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PROGRESS REPORT.

This progress report covers the period from March 1, 1987 to Feb. 28, 1988.

Our objectives as stated in the last progress report were to 1) synthesize a radioiodinated derivative of 4IQNB with a partition coefficient 10-fold less than that of 4IQNB, i.e., similar to that of QNB; and 2) to synthesize a high affinity analogue of QNB radiolabeled with F-18.

1. Papers and Abstracts.

In studies to better understand the nature of the m-AChR receptor subtypes, we have generated a manuscript which has been submitted for publication in Life Sciences entitled: The effect of chronic atropine and diisopropylfluorophosphate on rat brain muscarinic acetylcholine receptor subtype concentrations (see Appendix I). The abstract follows:

After chronic treatment of rats with either atropine or diisopropylfluorophosphate (DFP), muscarinic acetylcholine receptor (m-AChR) concentrations and subtype distribution were determined in seven brain regions by competitive binding assay utilizing [^{3}H] 3-quinuclidinyl benzilate as the radioligand and 3-quinuclidinyl xanthene-9carboxylate (QNX), which shows a 16-fold selectivity for the M_1 receptor) as the competing ligand. The response of receptor populations in various regions of the brain to chronic drug treatment is complex and differential, the extent and direction of the response varying in each structure examined. Following atropine treatment, there were significant increases in the QNX high affinity binding site in the frontal cortex, hippocampus, corpus striatum, and superior colliculus. After treatment with DFP, frontal cortex, corpus striatum and hippocampus showed significant decreases in both the high and the low affinity QNX binding sites. Receptor concentrations in the thalamus varied in the opposite direction with a significant downregulation of the low affinity QNX binding sites upon chronic atropine treatment and upregulation of the high affinity QNX binding sites following chronic DFP. These effects are different from those seen with pirenzipine which indicate that QNX and pirenzipine identify different subtype populations of m-AChR.

We have also developed a more direct synthesis of 3-quinuclidinyl 4-iodobenzilate and its analogues. Initial studies on this latter goal were presented in an earlier Progress Report (March 1, 1986 to Feb. 28, 1987) in which QNB was iodinated through an organothallium intermediate (J. Nucl. Med. 27 (1986) 1045). The importance of this abstract is not for the production of a radiopharmaceutical for use in man, but for the rapid radioiodination of the many analogues of QNB which we originally proposed. The synthesis of the iodinated derivative of each compound through the preparation of a 4-nitrophenyl analogue, followed by reduction to the 4-amino derivative and conversion to the 4-triazeno adduct is a lengthy procedure not amenable to the screening of new compounds, even when considering only three interesting candidates. This has been well evidenced by the time required for synthesis of 3-quinuclidinyl α-hydroxy-α-(4-iodophenyl)-α-methylacetate (4IQNA) from its α-(4-triazenophenyl) intermediate. In addition, we suggest that 4IQNA will define the lower limits of affinity with regards to localization of iodinated muscarinic antagonists.

The initial results for the iodination of 3-quinuclidinyl atrolactate (QNA) were obtained through the thallation reaction, followed by reaction with iodide as des-

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cribed above, to provide an initial screening of the compound. The following is an abstract which was submitted to the Society of Nuclear Medicine by H.S. Lee, R.E. Gibson, V. Cohen, and R.C. Reba:

3-Quinuclidinyl atrolactate (QNA) is an analogue of 3-quinuclidinyl benzilate (QNB) which exhibits a 25-fold higher affinity for the muscarinic acetylcholine receptor (m-AChR) from the corpus striatum than for the m-AChR from ventricular muscle. A radioiodinated derivative of QNA can be used to bind high affinity m-AChR selectively in the CNS.

A simple method was developed to radioiodinate QNB which has been applied to QNA, using a milligram amount of QNA as substrate. Two reaction steps were involved: QNA was reacted with thallic trifluoroacetate at 60° C for 24 hours in trifluoroacetic acid, then radioactive I-125/NaI and aluminum chloride were added to the reaction mixture and the reaction was allowed to continue at 70° C for another 24 hours. The above reactions were carried out in a sealed ampule. High performance liquid chromatography was employed for the seperation of reaction products. The mole ratio of QNA:aluminum chloride:thallic trifluoroacetate was 1:1:1. The radiochemical yield of [3-I-125]QNA was 8%. The specific activity was 35 Ci/mmole and the equilibrium association constant was 2.36 x 10^7 M^{-1}. This low affinity indicates a steric interaction in the 3-position of the phenyl ring of QNA.

2. Work in Progress.

a) CHEMISTRY.

i) Iodinated and Reduced Lipophilic Analogues

During this contract period, we have been involved primarily with the difficult synthesis of analogues of 3-quinuclidinyl benzilate (QNB) which are lower in lipophilicity. The development of second generation compounds is considerably more difficult than the initial investigations since we are restricted to specific structures to satisfy the greater physicochemical requirements for properties such as reduced lipophilicity while maintaining a high affinity. We are beginning to make significant progress on each of these goals.

Our distribution studies have indicated that localization of [^{125}I] 4IQNB in the CNS is reduced compared to [^{3}H] QNB as the result of systemic losses (1) which we believe results from the increase in lipophilicity caused by introduction of iodine (log P increases by 1). We have calculated that the lipophilicity can be decreased by replacing the second phenyl ring of 4IQNB with a methyl group (compound II in Table 5 of the original proposal). The initial results obtained from the iodination of 3-quinuclidinyl atrolactate (QNA) through the thallation reaction followed by reaction with iodide was pursued as described above to provide an initial screening of the compound. The product obtained from this reaction is assumed to be iodinated in the meta-position because the reaction has been reported to exhibit thermally determined control over the thallation isomer distribution, with the para-substituted product being favored at low temperatures and the meta-substituted product being obtained at 70° C. We have determined that 3-quinculidinyl 3-iodobenzilate (3IQNB) exhibits a lower affinity for the m-AChR than does 4IQNB, and thus prepared the 4-iodo analogue of QNA by the more laborious route of preparing the triazene

The triazene derivative of QNA has been synthesized in a mutiple step sequence

which involved: 1) the synthesis of 4-nitroacetophenone; 2) the reaction of trimethylsilyl cyanide with 4-nitroacetophone followed by acid hydrolysis to provide α-hyroxy-α-(4-nitrophenyl)acetic acid; 3) the esterification of the acetic acid derivative with ethanol followed by transesterification with the sodium salt of 3-quinuclidinol to provide 4-nitroQNA; 4) catalytic reduction of the nitro-group to provide 4-aminoQNA; 5) diazotization of the amine followed by reaction with 3-methylpiperidine to provide the triazene intermediate (4-triazenoQNA). From the triazene derivative, we have prepared the radioiodinated analogue of QNA, [^{125}I] 3-quinuclidinyl α-hydroxy-α-(4-iodophenyl)-α-methyl-acetate, by the acid catalyzed decomposition of the triazene in the presence of [^{125}I] NaI. The determination of the in vitro affinity is presented in the Biochemistry section.

From Table 5 of the original proposal, we suggested that pyridyl analogues of QNB represent a second class of compounds which would be sufficiently low in lipophilicty to offset the increased lipophilicity from iodination. To determine the affinity of a pyridyl analogue, we attempted the preparation of compound IX of Table 5 in the last proposal: 3- quinuclidinyl α-hydroxy-α-phenyl-α-(4-pyridyl)acetate. In a one of our progress reports (Oct. 1986), we reported an apparently successful synthesis of compound IX via the benzil-benzilic acid rearrangement using the pyridyl analogue of benzil and the sodium salt of 3-quinuclidinol. The reaction product did not provide the correct elemental analysis, but an in vitro assay of the affinity of the material indicated a very high affinity for the m-AChR. Subseoeunt attempts to reproduce that synthesis have been completely unsuccessful. Further careful examination of the product indicated that multiple compounds are present in the material sent for analysis. The major side products from this reaction should be 3-quinuclidinyl benzoate and 3-quinuclidinyl (4-pyridyl)formate. We have synthesized these two materials and find that neitner compound has sufficient affinity ($K_D < 10^7$ M^{-1}) to provide the results obtained with the reaction product we originally examined. A small contaminant of QNB obtained by intermolecular exchange during the rearrangement may be responsible for the high affinity of the mixture. NMR analysis of the mixture could neither confirm nor refute the presence of the desired product.

Inasmuch as we consider this particular compound of significant interest, we have attempted the synthesis of compound 11 via three different routes: 1) the reaction of phenylpyridyl ketone with trimethylsilyl cyanide as described above did not provice significant reaction; a mixture of 4-pyridyl lithium with either 2) 3-quinuclidinyl benzoylformate or 3) ethyl benzoyl formate did not provide the desired reaction. We were able to obtain the benzil-benzilic acid rearrangement using NaOH or sodium t-butoxide, but the isolated products could not be subsequently converted to the desired 3-quinuclidinyl ester nor ethyl or methyl esters. We have discontinued attempts to synthesize this compound!

The third class of compounds which we attempted to synthesize are alkoxy analogues as typified by compound VII in Table 5 of the previous contract submission. We have expanded the alkoxy analogues to incluce 1) α-hydroxy-α-methoxymethyl-α-phenyl-acetate, a hydrophilic analogue of compound V, table 5; and 2) α-ethoxymethyl-α--hydroxy-α-phenylacetate, an isolipophilic analogue of compound VII of table 5, and 3) α-hydroxy-α-phenyl-α-(propoxymethyl)acetate. The first of these was synthesized in a manner analagous to 4-nitroQNA, i.e., the reaction of trimetylsilyl cyanide with α-methoxyacetophenone followed by acid hydrolysis, esterification with EtOH and trans-esterification with 3-quinuclidinol. The synthesis of this compound required five months of effort to obtain the desired product. The other alkoxy derivatives have been more recalcitrant in synthesis partly due to the difficulty in synthesizing the starting acetophenone analogues. We have successfully prepared the α-etnoxy and α-

propoxy derivatives of acetophenone by preparation of benzoyldiazomethane followed by reaction with either EtOH or propanol. Subsequent convertion to the desired products is obtained as described for the α-methoxy analogue.

The one derivative whose synthesis has thus far eluded us is methoxyethyl analogue of QNB (Comp'd VII of Table 5 in the previous proposal). None of the above procedures provide the methoxyethyl phenyl ketone intermediate.

The affinity of these new compounds has been determined and are presented in Table 1.

ii) Fluorinated Analogues.

In addition to the iodinated products, we suggested that the synthesis of an F-18 analogue of QNB is possible, specifically, 3-quinuclidinyl flurodiphenylacetate (QFDPA). In order to prepare this compound we have attempted the synthesis of two possible precursors: 3-quinuclidinyl α-bromo-α,α-diphenylacetate and 3-quinuclidinyl α-chloro-α,α-diphenylacetate via the reaction of QNB with either thionyl chloride or thionyl bromide.

^{18}F is available from the Department of Nuclear Medicine at NIH (see letter of collaboration, Appendix VIII) as tetraethylammonium fluoride (TEAF). We examined the reaction of unlabeled TMAF with the α-chloro precursor in acetonitrile and DMSO. We observed no reaction in acetonitrile, but obtained a product in DMSO which co-eluted with an authentic sample of QFDPA in HPLC analysis. Attempts to incorporate F-18 using TEAF were not successful, however. The mass spectra of the isolated unlabeled material from HPLC does not correspond to that of authentic QFDPA. There are references in the literature which suggest that the reaction of fluoride with alkylbromides is successful but not the reaction with alkyl chlorides. We are currently attempting the synthesis with the α-bromo derivative.

b) BIOCHEMISTRY.

i) In Vitro Binding Affinities.

We have determined the affinity constants of the various compounds synthesized this year for the muscarinic receptor from rat corpus striatum (M_1). The affinity constants of QNB, 3-quinuclidinyl 4-nitrobenzilate (4-NO_2QNB), 3-quinuclidinyl 4-triazenobenzilate (4-TQNB), and QNA are provided in Table I for comparison.

The substitution of 4-nitro on QNB did not lead to a reduction in affinity to the m-AChR from CNS. In the case of QNA, a large reduction is observed (compare 4-NO_2-QNA to QNA). The affinity of 3IQNA is 5-fold higher than that of 4-NO_2-QNA indicating that the more lipophilic iodine is better tolerated than the nitro group. Nonetheless, the affinity constant of 3IQNA is too low for good receptor binding in vivo. Surprisingly, the compound synthesized via the triazene reaction, 4IQNA, is so low in affinity that we were not able to obtain a specific binding curve.

The methoxy- and propoxy-analogues of QNA indicate that the oxygen atom is not well tolerated in the beta-position (Table I). A 5-fold loss in binding is observed with the methoxy substituent. The addition of two methylene groups recovers 3-fold of the binding. However, these two compound should be compared with QNB analogues in

which a phenyl ring is replaced by n-butyl and n-propyl. The alkyl derivatives have essentially the same affinity as QNB, while the oxo-cogeners exhibit at least ten-fold lower affinity.

Table 1.

Affinity constants for QNB and analogues for the M_1 and M_2 Receptors.

	K_A (M^{-1})
	Rat Corpus Striatum
QNB	3.71×10^{9}
4-NO_2QNB	2.55×10^{9}
QNA	4.95×10^{8}
4-NO_2-QNA	5.57×10^{6}
4-TQNB	3.43×10^{9}
3IQNA	2.4×10^{7}
4IQNA	ND[1]
MeO-QNA	1.0×10^{8}
PrO-QNA	3×10^{8}

[1] Not Determinable.

ii) Studies in Pancreas.

We have continued our investigation of the m-AChR in pancreas. In addition to the abstract presented in the progress report above, we have continued studies to characterize the 4IQNB binding siter. To summarize, we find that there is a receptor-like protein in the pancreas which binds 4IQNB with high affinity and negative cooperativity. The drug profile appears to be cholinergic, but does not correspond to that expected for the muscarinic receptor. The interaction of QNB with this population is with a low affinity. In addition, 4IQNB exhibits negative cooperativity in the interaction with this protein. In studies obtained since the presentation of this work, we have found that the concentration of the 4IQNB protein is from 10 to 100-fold higher than that which binds [^{3}H] QNB. We have observed significant binding of 4IQNB in preparations devoid of QNB binding activity. We previously suggested that the difference in receptor concentrations using [H-3] QNB and [I-125] 4IQNB (a difference of 50- to 100-fold) may result from partial enzymatic degradation of the muscarinic receptor such that QNB does not recognize the receptor antagonist binding site while 4IQNB does. Nonetheless, the properties of the binding site for 4IQNB are different than those for sites obtained from the CNS or myocardium. We have prepared the pancreatic acinar cells in the presence of a protease cocktail containing leupeptin, pepstatin, phenylmethylsulfonyl fluoride and EDTA. The ratio of receptor concentrations determined using the two radioligands remained unchanged at 50-fold, thus indicating that the species which binds 4IQNB is not a protease altered receptor. The site does not have the stereoselectivity of the m-AChR: the affinities of R-QNB and S-QNB are identical, while for the m-AChR these stereoisomers differ in affinity by at least 100-fold.

In recent work using material synthesized under support from this contract (see

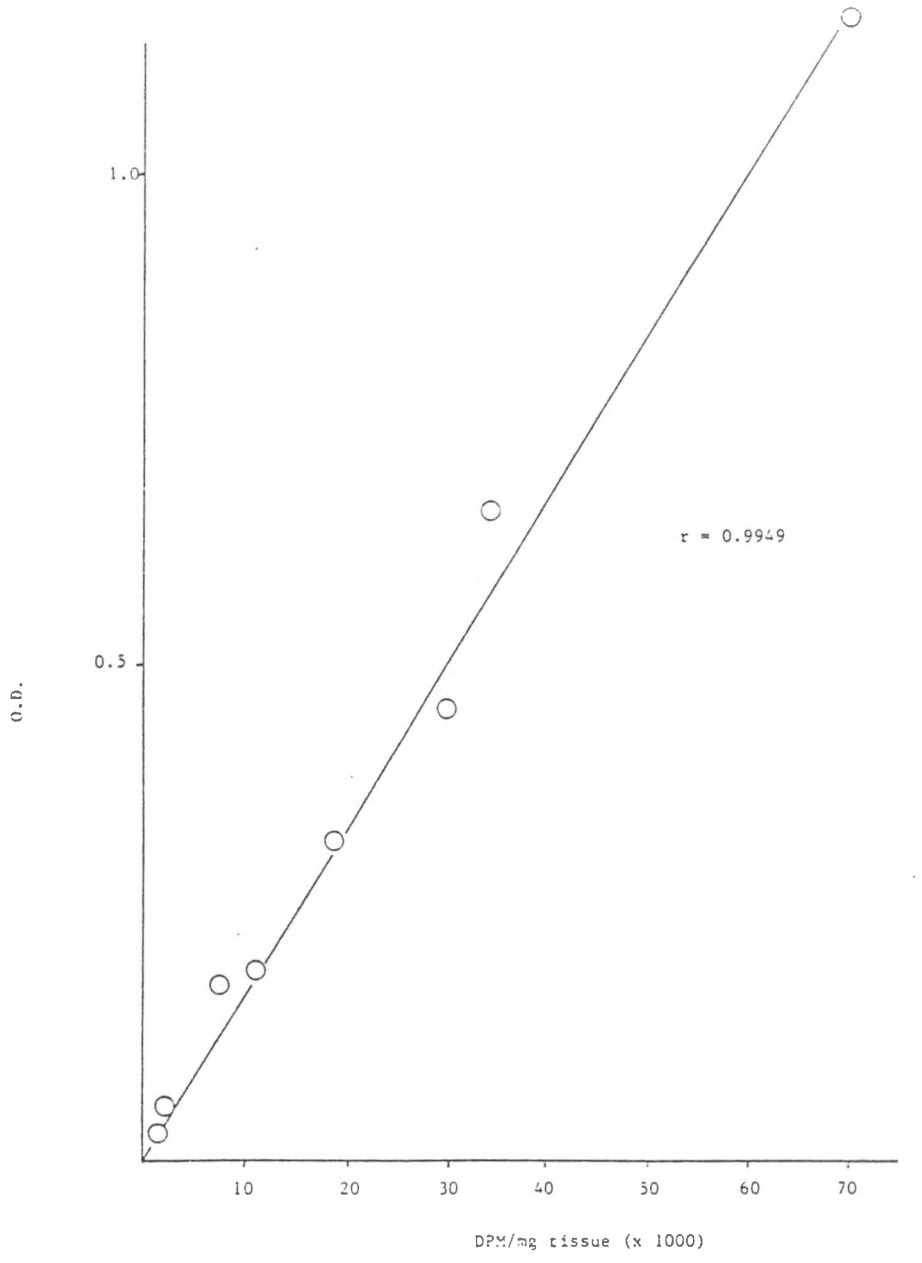
manuscript, Appendix II), Dr. Joad and Dr. Casale identified a 4IQNB binding protein in peripheral rat lung which did not have the properties expected of the m-AChR. The results they report are consistent with our observations in the rat pancreas. We have conducted similar studies on rabbit lung in collaboration with Dr. John Bloom and Dr. Henry Yamamura of the University of Arizona, Tucson, and find that the receptor identified in rabbit peripheral lung is the same as that labeled by [^{3}H] QNB, i.e., the muscarinic receptor. This species difference in lung is interesting and suggests that the results obtained with 4IQNB in rat pancreas must be extended to include other species, including man. We would be remiss to discard 4IQNB and its analogues as potential pancreas imaging agents based on the unusual results from one species.

iii) Autoradiography.

Using supplemental funds provided to this contract, we have established and become familiar with the basic techniques requisite to conduct quantitative autoradiographic studies. Although the supplimental funds were not continued, we have continued in developing this valuable technique for assessing in vitro and in vivo interactions of radioligands with receptors.

The primary addition since our last progress report is the development of good quantitative standards using brain-paste cylinders containing known quantities of I-125 activity. The standard is very linear over eight of eleven of the standard samples (Fig 1). Two samples saturated the film while one sample contained concentrations of I-125 too low to provide an adequate grain density for visualization.

Fig. 1



THE EFFECT OF CHRONIC ATROPINE AND DIISOPROPYLFLUOROPHOSPHATE ON RAT BRAIN MUSCARINIC ACETYLCHOLINE RECEPTOR SUBTYPE CONCENTRATIONS.

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SUMMARY

After chronic treatment of rats with either atropine or diisopropylfluorophosphate (DFP), muscarinic acetylcholine receptor (m-AChR) concentrations and subtype distribution were determined in seven brain regions by competitive binding assay utilizing [^{3}H]3--quinuclidinyl benzilate ([^{3}H]QNB) as the radioligand and 3-quinuclidinyl xanthene-9-carboxylate (QNX), which shows a 16-fold selectivity for the M_1 receptor, as the competing ligand. The response of receptor populations in various regions of the brain to chronic drug treatment is complex and differential, the extent and direction of the response varying in each structure examined. Following atropine treatment, there were significant increases in the concentration of QNX high affinity binding site in the frontal cortex, hippocampus, corpus striatum, and superior colliculus. After treatment with DFP, frontal cortex, corpus striatum and hippocampus showed significant decreases in both the high and the low affinity QNX binding sites. Receptor concentrations in the thalamus varied in the opposite direction with a significant down-regulation of the low affinity QNX binding sites upon chronic atropine treatment and up-regulation of the high affinity QNX binding sites following chronic DFP. These effects are different from those seen with pirenzipine which indicate that QNX and pirenzipine identify different subtype populations of m-AChR.

INTRODUCTION

Chronic treatment with the cholinergic antagonists atropine or scopolamine has been shown to increase [^{3}H]QNB binding in the brains of rats (1-5) and guinea pigs (6). This increase in binding was found to be due to an increase in B_{max} without a change in the equilibrium dissociation constant, K_d (1,2,6) indicating that only the concentration of receptors is affected. Furthermore, several groups have demonstrated that chronic treatment with acetylcholinesterase inhibitors, in particular diisopropylfluorophosphate (DFP), or muscarinic agonists produce down regulation of muscarinic receptors. A decrease in the amount of [^{3}H]QNB binding after such treatment has been seen in

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peripheral tissues of guinea pigs (7) and rats (8), and in the CNS in guinea pigs and rats (1,6,7,9-12) as well as mice (13). In studies designed to demonstrate up-regulation of the receptor, cortex, hippocampus and corpus striatum exhibited increased [^{3}H]QNB binding while hypothalamus, thalamus, cerebellum and brainstem were unchanged. In studies designed to show down-regulation of the m-AChR, the cortex, hippocampus, and corpus striatum were the tissues in which the concentration of receptor was most greatly affected while in the thalamus, brainstem, hypothalamus, and cerebellum, the concentration of receptor was less affected or unchanged.

The muscarinic receptor exhibits multiple subtypes (14,15) which are differentially affected by chronic drug treatment (16). To eludicate further the characteristics of up- and down-regulation of muscarinic receptors and receptor subtypes, we measured the effect of chronic treatment with atropine or DFP on the concentrations of receptors and determined the concentrations of receptor-subtype by competition assay using the antagonist 3-quinuclidinyl xanthene-9-carboxylate (QNX). These results indicate that QNX, which has been shown to exhibit receptor selectivity (17), and the M_1 selective drug pirenzipine identify different m-AChR subtypes in the brain.

METHODS.

Chronic Atropine Adminstration. Twelve Sprague Dawley male rats (200-250 g) were treated with atropine sulfate (6 animals) or control saline (6 animals) via osmotic pump implant. ALZET (model 202) 200N/osmotic pumps (Alza Corp., Palo Alto, CA) which delivered 0.5 ul/hr of either 0.9% saline or atropine at a dose of 12 mg/day for 14 days were implanted subcutaneously between the scapulae of rats. Twenty-four hours after removal of the pumps, the rats were sacrificed by decapitation, whole brains removed and placed on ice for dissection. Frontal cortex, cerebellum, corpus striatum, hippocampus, inferior and superior colliculi, and thalamus were removed, frozen on dry ice and stored at -70° C until assay.

Chronic Diisopropylfluorophosphate Administration. 15 female Sprague Dawley rats received i.m. injections of DFP suspended in clarified corn oil for 10 days by the following schedule: day 1 - 1 mg/Kg, day 2 through 5, 0.5 mg/kg, day 6 - 0.25 mg/kg, day 7, no injection, day 8 through 10, 0.25 mg/kg. Six rats survived the treatment in good condition although all exhibited classic parasympathomimetic symptoms after the initial dose. Twenty-four hours after the last dose, the rats were sacrificed by cervical dislocation, brains removed and dissected as above with the exceptions that pons was removed from the brain stem and cerebellum was omitted.

Binding Studies. The details of the tissue preparation and competition and saturation binding assays have been previously described (17,18). Frozen tissue was weighed and homogenized in 3-5 ml (depending on tissue weight and *a priori* knowledge of receptor concentrations in the tissues) of ice cold 0.9% saline, containing 10 mM Tris buffer (pH 7.4) and 10% sucrose to maintain a uniform suspension, using a Brinkmann Polytron PC-U (medium speed, 1 burst of 15 sec). The receptor was used without further preparation.

For the saturation analysis, 0.1 ml aliquots of the tissue preparation were added to 5 ml of 6 concentrations of [^{3}H]QNB in duplicate. Binding to non-receptor proteins and filter papers at each radioligand concentraion was determined in duplicate in the presence of 10^{-5} M atropine. Incubation for 2 hrs was sufficient to achieve equilibrium, at which time the incubation mixtures were filtered over GF/C filters using a cell harvester apparatus, followed by two washes with buffer (5 mls each), all at ambient temperature.

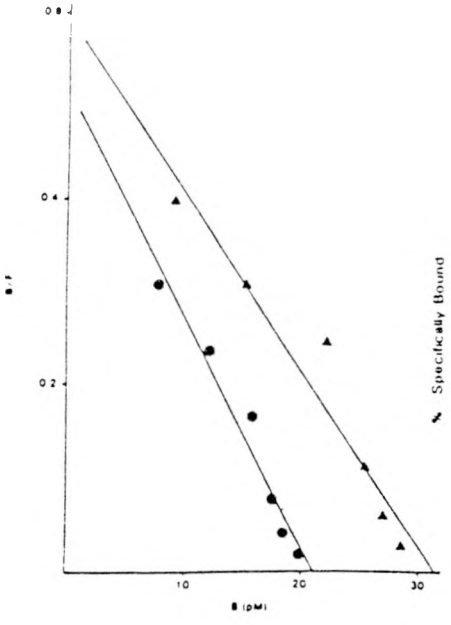


FIG. 1.

Scatchard plot of specific [^{3}H]QNB binding to rat corpus striatum. (●) saline treated control, K_A = 2.4 x 10^{10} M^{-1}, R_o = 1.3 x 10^{-7} M; (▲) chronic atropine treated, K_A = 1.87 x 10^{10} M^{-1}, R_o = 1.8 x 10^{-7} M.

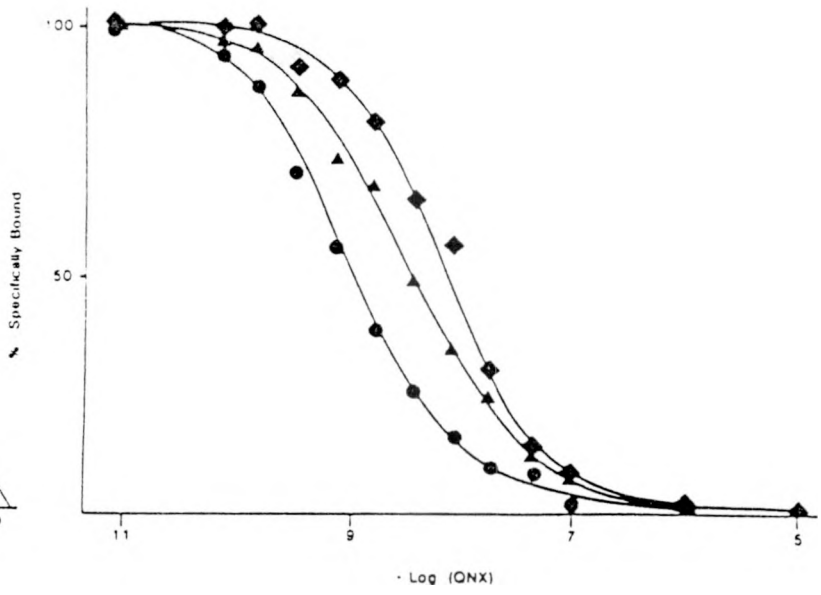


FIG. 2.

Equilibrium competition of QNX for [^{3}H]QNB binding sites in cortex (●), thalamus (▲), and cerebellum (◆). (Thalamus curve is shifted to the right by 1/3-log. unit for visual clarity). Individual parameters obtained from Scafit are: cortex - R_1 = 9.14 x 10^{-8} M, K_{21} = 4.57 x 10^9 M^{-1} and R_2 = 2.39 x 10^{-8} M, K_{22} = 1.67 x 10^8 M^{-1}; thalamus - R_1 = 2.64 x 10^{-8} M, K_{21} = 1.1 x 10^{10} M^{-1} and R_2 = 2.69 x 10^{-8} M, K_{22} = 6.0 x 10^8 M^{-1}.

To analyze the data with respect to the concentration of each class of receptor present within a given brain structure and how it varies with chronic drug treatment, we assumed that QNX has only a high and low affinity site in competition with the radioligand which are common to all brain structures examined. However, for the tissues in which higher values of K_{21} and K_{22} were observed, we used these higher values of K_{21} and K_{22} (maintaining the affinity ratio at 14) to determine the relative proportions of high and low affinity sites. To insure the generality of the conclusions obtained from this analysis, we analyzed the data using different values for the assumed affinities of QNX, in the range of K_{21} = 3.5 x 10^9 M^{-1} to 1.9 x 10^{10} M^{-1}, with the value of K_{22} set as $K_{21}/14$. The absolute concentrations of the two sites determined within each structure varies with the assumed values of K_{21} and K_{22}, but the observation of either a significant increase, decrease or no significant change in the receptor concentrations does not vary.

The results from chronic atropine treatment of animals are presented in Tables II and III. Control values are from animals treated with saline in the same experiment. Significant increases in total receptor concentration are observed in all tissues except thalamus and cerebellum. Tissue concentrations

for the high affinity QNX receptor subtype range from 1.28×10^{-9} M in the cerebellum to 1.28×10^{-7} M in the corpus striatum. By contrast, the concentrations of the low affinity component range from 5.92×10^{-9} M in the cerebellum to 4×10^{-8} M in the superior colliculus. Examination of the receptor concentrations from the atropine treated animals indicates that cortex, corpus striatum, hippocampus, and superior colliculus have significant increases in the high affinity QNX binding site. There is a large increase in the high

TABLE I.

Detemination of QNX High Affinity (K_{21}) and Low Affinity (K_{22}) Constants in Different Brain Regions Following Chronic Atropine Treatment.

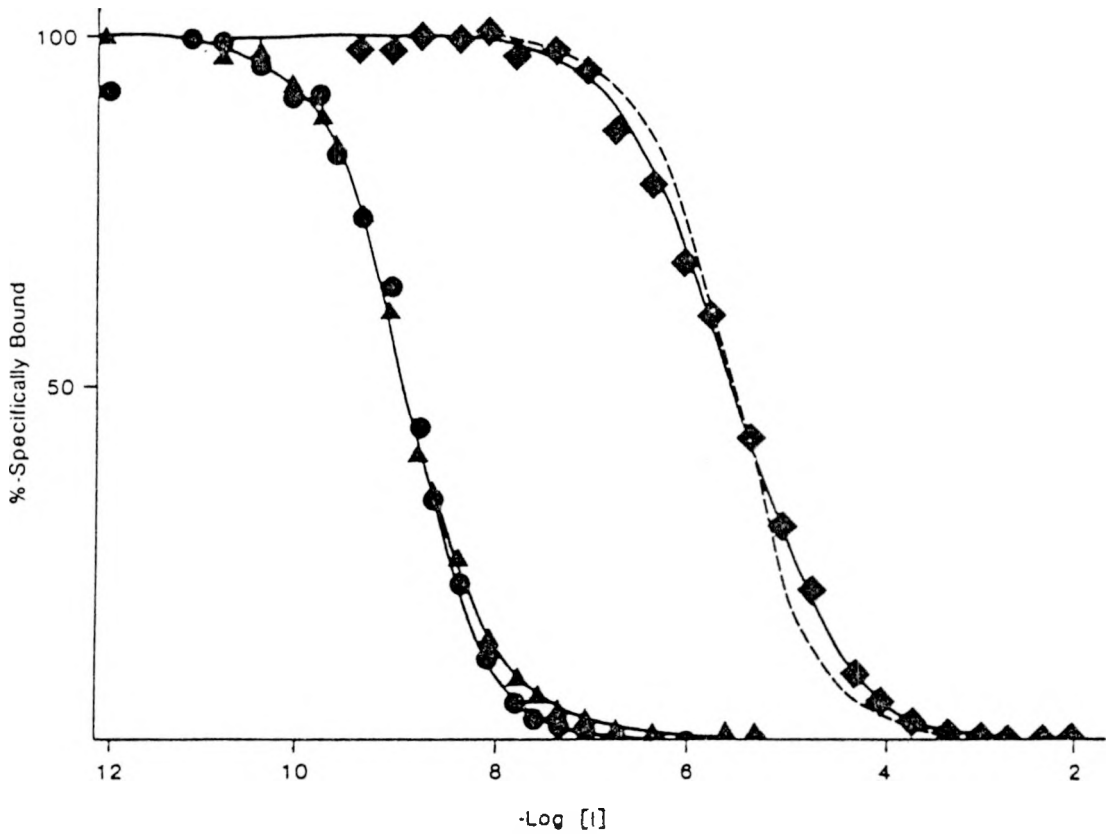
		Equilibrium Association Constant (coeff. var.)			
TISSUE		CONTROL		TREATED	
Corpus Striatum	K_{21}	3.37×10^{9} M^{-1}	(20)	3.74×10^{9} M^{-1}	(35)
	K_{22}	5.92×10^{8} "	(148)	8.10×10^{8} "	(170)
Cortex[a]	K_{21}	4.96×10^{9} "	(24)	4.01×10^{9} "	(19)
	K_{22}	3.49×10^{8} "	(35)	2.29×10^{8} "	(63)
Hippocampus	K_{21}	3.57×10^{9} "	(37)	5.54×10^{9} "	(92)
	K_{22}	4.70×10^{8} "	(157)	1.01×10^{9} "	(116)
Superior Colliculus	K_{21}	3.17×10^{10} "	(151)	2.67×10^{10} "	(903)
	K_{22}	6.82×10^{8} "	(173)	8.04×10^{8} "	(10)
Inferior Colliculus	K_{21}	9.99×10^{8} "	(1101)	4.65×10^{10} "	(903)
	K_{22}	4.00×10^{8} "	(173)	4.99×10^{8} "	(10)
Thalamus[a]	K_{21}	1.44×10^{10} "	(36)	1.35×10^{10} "	(67)
	K_{22}	9.51×10^{8} "	(14)	1.05×10^{9} "	(35)
Cerebellum	K_{21}	4.05×10^{9} "	(216)	1.44×10^{9} "	(347)
	K_{22}	3.84×10^{8} "	(14)	3.65×10^{8} "	(35)
Pons	K_{21}	1.90×10^{9} "	(163)	3.32×10^{9} "	(309)
	K_{22}	3.78×10^{8} "	(52)	4.08×10^{8} "	(54)

a) Two-site model significantly better than one-site ($p<.001$)

affinity site in the cerebellum. However, this is not statistically significant most likely due to the poor precision achievable at the low receptor concentrations of the cerebellum. With the exception of inferior colliculus, these tissues do not exhibit an increase in the concentration of low affinity QNX site. Thalamus shows a significant decrease in the concentration of the QNX low affinity site.

The results of chronic treatment with DFP are summarized in Tables IV and V, and are compared with injection vehicle controls. Significant reduction in receptor concentration is observed with the corpus striatum, frontal cortex, and hippocampus. By contrast, there is a significant increase in the concentration of m-AChR in the thalamus. With respect to subtype populations seen in the corpus striatum and cortex, there are significant decreases in the high and

low affinity QNX sites, while there is a significant decrease in only the high affinity site in the hippocampus. The decrease in the concentration of the low affinity QNX site in the hippocampus is similar to that seen for the above tissues, and is not significant for the same reasons as cited for the cerebellum. The thalamus exhibits a different effect: there is a large increase in the concentration of high affinity QNX binding site and a smaller increase in the concentration of low affinity QNX binding site. Although neither increase is statistically significant due to the scatter in the data, the significant increase in the concentration of total receptors in the thalamus (Table IV) lends validity to the increase in at least the high affinity site.



Inhibition of [^{3}H]QNB binding to m-AChR from corpus striatum by QNB (●), QNX (▲), and pirenzipine (◆). The solid lines are curve fits based on minimized residuals for QNB (1-site), QNX (2-sites) and pirenzipine (2-sites); the dashed line is the theoretical curve for a single site having an IC_{50} the same as pirenzipine. The parameters obtained by LIGAND analysis are: QNB - K_{21} = 5 x 10^9 M^{-1} (100%); QNX - K_{21} = 4.2 x 10^9 M^{-1} (94%), K_{22} = 4.8 x 10^8 M^{-1} (6%); pirenzipine - K_{21} = 8.7 x 10^6 M^{-1} (50%), K_{22} = 5.4 x 10^5 M^{-1} (50%).

Inhibition of [^{3}H]QNB binding to m-AChR from corpus striatum by QNB, QNX, and pirenzipine is shown in Fig. 3. The inhibition curve by the nonselective anatgonist, QNB, is fit very well by a single site model. The QNX inhibition curve exhibits a consistent deviation from a single site in the concentration range of 10^{-9} to 10^{-6} M QNX which represents a 5% contribution by a lower affinity site; however, a two site model does not provide a statistically

significant better fit of the data than the one site model. Pirenzipine, by contrast, exhibits as least two binding sites in competition for [^{3}H]QNB. Assumption of a two-site model provides a good fit of the data with equal concentrations of the high (M_1) and low (M_2) affinity sites. The binding parameters obtained for an average of 4 curves is presented in the legend of Fig. 3.

DISCUSSION

We previously defined QNX as selective for the M_1 AChR from competition studies using the corpus striatum as a source of M_1 receptors and ventricular muscle as the source of M_2 receptors (17). The results indicated a 16-fold selectivity for the receptor obtained from the corpus striatum, a selectivity similar to that obtained in competition studies using the M_1-selective drug pirenzipine. We therefore suggested that QNX exhibits the same selectivity as pirenzipine but with an affinity for the M_1-subtype equal to that of QNB (18).

TABLE II.

The Effect of Chronic Atropine Treatment on m-AChR Concentration* in Rat Brain Tissue.

TISSUE	CONTROL	ATROPINE	%-CONTROL
Corpus Striatum	1.28×10^{-7} M	1.64×10^{-7} M	128**
Cortex	8.83×10^{-8} M	1.27×10^{-7} M	144**
Hippocampus	9.58×10^{-8} M	1.22×10^{-7} M	127***
Superior Colliculus	4.80×10^{-8} M	5.79×10^{-8} M	121**
Inferior Colliculus	3.55×10^{-8} M	5.13×10^{-8} M	144**
Thalamus	4.63×10^{-8} M	4.45×10^{-8} M	96
Cerebellum	6.86×10^{-9} M	7.15×10^{-9}	104

* results are expressed as moles receptor/g tissue x 1000 to provide the Molar concentration of receptor.
** denotes significant difference from control at $p<.001$.
*** denotes significant difference from control at $p<.02$.

The data presented demonstrate that the effects of chronic atropine and chronic DFP treatment on receptor subtypes within the CNS as measured by QNX competition differ depending on the region examined. In the case of receptor changes effected by chronic atropine treatment, the total receptor concentration is increased, consistent with previous reports (1-5). However, this study demonstrates that chronic atropine treatment differentially affects muscarinic receptor subtypes. The predominant increase was in the high affinity QNX binding site (R_1); but in the case of the inferior colliculus and thalamus, a significant decrease in the low affinity QNX binding site (R_2) was the only statistically significant change observed. In the case of chronic DFP treatment, the total receptor concentration was reduced in cortex, corpus striatum and hippocampus, also similar to previous reports, but unlike the atropine effect.

both high and low affinity QNX sites are affected.

The thalamus provides results which were in contrast to those observed in the cortex, corpus striatum and hippocampus, i.e., atropine treatment led to a modest increase in the high affinity QNX site (not significant) but a signficant decrease in the low affinity QNX site, whereas DFP treatment produced an increase in both the high and low affinity QNX sites rather than the expected decrease. When analyzed for two sites, the increases were not significant, but there was a significant change in the total concentration of receptor.

TABLE III.

The Effect of Chronic Atropine Treatment on the Concentration* of QNX High Affinity (R_1) and Low Affinity (R_2) Binding Sites in Rat Brain Tissue.

TISSUE		CONTROL	ATROPINE	%-CONTROL
Corpus Striatum	R_1	1.28×10^{-7} M	1.64×10^{-7} M	128**
Cortex	R_1	8.31×10^{-8} M	1.27×10^{-7} M	153**
	R_2	1.79×10^{-8} M	1.78×10^{-8} M	99
Hippocampus	R_1	9.76×10^{-8} M	1.23×10^{-7} M	126***
	R_2	1.08×10^{-8} M	1.35×10^{-8} M	126
Superior Colliculus	R_1	5.18×10^{-9} M	1.71×10^{-8} M	330**
	R_2	4.76×10^{-8} M	4.55×10^{-8} M	96
Inferior Colliculus	R_1	1.90×10^{-8} M	1.52×10^{-8} M	80
	R_2	3.43×10^{-8} M	4.07×10^{-8} M	119***
Thalamus	R_1	2.63×10^{-8} M	3.14×10^{-8} M	119
	R_2	2.71×10^{-8} M	2.08×10^{-9} M	77****
Cerebellum	R_1	1.27×10^{-9} M	2.18×10^{-9} M	172
	R_2	5.93×10^{-9} M	5.58×10^{-9} M	94

* results are expressed as moles receptor/g tissue x 1000 to provide the Molar concentrations of receptor.
** denotes significant difference from control at $p<.001$.
*** denotes significant difference from control at $p<.05$.
**** denotes significant difference from control at $p<.005$.

Two caveats must be considered in the above results. First, the affinity constants of QNX used for the analysis had to be set as constants (rather than being derived from the experimental data) to provide statistically significant determinations of receptor subtype concentrations. This assumption is valid provided that the affinity constants for QNX are the same in all tissues and under all of the conditions studied. We observed differences, however, in the high and low affinity components for QNX in the thalamus which suggest that this assumption may not be entirely correct. The ratio of the high to low affinity constants remained, however, at 14. Equal shifts in the affinity constants of the high and the low affinity sites may result from systematic errors, e.g. an error in the preparation of the inhibitor concentration. In this case, the higher affinities may represent real differences in these para-

meters in the thalamus. To insure that the observed increases or decrease in receptor conentrations did not result from the assumptions made in the calculations, we examined the data using a range of assumed QNX affinities (maintaining a constant ratio of 14) and obtain the same results with respect to changes in subtype concentrations.

TABLE IV.

The Effect of Chronic Diisopropylfluorophosphate Treatment on m-AChR Concentration* in Rat Brain Tissue.

TISSUE	CONTROL	DFP	%-CONTROL
Corpus Striatum	1.49×10^{-7} M	9.15×10^{-8} M	61.6*
Cortex	8.28×10^{-8} M	6.22×10^{-8} M	74.6*
Hippocampus	7.06×10^{-8} M	4.90×10^{-8} M	69.4*
Superior Colliculus	7.06×10^{-8} M	6.58×10^{-8} M	93.2
Inferior Colliculus	3.50×10^{-8} M	3.55×10^{-8} M	101
Thalamus	2.92×10^{-8} M	3.54×10^{-8} M	121**
Pons	2.64×10^{-8} M	2.62×10^{-8} M	99.3

* results are expressed as moles receptor/g tissue x 1000 to provide receptor in Molar conc.
* denotes significant difference from control at $p<.001$.
** denotes significant difference from control at $p<.05$.

Second, there are considerable differences in the concentrations of m-AChR present in several of the brain structures of the control populations from the two studies. The values obtained for the atropine study are in good agreement with concentrations reported previously (1-5). But the concentration of m-AChR determined in the thalamus of the control animals for the DFP study is 63% that of the controls for the atropine study. In addition, the QNX low affinty site is 51% in the DFP study, instead of 71% of the total population in the control group of the atropine study. We used male rats for the atropine study and female rats for the DFP study so it is possible that the differences observed reflect the different sex of the animals. Alternatively, it is possible that differences in the protocols of the two studies could lead to alterations in the receptor concentration, e.g., atropine was administered via osmotic pumps so animals were not handled from the time of the surgery until the animals were sacrificed; by contrast, the animals in the DFP study were given subcutaneous injections twice daily, either with DFP or the vehicle for control animals. This procedure was quite distressing to the animals as reflected by their strong avoidance responses and aggressive behavior as the injection schedule proceeded. However, in both groups, the control animals were handled the same as the drug-treatment group and the drug-induced changes observed for the total receptor concentrations in this study with respect to the appropriate control groups are consistent with previously reported studies.

Perhaps the most interesting feature of our study is the contrast it pre-

sents with a recent study presented by Lee and Wolfe (16) in which receptor subtype populations were determined by saturation analysis using [^{3}H]pirenzipine and [^{3}H]QNB to define the M_1 receptor population and total receptor populations, repectively, and M_2 receptor populations were determeined by subtraction. In their study, chronic atropine treatment increased the total concentration of mAChR in cortex but did not change the concentration of

TABLE V.

The Effect of Chronic DFP Treatment on the Concentration* of QNX High Affinity (R_1) and Low Affinity (R_2) Binding Sites in Rat Brain.

TISSUE		CONTROL	DFP	%-CONTROL
Corpus Striatum	R_1	1.58×10^{-7} M	9.55×10^{-8} M	60.5**
	R_2	1.17×10^{-8} M	6.62×10^{-9} M	56.4**
Cortex	R_1	8.31×10^{-8} M	6.25×10^{-8} M	75.2**
	R_2	1.03×10^{-8} M	6.46×10^{-9} M	61.9***
Hippocampus	R_1	6.93×10^{-8} M	4.72×10^{-8} M	68.1**
	R_2	5.08×10^{-9} M	3.58×10^{-9} M	70.5
Superior Colliculus	R_1	1.25×10^{-8} M	1.38×10^{-8} M	110
	R_2	6.21 X 10^{-8} M	6.16×10^{-8} M	99.2
Inferior Colliculus	R_1	5.76×10^{-9} M	5.48×10^{-9} M	95.1
	R_2	3.17×10^{-8} M	3.11×10^{-8} M	98.1
Thalamus	R_1	9.54×10^{-9} M	1.25×10^{-8} M	131
	R_2	2.26×10^{-8} M	2.67×10^{-8} M	118
Pons	R_1	7.96×10^{-9} M	8.24×10^{-9} M	104
	R_2	2.01×10^{-8} M	1.97×10^{-8} M	98.3

* results are expressed as moles receptor/g tisse x 1000 to provide receptor in Molar concentrations.
* denotes signficantly different from control at $p<.001$.
** denotes signficantly different from control at $p<.005$.

[^{3}H]pirenzipine bound. Thus, by implication, the M_2 sites which are not labelled by pirenzipine were responsible for the increase. Our results obtained using the corpus striatum are consistent with predominantly one class of binding site (see Fig 3. and Table V): 7% of a QNX low affinity site is obtained which is not, however, statistically significant. By contrast, estimations of the fraction of M_2 receptor in corpus striatum using [^{3}H]pirenzipine binding are as large as 50% (21). Similar differences between QNX and pirenzipine have been observed in peripheral tissues: the affinity of pirenzipine for the muscarinic receptor from pancreatic acinar cells suggests that the receptor is of the M_2 subtype (22,23). By contrast, the affinity constant of QNX for the same receptor population is that same as that of QNB, i.e., consistent with an assignment of M_1 receptors (R.E. Gibson, et al., unpublished result).

One explanation for the different results obtained with pirenzipine and QNX is that the two ligands identify different receptor subtypes. Three puta-

tive subtypes have been implicated by either kinetic studies (14) or equilibrium binding studies (24), and four receptor subtypes have been indentified by cloning techniques (15); it is not unlikely that these two ligands, QNX and pirenzipine, are identifying different subtypes. The above results are consistent with the hypothesis that QNX does not differentiate between the receptor subtypes in the CS identified by pirenzipine, while the low affinity component of QNX inhibition, perhaps the M_3 receptor, cannot be discerned in the pirenzipine competition curve, i.e., Fig. 3 is well fit by two components but may comprise three or four components. The atropine-induced increase in M_2 receptor observed by Lee and Wolfe (16) of as much as 65-95% would appear as a 20-30% increase in the high affinity component of the QNX curve. It is also possible that the low affinity QNX binding site is the same as the high affinity agonist binding sites which are identified by [^{3}H]acetylcholine binding. A comparison in different brain regions of the proportion of low affinity QNX sites in the structures used in this study with the concentration of high affinity [^{3}H]acetylcholine binding sites expressed as a percentage of [^{3}H]QNB binding sites (25) supports this view. For example, the % ACh sites in the cortex (15%), corpus striatum (9%) and hippocampus (13%) are in good agreement with the % QNX low affinity site for those structures (18%, 7%, and 10%, respectively from Table III). Additionally, in a study similar to this one by Dr. K.J. Kellar, et al. (personal communication), atropine resulted in an increase in the total number of QNB binding sites in the cortex with no increase in the concentration of ACh binding sites while DFP treatment resulted in a decrease in the concentration of both QNB and ACh binding sites, precisely in concordance with our results with the low affinity QNX binding site.

This is not a completely adequate description of QNX binding inasmuch as the agreement between the ACh results and those with QNX is only approximate: the ACh binding sites as a percentage of QNB sites in the thalamus, pons, and cerebellum differ from the % low affinity QNX binding sites in this study with the largest difference being in the cerebellum (51% for ACH, 82% for QNX). The percentage of ACh binding sites in the rat atrial muscle (62%) is also different from our results with QNX in rat and dog myocardium in which a high affinity component was not evident, i.e., we would predict an equal number of high affinity agonist sites to QNB binding site rather than 62% of the QNB binding sites. Although this study supports the presence of multiple receptor subtypes which may exhibit different affinities and selectivities for muscarinic anatgonist, a proper understanding the complexities of receptor function and regulation by atropine and DFP will require investigations with ligands with greater selectivity for the multiple receptor subtypes that are present in the CNS.

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