentration of 2 nM was selected to estimate the B_{max} values because it is approaching saturation while nonspecific binding is relatively low. Nonspecific binding was measured at each concentration of [^{3}H]QNB by adding an excess of unlabeled atropine. At the end of the incubation period, the slides were removed from the jars and rinsed twice in phosphate buffer for 10 min to remove any unbound drug. One section was then wiped off each slide with filter paper and placed in a Nalge bag for standard scintillation counting. In the indirect binding assays, the binding of

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gallamine was inferred from the displacement of [^{3}H]QNB. The slides were incubated with 0.2 nM [^{3}H]QNB in the presence of increasing concentrations of gallamine (0.5 μM to 0.1 mM) for 1.5 hr. The procedures for the direct and indirect assays were the same. The final concentration of [^{3}H]QNB was measured by removing aliquots from each jar for scintillation counting. The slides were allowed to dry and returned to storage at -20°.

Autoradiography. The slides, previously incubated in [^{3}H]QNB with various concentrations of gallamine in the direct and indirect assays and which now contained three sections, were apposed to LKB [^{3}H]-sensitive Ultrofilm. Apposition took place in the dark; care was taken that the film was firmly pressed against the sides of the lead cassette to ensure complete exposure. The cassette was wrapped in a dark cloth and stored at -20° for 7 days. At the end of the exposure period, the film was developed in Kodak D-19 developer for 5 min, rinsed in a stop bath for 30 sec, and fixed in Kodak rapid fixer for another 5 min. After a 20-min rinse in water, the film was dipped in a Photo-flo solution for 30 sec and air-dried.

Microdensitometry. Quantification of the autoradiograms was achieved using a modification of techniques outlined by Unnerstall *et al.* [12]. Images generated by autoradiographic procedures were examined using a Perkin–Elmer (Micro-10) microdensitometer interfaced with a Sun computer utilizing a UNIX operating system. Scanned images were stored in digital form as files on backup tapes. Images contained on individual slides were displayed on a cathode ray tube, and 150 × 150 μm areas were selected for density analysis using a mouse-controlled cursor. Quadruplicate measures of density were taken for each structure examined within each image of rat brain section. Density readings for [^{3}H]QNB in the same tissue of known radioactivity were also taken for comparison with each sheet of film. Optical density readings were converted to measurements of fmoles per mg tissue by comparison with the standards so that percent inhibition could be calculated. Background readings of optical density and levels of nonspecific binding were used in determining the relative amount of drug specifically bound to each section for the indirect assays [12].

RESULTS

Binding assay on whole slices. The occupancy curves in Fig. 1 reveal the inhibition by gallamine of the binding of [^{3}H]QNB to central muscarinic receptors. A wide range (10–90%) of inhibition which increased with increasing concentrations of gallamine is evident. These plots (log-dose response and Scatchard) illustrate the presence of more than one population of receptors in the rat brain, through their respective sigmoidal and concave shapes. The log-dose response curve saturates at approximately 1×10^{-4} M gallamine and possesses an inflection point at between 5×10^{-6} M and 2×10^{-6} M gallamine. The parameters for high and low affinity sites were obtained for the whole brain using a two-site model as described previously [2]; alpha (α), the fraction of sites having a high affinity for gallamine, was approximately 0.43; K_h was approximately 6×10^{-7} M gallamine; and K_l was approximately 1×10^{-5} M gallamine. Based on these data, concentrations of gallamine that predominantly inhibit [^{3}H]QNB binding to high affinity sites (10^{-6} M) or inhibit binding to both sites (10^{-5} M) were selected for the autoradiographic analysis described below.

Autoradiography. Figures 2–4 present autoradiographic images from three levels of the brain incubated with increasing concentrations of gallamine. The photographs were printed using the autoradiograms as negatives so that light areas represent [^{3}H]QNB binding. Figure 2 shows cortex, the caudate near the anterior commissure and the septal nodes; Fig. 3 shows cortex, hippocampus and diencephalon; and Fig. 4 shows cortex, caudal hippocampus near the superior colliculus, some diencephalon, and brainstem. The concentrations of

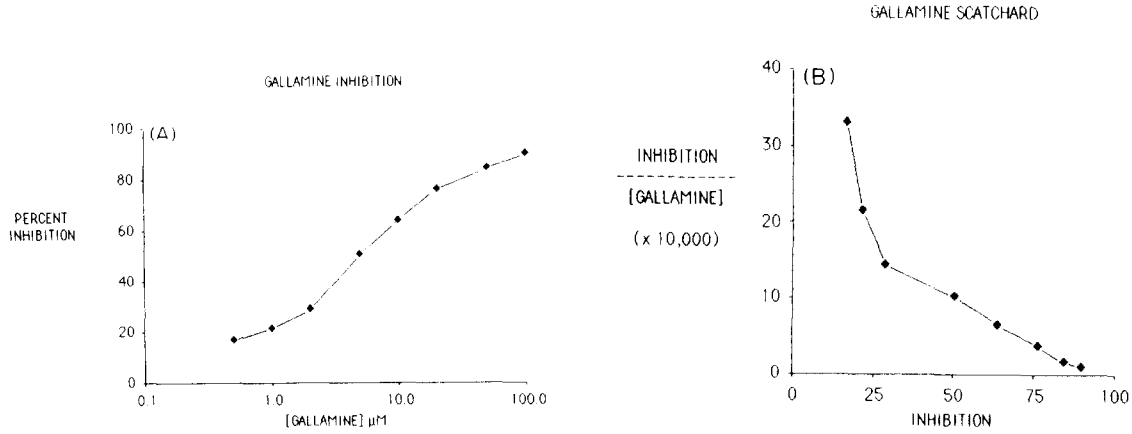
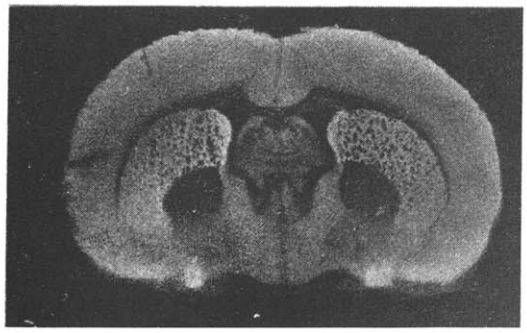
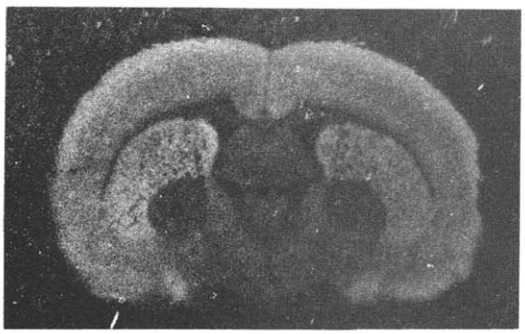


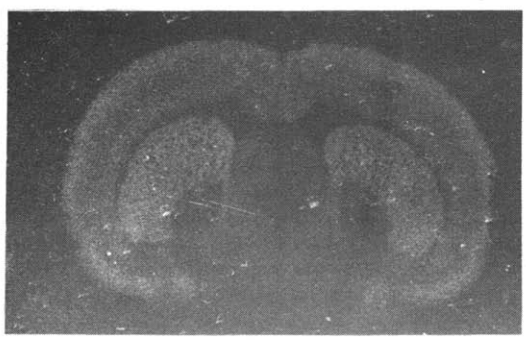
Fig. 1. Inhibition of 0.2 nM [^{3}H]-*l*-QNB binding by gallamine in whole brain sections. A two-site analysis of the data gave $K_h = 6 \times 10^{-7}$ M and $K_l = 1 \times 10^{-5}$ M. The data are representative of two additional experiments. Slide mounted coronal sections (12 μm) were incubated with 0.2 nM [^{3}H]QNB in the presence of eight different concentrations of gallamine for 1.5 hr at room temperature. After washing to remove unbound [^{3}H]QNB, sections were removed from the slides for scintillation counting.



(A)

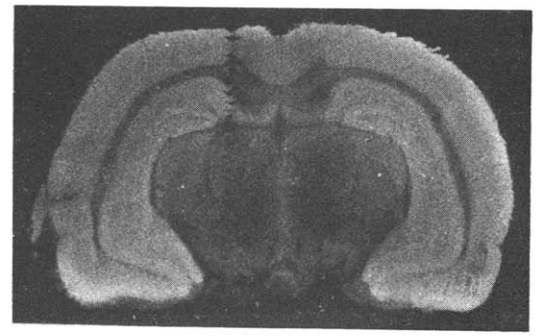


(B)

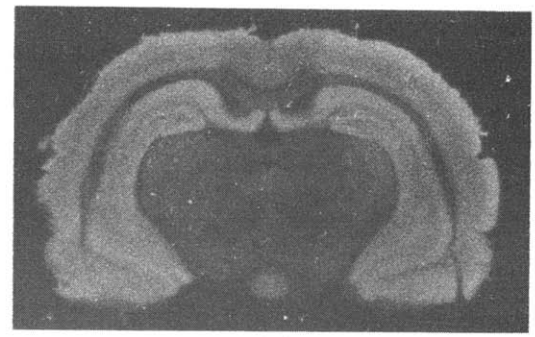


(C)

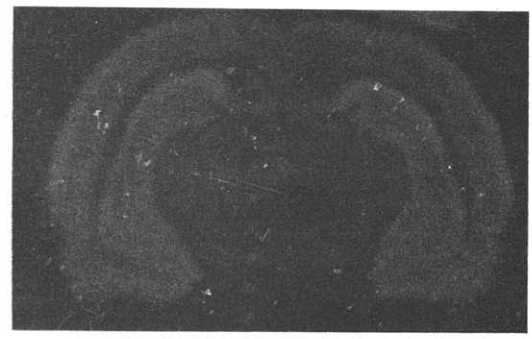
Fig. 2. Autoradiograms of 0.2 nM [^{3}H]-*l*-QNB binding to rostral forebrain regions (A) and its inhibition by 10^{-6} M (B) and 10^{-5} M (C) gallamine. Coronal sections (12 μm) were incubated with 0.2 nM [^{3}H]QNB for 1.5 hr at room temperature in the presence and absence of gallamine as indicated. After washing to remove unbound [^{3}H]QNB, slides were apposed to tritium sensitive film for 7 days at $-20°$ and subsequently developed. The data are representative of two additional experiments.



(A)



(B)



(C)

Fig. 3. Autoradiograms of 0.2 nM [^{3}H]-*l*-QNB binding to mid-forebrain and diencephalic regions (A) and its inhibition by 10^{-6} M (B) and 10^{-5} M (C) gallamine. Lighter areas represent higher concentrations of receptors. Additional details can be found in the legend to Fig. 2.

gallamine (1×10^{-6} M, 1×10^{-5} M) were selected on the basis of the inhibition data from the binding assays on slices and were chosen to approximate the values of K_h and K_l. These autoradiograms qualitatively show the regional distribution of the muscarinic receptors by the differential increase in inhibition with the corresponding increase in gallamine concentration. In the forebrain, the only region where appreciable inhibition occurred at 1×10^{-6} M gallamine was the septal nucleus. The remaining regions of the forebrain appear to contain those receptors that possess low affinity for the drug. This is reflected in the noticeable increase in inhibition with 1×10^{-5} M gallamine. Binding to the diencephalon and the brainstem was inhibited at the lower concentration of gallamine, indicating that these regions possessed high affinity receptors.

Microdensitometry. Quantification of the autoradiograms is presented in Tables 1–3. The regional inhibition data obtained agrees with the qualitative inhibition seen in Figs. 2–4. The septal nucleus was shown to be a region of high affinity in the forebrain with 51.2% inhibition at 1×10^{-6} M gallamine. The remaining regions of the forebrain, cortex, and hip-

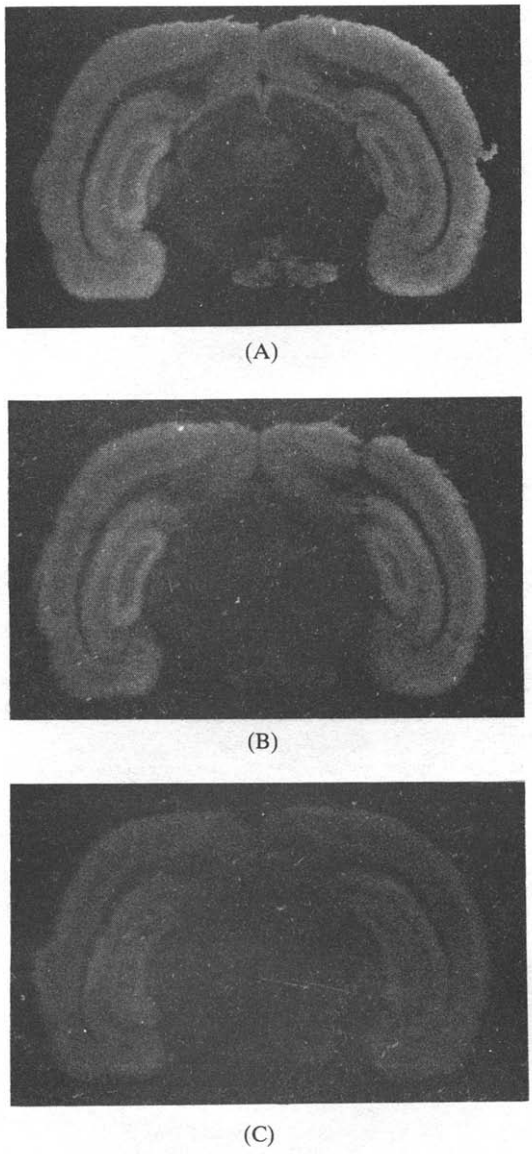


Fig. 4. Autoradiograms of 0.2 nM [^{3}H]-*l*-QNB binding to caudal forebrain, diencephalic and brainstem regions (A) and its inhibition by 10^{-6} M (B) and 10^{-5} M (C) gallamine. Lighter areas represent higher concentrations of receptors. Additional details can be found in the legend to Fig. 2.

pocampus possessed primarily low affinity receptors with approximately one-half or less of that inhibition. At the higher concentration of gallamine, the low affinity sites for gallamine in the forebrain were occupied. Overall, the diencephalon was shown to be a region of high affinity sites. The brainstem was the region inhibited most dramatically at the low concentration of gallamine and, therefore, contained mostly high affinity receptors. The superior colliculus of the brainstem was the region in the brain where the highest percentage of inhibition occurred, 65.1% at 1×10^{-6} M and, therefore, of the regions examined, must have contained the highest proportion of muscarinic receptors having high affinity for gallamine.

DISCUSSION

This study employed binding assays on whole brain slices, autoradiography, and microdensitometry to investigate the regional distribution of the muscarinic receptors that selectively bind gallamine. The combination of these techniques for receptor identification yielded complementary qualitative and quantitative results. Ellis and Hoss [5] reported that gallamine discriminates at least two subtypes of central muscarinic receptors by differentially inhibiting the binding of [^{3}H]QNB to these receptors in membrane preparation from the rat brain. The binding assays performed in this study confirm and extend their findings. The inhibition data obtained in the assays were measures of the total inhibition of QNB by gallamine per whole slice and best describe the muscarinic receptors as consisting of two subpopulations. Alternatively, the data could represent a continuum of affinities for gallamine. The concentrations of gallamine that bind to central muscarinic receptors are comparable to concentrations required to inhibit physiological responses in the superior cervical ganglion [8].

The mechanism by which gallamine interacts with the muscarinic receptor is still unknown, although several different models ranging from competitive binding to allosteric interaction have been proposed. In a study examining the effects of gallamine on the binding properties of [^{3}H]*N*-methylscopolamine ([^{3}H]NMS), Stockton *et al.* [6] reported that gallamine modulates the binding of ligands through an allosteric mechanism. They showed that the associ-

Table 1. Inhibition of [^{3}H]QNB binding by gallamine in the rostral forebrain regions

	% Inhibition of QNB binding	
Region	1.0×10^{-6} M	1.0×10^{-5} M
Forebrain		
Cerebral cortex, ext.	21.6 ± 2.03	68.1 ± 2.63
Cerebral cortex, cen.	26.4 ± 2.97	67.1 ± 2.49
Cerebral cortex, int.	15.8 ± 2.13	66.3 ± 2.28
Cingulate cortex	27.5 ± 4.21	65.3 ± 1.79
Dorsal striatum	13.6 ± 2.34	62.9 ± 1.44
Ventral striatum	13.0 ± 4.73	49.7 ± 4.56
Olfactory bulb	27.9 ± 3.24	66.4 ± 2.02
Septal nucleus	51.2 ± 2.16	76.3 ± 0.86
Whole slice	18.1 ± 12.1	59.9 ± 1.65

Table 2. Inhibition of [^{3}H]QNB binding by gallamine in mid-forebrain and diencephalic regions

Region	% Inhibition of QNB binding	
	Gallamine	
	1.0×10^{-6} M	1.0×10^{-5} M
Forebrain		
Cerebral cortex, ext.	23.3 ± 7.62	73.8 ± 7.36
Cerebral cortex, cen.	32.6 ± 5.43	76.3 ± 3.14
Cerebral cortex, int.	26.4 ± 3.70	76.3 ± 2.64
Cingulate cortex	29.6 ± 4.65	77.7 ± 4.65
Dorsal CA1	11.4 ± 4.86	64.6 ± 2.68
Dorsal GD ect	14.9 ± 4.25	70.0 ± 3.85
Dorsal DG end	20.3 ± 3.51	71.8 ± 3.65
Dorsal CA3	24.2 ± 2.38	76.3 ± 2.91
Ventral CA	24.2 ± 5.40	77.2 ± 0.65
Ventral DG	32.2 ± 1.72	77.7 ± 1.15
Diencephalon		
Pretectal area	44.5 ± 5.00	84.2 ± 3.07
Medial thalamic nucleus	39.9 ± 4.11	76.3 ± 2.85
Centro-medial thalamic nucleus	57.8 ± 3.68	88.9 ± 3.90
Hypothalamus	37.7 ± 4.91	87.5 ± 5.40
Whole slice	19.9 ± 4.80	62.6 ± 0.20

ation and dissociation rates of NMS were decreased by the presence of gallamine in a dose-dependent manner. When this study was repeated, similar results were obtained; the on- and off-rates of NMS binding to the muscarinic receptor were reduced, suggesting noncompetitive regulation by gallamine [13]. However, no change was observed when the on- and off-rates were measured for the QNB binding in the presence of gallamine [13], indicating a difference between the interaction of QNB and NMS with the receptor. The finding that NMS distinguishes subpopulations of receptors seen uniformly by QNB is consistent with this idea [14]. Although the mechanism of binding to the muscarinic receptor is still controversial, there is general agreement that gallamine discriminates subtypes of receptors [5, 15].

Ellis and Hoss [5] proposed that gallamine possesses greater overall affinity for the brainstem than for the forebrain. Visualization of the regional variations of the subtypes of central muscarinic receptor distinguished by gallamine in a whole slice is possible through autoradiographic techniques. The three levels presented here represent most of the major regions of the brain where muscarinic receptors are found in appreciable quantity (Figs. 2–4) and include

Table 3. Inhibition of [^{3}H]QNB binding by gallamine in caudal forebrain, diencephalic, and brainstem regions

Region	% Inhibition of QNB binding	
	Gallamine	
	1.0×10^{-6} M	1.0×10^{-5} M
Forebrain		
Cerebral cortex, ext.	9.7 ± 6.13	61.7 ± 4.01
Cerebral cortex, cen.	16.8 ± 3.95	68.4 ± 3.20
Cerebral cortex, int.	11.4 ± 3.73	64.8 ± 3.01
Cingulate cortex	22.4 ± 3.17	73.2 ± 1.48
Dorsal CA1	1.2 ± 6.70	49.9 ± 4.58
Dorsal DG ect	9.4 ± 3.06	65.4 ± 2.85
Dorsal DG end	4.9 ± 4.94	62.8 ± 1.98
Dorsal CA3	13.5 ± 4.20	63.3 ± 0.76
Ventral CA	13.7 ± 5.85	64.3 ± 2.55
Ventral DG	17.0 ± 4.16	65.7 ± 1.55
Diencephalon		
Pretectal area	38.8 ± 2.64	75.0 ± 1.79
Brainstem		
Periaqueductal gray	52.2 ± 3.36	86.0 ± 3.92
Pontine nucleus	57.6 ± 1.20	88.5 ± 0.30
Superior colliculus	65.1 ± 5.34	89.1 ± 0.75
Whole slice	23.7 ± 3.25	63.5 ± 3.13

the cortex, striatum, hippocampus, diencephalon, and brainstem. It can be seen that the brainstem and diencephalon contained those receptors that possessed a high affinity for gallamine; only a low concentration of gallamine (1×10^{-6} M) was necessary to inhibit the binding of QNB. However, the forebrain, which includes the cortex, striatum, and hippocampus, required a higher concentration of gallamine (1×10^{-5} M) to achieve a similar degree of inhibition; these are regions which contain low affinity receptors. When the autoradiograms were then analyzed using microdensitometry (Tables 1–3), agreement was obtained between the pictorial and the quantitative results. The regions of the brainstem consistently contained receptors which possessed higher overall affinity for gallamine than did the regions of the forebrain. Approximately 50% inhibition was achieved in the brainstem for the low gallamine concentration (1×10^{-6} M), whereas the forebrain, which contains predominantly low affinity sites, achieved less than 20% inhibition at that same concentration.

Gallamine displays no agonist or partial agonist activity [16, 17], yet it discriminates among the subpopulation of muscarinic receptors, a characteristic once attributed solely to agonists. It is of interest to compare the similarities of the anatomical distributions of the muscarinic receptors having high affinities for gallamine and the agonist carbachol. In general, forebrain sites have low affinities and brainstem high affinities for both gallamine and carbachol [5, 13]. Conversely, pirenzepine, which selectively labels M1 receptors, binds with greater affinity in the forebrain compared with the brainstem [4, 18].

In conclusion, the anatomical distribution of muscarinic receptors preferring gallamine was determined in the rat brain by autoradiography. Receptors in brain regions, primarily including diencephalon and brainstem structures in addition to the septal nuclei, that displayed high affinity for gallamine, also displayed high affinity for carbachol, but not pirenzepine. Although there remains some controversy regarding the nature of the binding of gallamine, the distribution of high affinity binding sites in the CNS suggests that gallamine is a selective M2 antagonist.

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