0026-895X/89/050749-09$02.00/0

MOLECULAR PHARMACOLOGY, 36:749-757



# Regulation of Muscarinic Receptor Subtypes and Their Responsiveness in Rat Brain Following Chronic Atropine Administration

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Received September 29, 1988; Accepted August 2, 1989

**SUMMARY**

Chronic administration of *l*-atropine to rats caused a dose-dependent increase (30%) in the density of muscarinic receptors, as measured with [$^3$H]quinuclidinyl benzilate ([$^3$H]QNB), in cortex (CTX), dorsal hippocampus (DH), and heart but not the corpus striatum. Serum concentrations of *l*-atropine reached 80 to 160 nM within 6 hr, whereas densities of binding sites for [$^3$H]QNB did not show a significant increase until after the second day of infusion and receptor densities did not reach new steady state levels until after the fourth day of infusion. The density of binding sites for the M1-selective muscarinic receptor antagonist, [$^3$H] pirenzepine ([$^3$H]PZ) was also measured. As noted previously, the density of binding sites for [$^3$H]PZ (defined as M1) was lower than the density of binding sites for [$^3$H]QNB (defined as M1 plus M2). When the densities of binding sites for [$^3$H]QNB and [$^3$H] PZ in CTX plus DH were determined after 14 days of treatment, [$^3$H]QNB binding sites showed a 28% increase, whereas [$^3$H]PZ binding sites did not show any increase. The difference between the densities of binding sites for [$^3$H]QNB and [$^3$H]PZ, an estimate of the density of M2 sites, doubled. The density of binding sites for [$^3$H]QNB appeared to be stable for at least 64 hr after the withdrawal of the drug. The increase in the density of binding sites for [$^3$H]QNB was not reflected in the binding of [$^3$H]oxotremorine-M ([$^3$H]OXO-M), a muscarinic agonist, which was unchanged by *l*-atropine administration. Because the binding of [$^3$H]OXO-M is sensitive to GTP, this observation suggests that the "induced" receptors may not be coupled to a guanine nucleotide-binding protein. In spite of the fact that there was a doubling of the density of M2 sites, no significant differences in dose-response curves for carbachol-induced inhibition of [$^3$H] cAMP accumulation were observed in slices of CTX plus DH from control and *l*-atropine-treated rats. Similarity, acetylcholine-stimulated accumulation of [$^3$H]inositol phosphates in slices of CTX plus DH showed no significant differences between the tissues from control and treated rats. The above data show that 1) the density of high affinity binding sites for [$^3$H]PZ in CTX plus DH does not increase in response to chronic blockade, 2) although sites with low affinity for [$^3$H]PZ in CTX plus DH are up-regulated with chronic *l*-atropine treatment, there was no significant change in the ability of carbachol to inhibit forskolin-stimulated [$^3$H]cAMP accumulation or the ability of acetylcholine to stimulate phosphoinositide metabolism, and 3) the density of binding sites for [$^3$H]OXO-M is not affected by chronic blockade.

Subtypes of muscarinic cholinergic receptors have been proposed to exist, based on physiological (1, 2), radioligand binding (3), biochemical (4–7), and, most recently, molecular biological (8–11) studies. Subtypes were originally designated as either M1 or M2 depending whether they had high (10 nM) or low (300–1000 nM) affinity, respectively, for the selective drug PZ (2). Studies utilizing [$^3$H]PZ supported this concept and this ligand was shown to label only a subset of the total number of binding sites labeled by [$^3$H]QNB (12–14). Thus, sites labeled by [$^3$H]PZ are called M1 sites and sites labeled by [$^3$H]QNB are assumed to be the total number of muscarinic receptors (M1 plus M2). In light of the recent publications utilizing molecular biological approaches, it is clear that more than two subtypes of muscarinic receptors exist. For example, Bonner *et al.* (10, 11) have isolated and sequenced five distinct genes coding for proteins that appear to be muscarinic receptors. These authors have suggested naming these receptors of known sequence m1 through m5, noting the use of lower case "m." Some (m1 and m4) of these proteins have high affinity for PZ, whereas others have intermediate (m3 and m5) or low (m2) affinity for PZ. Thus, it is clear that high affinity binding sites for [$^3$H]PZ (M1 sites) are likely to represent more than one

This work was supported by National Institutes of Health Grant GM 31155. B.B.W. is an Established Investigator of the American Heart Association.

**ABBREVIATIONS:** PZ, pirenzepine; ACH, acetylcholine; $B_{max}$ maximal number of specific binding sites; CTX, cortex; DH, dorsal hippocampus; $IC_{50}$, concentration necessary to produce 50% of the maximal inhibition; IBMX, isobutylmethylxanthine; IP, inositol phosphates; $K_d$, equilibrium dissociation constant; OXO-M, oxotremorine-M; PI, phosphoinositide; QNB, quinuclidinylbenzilate; VH, ventral hippocampus; G protein, guanine nucleotide-binding protein.

molecular species (subtype of muscarinic receptor) and, similarly, M2 sites may represent more than one molecular species. At the current time there are no tools, pharmacological or immunological, that are selective enough to allow us to assay for a given molecularly defined subtype of muscarinic receptor.

Chronic blockade of muscarinic receptors by atropine has been shown to result in an increase in the density of binding sites for [$^3$H]QNB (15, 16). Presumably, this phenomenon occurs because normal stimulation of the receptor by ACH is inhibited and the cell responds to this decreased input by increasing the number of receptors. No one has, until recently (17), however, examined the possibility that not all muscarinic receptor binding sites are regulated similarly. The current experiments were designed to examine the possibility that "subtypes" of muscarinic receptors, as defined based on the affinity of PZ, can be regulated differentially following chronic blockade with atropine.

In addition to examining the regulation of high and low affinity binding sites for PZ, these studies were designed to determine the effects of chronic receptor blockade on the ability of cholinergic agonists to stimulate the breakdown of PI as well as inhibit the production of cyclic AMP. There have been suggestions that these second messenger systems are specifically related to high and low affinity binding sites for PZ (4, 5). This issue has, however, been the subject of some controversy (18–23) and, as yet, there is no universal simple relationship between the affinity of a receptor for PZ and the second messenger system that is modulated by that receptor. Nevertheless, in the rat forebrain it appears that muscarinic receptor-stimulated phosphoinositide breakdown is mediated by receptors having high affinity for PZ, whereas muscarinic receptor-mediated inhibition of adenylyl cyclase is mediated by receptors with low affinity for PZ (4, 23). Thus, we thought it worthwhile to examine the regulation of these receptor-mediated second messenger systems in response to chronic blockade.

## Experimental Procedures

### Materials

Tris, *l*-hyoscyamine hemisulfate, ACH, carbachol, and eserine were purchased from Sigma Chemical Co. (St. Louis, MO). *l*-Hyoscyamine is the active isomer of atropine and it will be referred to as *l*-atropine. [$^3$H]QNB (33.2–39.4 Ci/mmol), [$^3$H]PZ (76–85 Ci/mmol), [$^3$H]OXO-M (87 Ci/mmol), [$^3$H]inositol (12.8 Ci/mmol), [$^3$H]adenine (28 Ci/mmol), and [$\alpha^{32}$P]ATP were purchased from New England Nuclear (Boston, MA). Budget-Solve was purchased from Research Product Int. (Mt. Prospect, IL). All the other chemicals used in the present studies were reagent grade from our laboratory stock. Alzet osmotic minipumps (model 2002) were purchased from Alzet corp. (Palo Alto, CA).

### Methods

**Atropine treatment.** *l*-Atropine was infused at 0.5 μl/hr from Alzet osmotic minipumps that contained the drug in distilled water. Control rats were either implanted with pumps containing distilled water or, in some cases, sham operated without implantation of pumps. Filled pumps were primed by storage in 0.9% saline solution overnight before implantation, when treatment was under 1 week. Rats were anesthetized with ether and the osmotic minipumps were implanted in the subcutaneous space posterior to the scapulae (and between the shoulder blades). The incision was closed with wound clips. Minipumps were left in rats for indicated periods of time and, in all cases except for the time course in Fig. 1, minipumps were removed from the rats anesthetized with ether 24 hr before the rats were sacrificed for tissue collection. The distribution and elimination of *l*-atropine appeared to be rapid, because mydriasis and loss of light-induced pupillary response could be detected within 6 hr from the start of drug infusion and the pupillary response to light reappeared approximately 4–6 hr after the osmotic minipumps had been removed. The stability of *l*-atropine solution was examined in competition curves using both freshly made *l*-atropine solution and *l*-atropine solution that had been stored at 37° for 19 days. There was no difference in the $IC_{50}$ values of competition curves generated using the binding of [$^3$H]QNB (data not shown).

**Determination of serum levels of atropine.** Serum samples were obtained by collecting trunk blood after decapitation. Whole blood samples were left at room temperature for 30 min and centrifuged at 4000 × g for 5 min. The supernatant (serum) was transferred to a fresh tube and centrifuged at 4000 × *g* for 5 min to remove any remaining clots. The serum concentrations of *l*-atropine were estimated by measuring the ability of diluted serum samples to inhibit the binding of [$^3$H]QNB to a rat cortical homogenate that was prepared as described below. Increasing concentrations of atropine ($10^{-12}$ to $10^{-6}$ M) were used in constructing a standard curve from which the serum concentrations of *l*-atropine were determined. In these determinations, each assay contained 50 μg of cortical membrane protein, 150 pM [$^3$H]QNB , 50 μl of buffer alone or buffer containing varying concentrations of atropine, and 200 μl of diluted serum, in a final assay volume of 2 ml. Serum from untreated rats was added into assays for the standard curve, so that all assays contained an equal amount of serum.

**PI breakdown.** The breakdown of PI was monitored by measuring the accumulation of [$^3$H]IP, as reported by Berridge *et al.* (24) and modified by Gil and Wolfe (4). CTX plus DH slices were prepared by removing these brain regions from rats and slicing them with a McIlwain tissue chopper twice at 250-μm thickness, with 90° rotation each time. The slices from one rat brian were suspended in 4 ml of a pH 7.4 bicarbonate buffer containing (mM): glucose, 10; NaCl, 123; KCl, 5; $MgCl_2$, 1.3; $KH_2PO_4$, 1.4; $NaHCO_3$, 26; and were gassed with 95% $O_2$/5% $CO_2$. A portion of the tissue was resuspended in 10 mM Na-K phosphate buffer (pH7.4) and processed for receptor binding assays. Aliquots (200 μl containing 3–3.6 mg of protein) of the suspension of brain slices were preincubated with approximately 1 μCi of [$^3$H]inositol for 45 min. LiCl (10 mM final) was added 5 min before the end of the preincubation period to promote the accumulation of [$^3$H]IP (24). ACH (1 mM final) and eserine (10 μM final) were added. Final assay volume was 260 μl. The tissue was incubated at 37°, with shaking and gassing, for 30 min. [$^3$H]IP was isolated as described in detail by Gil and Wolfe (4). Protein concentration was measured by the method of Lowry *et al.* (25), using fraction V bovine serum albumin as a standard.

**[$^3$H]cAMP accumulation.** Inhibition of [$^3$H]cAMP accumulation by carbachol was carried out using a modification of the method of Shimizu *et al.* (26). CTX plus DH slices, prepared as described for studies of PI metabolism, were suspended in 20 ml (per rat) of the same gassed bicarbonate buffer. A portion of the brain slice suspension was taken out and processed for receptor binding, as described below. The tissue was preincubated for 20 to 30 min and then transferred to fresh buffer containing [$^3$H]adenine (2.5 μCi/ml) for 30 min. The slices were then washed three times before final resuspension (1–1.3 mg/ml; 40 ml/rat) in IBMX (1 mM)-containing buffer for the assay. Aliquots (1 ml of the tissues were incubated with 50 μM forskolin in the absence or presence of varying concentrations of carbachol at 37°, gassed with 95% $O_2$/5% $CO_2$, and shaken for 30 min. Reactions were stopped by the addition of trichloroacetic acid (5% final). [$^3$H]ATP and [$^3$H]cAMP were separated according to the method described by Salomon *et al.* (27). Results were first calculated as the percentage of conversion of [$^3$H]ATP (assumed to be the sum of [$^3$H]ATP and [$^3$H]cAMP) to [$^3$H]cAMP and were then expressed as a percentage of the value measured in the presence of forskolin alone.

**Adenylyl cyclase assay.** Muscarinic receptor-mediated inhibition of adenylyl cyclase activity in striatal homogenates was measured by monitoring the conversion of [$\alpha$-$^{32}$P]ATP to [$\alpha$-$^{32}$P]cAMP, using the method originally reported by Salomon *et al.* (27) and described in

detail by Gil and Wolfe (4). Tissues were prepared as follows. Striata were placed in cold 10 mM Tris·HCl (pH 7.5) containing 1 mM EDTA, homogenized with a Polytron, and centrifuged at 30,000 × *g* for 10 min. The resulting pellet was resuspended in 20 mM Tris·HCl (pH 7.5) containing 0.9% NaCl for the assay. Final protein concentration in the assay was 1.0–1.5 mg/ml and the incubation time was 10 min.

**Radioligand binding assays.** [$^3$H]QNB and [$^3$H]PZ binding assays. The densities of [$^3$H]QNB and [$^3$H]PZ binding sites were measured as described by Luthin and Wolfe (14). Tissues were homogenized in cold 10 mM Na-K phosphate buffer (pH 7.4) with a Brinkmann Polytron and were centrifuged at 30,000 × *g* for 15 min. The pellets were resuspended in the same buffer (approximately 10 mg/ml for brain tissues and 5 mg/ml for heart), of 10 mM Na-K phosphate (pH 7.4), disrupted with a Polytron, and stored at −80° until binding assays were performed. For the data shown in Fig. 1, when no time for drug clearance was allowed, tissues were washed twice by centrifugation and incubated at 32° for 10 min to promote the dissociation of residual *l*-atropine; they were then centrifuged and washed once more with the buffer before resuspension and freezing. A Polytron (at speed setting 6 for 5 sec) was used to disrupt the pellets after each centrifugation and after thawing just before the binding assays were performed.

Aliquots of tissue, 10–20 μg of protein for [$^3$H]QNB binding and 100–200 μg of protein for [$^3$H]PZ binding, were added to tubes containing varying concentrations of radioligands (10–600 pM for [$^3$H]QNB and 1–100 nM for [$^3$H]PZ), to initiate the reactions. All saturation experiments were performed with six concentrations in duplicate. The final assay volumes were 5.0 ml for [$^3$H]QNB binding and 0.25 ml for [$^3$H]PZ binding. Nonspecific binding was defined using 1 μM atropine. The assays were incubated for 1 hr at 32°. Binding reactions were terminated by the addition of 5 ml of cold 0.9% NaCl in 10 mM Tris·HCl (pH 7.5 at 4°) for [$^3$H]QNB binding assays and 10 ml of the same buffer for [$^3$H]PZ binding assays. The samples were filtered rapidly through glass fiber filters (Schleicher and Schuell No. 30), and filters were washed with 10 ml of buffer. Filters were dried by suction, placed in vials, and counted by scintillation counting at an efficiency of approximately 40% for $^3$H.

Saturation binding data were transformed using the method of Scatchard (28) and $K_d$ and $B_{max}$ values were estimated using unweighted linear regression analysis of the transformed data. $B_{max}$ values are expressed as fmol/mg of membrane protein.

[$^3$H]OXO-M binding assays. The density of binding sites for [$^3$H]OXO-M was measured using the method reported by Harden *et al.* (29), with some modifications. Tissues were homogenized in 10 mM Tris· HCl (pH 7.5 at 4°) containing 1 mM EDTA (1 CTX+DH/30 ml) and were centrifuged at 30,000 × *g* for 15 min. Pellets were resuspended in fresh buffer and disrupted with a Polytron. This washing step was repeated twice, with an incubation of 10 min at 32° between the second and the final wash. Tissues were resuspended in 10 mM Tris· HCl (pH 7.5 at 32°) for the assay. Aliquots of tissue containing 100–150 μg of protein were added to tubes containing $MgCl_2$ (5 mM final) and the radioligand (0.6–25 nM final) in a final volume of 0.25 ml. Nonspecific binding was defined using 1 μM atropine. Saturation binding was performed with six concentrations in duplicate. The reactions were incubated for 1 hr at 32° and were terminated by the addition of 10 ml of cold 10 mM Tris· HCl (pH 7.5 at 4°) before filtration. Filters were presoaked in a solution containing 1% bovine serum albumin and 1% Aquasil for 1 hr and were rinsed several times with distilled water before filtering. The collection of radioactivity and calculations were performed as described above.

Specific binding represented at least 70% for each ligand at all concentrations used and, in general, correlation coefficients for Scatchard (28) plots ranged from 0.950 to 0.999.

**Statistical analysis.** All data expressed as a percentage were analyzed by nonparametric tests, whereas all other data were analyzed by parametric tests. When data of one group were compared with data of another group in a single comparison, paired or unpaired *t* test (parametric) or Mann-Whitney rank sum test (nonparametric) was used. Dunnett's test was used when multiple sets of data were compared with a single set of data individually and when all sets of data follow a normal distribution. The Newman-Keuls multiple range test was used when multiple comparisons between sets of data following a normal distribution were made. The Kruskal-Wallis test was used when comparisons between sets of data following a nonnormal distribution (i.e., data presented as percentages) were made.

## Results

**Effects of chronic *l*-atropine administration on the density of binding sites for [$^3$H]QNB.** To determine whether chronic blockade of muscarinic receptors resulted in an increase in the density of binding sites for [$^3$H]QNB in various tissues, rats were treated with two doses of *l*-atropine for 7 days, at which time pumps were removed. Twenty-four hours later, the rats were sacrificed and the CTX, striatum, hippocampus, and heart were collected and processed for receptor binding. When rats were infused with *l*-atropine at 0.3 or 3 mg/day for 7 days, the CTX and hippocampus showed a 15 or 25% increase in the density of binding sites for [$^3$H]QNB, respectively, as shown in Table 1. The striatum, however, did not show a significant change in the density of binding sites for [$^3$H]QNB at either dose of *l*-atropine (data not shown). An additional 7 days of treatment at 3 mg/day did not cause a further increase in the densities of binding sites for [$^3$H]QNB in both CTX and hippocampus (data not shown). The heart showed a significant increase in the density of binding sites for [$^3$H]QNB only at a dose of 3 mg/day (Table 1).

When rats were infused with *l*-atropine at 5 mg/day for 14 days, the CTX and DH from the treated rats showed similar increases of 29 and 32%, respectively, in the density of binding sites for [$^3$H]QNB, whereas VH from the treated rats showed a 21% increase in the density of binding sites for [$^3$H]QNB (Table 2). However, at a dose of 3 mg/day for 14 days, the density of the binding sites for [$^3$H]QNB in VH did not show a significant change, whereas both the CTX and DH showed significant increases (29 ± 1% for CTX and 35 ± 4% for DH; $n = 3$) in the density of binding sites for [$^3$H]QNB at $p < 0.01$ (data not shown). Thus, in all subsequent studies, the CTX and DH were combined and used. Because the increase in the density of binding sites for [$^3$H]QNB that resulted from treatment with 5 mg of *l*-atropine/day was similar to, or perhaps larger than, that seen with treatment with 3 mg of *l*-atropine/day, the former was chosen as the dosage for all subsequent experiments.

TABLE 1

**Binding of [$^3$H]QNB to tissues from control and *l*-atropine-treated rats: effect of doses**

The infusion of *l*-atropine (0.3 and 3 mg/day for 7 days) was discontinued 24 hr before collection of tissues. The density of binding sites for [$^3$H]QNB was measured as described under Experimental Procedures. All results shown are the mean ± standard error of duplicate determinations on each rat from Scatchard (28) analysis of saturation curves. The percentage increase above control is shown in parentheses. Dunnett's test was used in the statistical analysis.

| | $B_{max}$ | | | |
|---|---|---|---|---|
| | Cortex | Hippocampus | Heart | *n* |
| | *fmol/mg* | | | |
| Control | 2668 ± 62 | 2236 ± 65 | 232 ± 13 | 4 |
| 0.3 mg/day | 3063 ± 83[a] (15%) | 2598 ± 59[a] (16%) | 259 ± 7 | 3 |
| 3.0 mg/day | 3327 ± 120[b] (25%) | 2813 ± 34[b] (26%) | 280 ± 13[a] (21%) | 4 |

[a] $p < 0.05$ and [b] $p < 0.01$ when compared with respective control groups.

TABLE 2

**Binding of [$^3$H]QNB to tissues from control and *l*-atropine-treated rats**

*l*-Atropine was infused at 5 mg/day for 14 days. The infusion was discontinued 24 hr before the collection of tissues. The density of binding sites for [$^3$H]QNB was measured as described under Experimental Procedures. All results shown are the mean ± standard error of duplicate determinations on each rat from Scatchard (28) analysis of saturation curves. The percentage increase above control is shown in parentheses. Data were pooled from two individual experiments with four control and four treated rats in each experiment. An unpaired *t* test was used in the statistical analysis.

| | $B_{max}$ | | | |
|---|---|---|---|---|
| | Cortex | Dorsal hippocampus | Ventral hippocampus | *n* |
| | fmol/mg | | | |
| Control | 2599 ± 75 | 2224 ± 62 | 2137 ± 46 | 8 |
| Treated | 3360 ± 54 (29%)* | 2926 ± 69 (32%)* | 2595 ± 107 (21%)* | 8 |

*$p < 0.01$ compared with respective control groups.

**Time course of the effects of chronic administration of *l*-atropine on the density of binding sites for [$^3$H]QNB.** To determine the relationship between the increase in density of binding sites for [$^3$H]QNB and serum levels of atropine, rats were infused with *l*-atropine at 5 mg/day for up to 2 weeks. Serum concentrations of *l*-atropine reached levels of nearly 100 nM within 6 hr of infusion (Fig. 1A), whereas the densities of binding sites for [$^3$H]QNB in CTX plus DH did not show a significant increase ($p < 0.01$) until after the second day of infusion and took at least 4 days to reach the new steady state levels (Fig. 1B). There were no significant changes in the serum levels of *l*-atropine during the 2-week infusion period. Serum levels of *l*-atropine were undetectable (<2 nM) 24 hr after the removal of the minipumps, and the increase in the density of binding sites for [$^3$H]QNB in CTX plus DH was similar whether measured before or 24 hr after the removal of drug (Fig. 1B).

**Selective up-regulation of the M2 binding/sites with chronic muscarinic receptor blockade.** To examine the possibility that subtypes of muscarinic receptors respond differently to chronic blockade, the densities of binding sites for [$^3$H]QNB and [$^3$H]PZ in CTX plus DH were determined after 14 days of *l*-atropine infusion and 1 day of drug clearance time. The density of binding sites for [$^3$H]QNB (M1 plus M2) showed a 28% increase, whereas the density of binding sites for [$^3$H]PZ (M1) did not show a significant increase (Fig. 2). The difference between [$^3$H]QNB and [$^3$H]PZ binding sites, an estimate of the density of M2 sites, showed a 102% increase (Fig. 3).

Densities of binding sites for both [$^3$H]QNB and [$^3$H]PZ were measured at 24, 40, and 64 hr following the removal of osmotic minipumps (Table 3). The changes in the density of binding sites for [$^3$H]QNB appeared to be stable for at least 64 hr after the withdrawal of drug, and no changes in the density of binding sites for [$^3$H]PZ were observed at any time, as shown in Table 3.

**[$^3$H]IP and [$^3$H]cAMP accumulation in slices of CTX plus DH.** To examine the possibility that the biochemical responses mediated by stimulation of muscarinic receptors are affected by chronic blockade of the receptors, ACH-stimulated PI breakdown, a response that has been suggested to be mediated by receptors having high affinity for PZ (4, 5), was measured. Tissues from treated rats produced 86% as great a PI response to 1 mM ACH as control tissues and this difference was not statistically significant [control = 100 ± 8% ($n = 20$), atropine treated = 86 ± 7% ($n = 18$), $p > 0.05$].

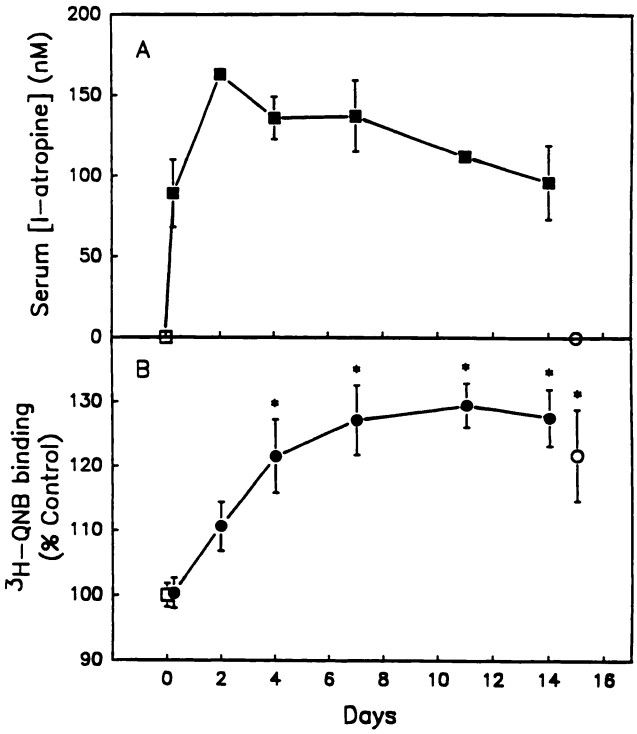


**Fig. 1.** Effects of *l*-atropine treatment on serum concentration of *l*-atropine and the density of binding sites for [$^3$H]QNB. Rats were treated with *l*-atropine at 5 mg/day for up to 14 days via Alzet osmotic minipumps. Except for the last time point (15-day) (*open circle* in both A and B), pumps were not removed before the collection of tissues. The pumps were removed on the 14th day for the last time point. Serum concentration of *l*-atropine and the density of binding sites for [$^3$H]QNB in CTX+DH were measured for each rat. There were 3 rats for each time point and 11 rats for control (0-day) (*open square* in both A and B). A, Serum concentrations of *l*-atropine were measured as described under Experimental Procedures. Serum concentration of *l*-atropine at 24 hr after the withdrawal of pumps were not measurable (<2 nM). Results shown are the mean ± standard error of triplicate determinations for each rat. B, The density of binding sites for [$^3$H]QNB was measured as described. Results shown are the mean ± standard error of $B_{max}$ values (Scatchard analysis of specific binding) measured in duplicate for each time point and are expressed as percentage of the mean of control values (2626 ± 51 fmol/mg). *$p < 0.01$ by Dunnett's test.

Inhibition of adenylyl cyclase activity by muscarinic agonists is a response that has been suggested to be associated with receptors having low affinity for PZ (4, 5). To determine whether the increase in the density of M2 binding sites caused by chronic administration of *l*-atropine is associated with an increase in the ability of a muscarinic agonist to modulate cAMP levels, slices from control and *l*-atropine-treated CTX plus DH were used to measure carbachol-mediated inhibition of forskolin-stimulated cAMP accumulation. Although there was a doubling of the density of the M2 binding sites, the dose-response curves for carbachol-mediated inhibition of [$^3$H]cAMP accumulation showed no significant differences in either maximal effect or the $EC_{50}$ value for carbachol (Fig. 4A).

**Effects of a 6-hr infusion of *l*-atropine on the dose-response curves of [$^3$H]cAMP accumulation to carbachol in CTX plus DH slices.** To examine the possibility that residual *l*-atropine (if there was any) remaining in the tissue during the assay could have masked a potential shift to the left of the dose-response curve to carbachol for treated tissues, shown in Fig. 4A, rats were infused with *l*-atropine at 5 mg/day for 6 hr. At this time, serum levels of atropine have reached

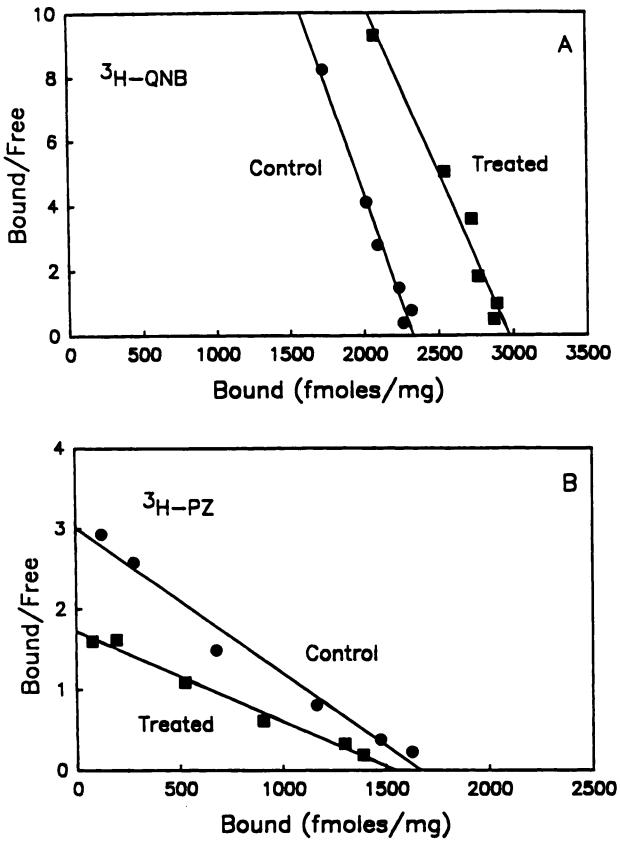


**Fig. 2.** Effect of chronic *l*-atropine treatment on the densities of binding sites and equilibrium dissociation constants for [$^3$H]QNB and [$^3$H]PZ. Scatchard (28) analysis of specific binding for [$^3$H]QNB (A) and [$^3$H]PZ (B) on control and *l*-atropine-treated (5 mg/day for 14 days and 1 day of clearance time) rats were carried out as described. Control and treated are represented by *filled circles* and *filled squares*, respectively. These are representative Scatchard (28) plots. $K_d$ values for the binding of [$^3$H]QNB: control, 7.5 pM; treated, 9.4 pM; for the binding of [$^3$H]PZ: control, 5.6 nM; treated, 8.9 nM. $K_d$ values for the binding of [$^3$H]QNB ranged from 6 to 14 pM and from 7 to 20 pM for control and treated, respectively; $K_d$ values for the binding of [$^3$H]PZ ranged from 4 to 16 nM and from 7 to 18 nM, respectively. The units for *B/F* are mol/mg/M × 100 for A and mol/mg/M × 10,000 for B.

steady state but no changes in receptors have occurred (Fig. 1). Serum concentrations of *l*-atropine were measured in these rats and were comparable to those shown in Fig. 1A (data not shown). No drug clearance time was allowed in these rats; therefore, residual *l*-atropine (if there was any) would be maximal. In tissues from these rats, the dose-response curves to carbachol showed no significant difference between control and treated tissues (Fig. 4B) and looked remarkably like those shown in Fig. 4A. Furthermore, similar dose-response curves to carbachol were obtained when rats were treated for 2 weeks and allowed no time for drug clearance before the collection of tissues (data not shown).

**Effects of chronic *l*-atropine administration on the density of binding sites for [$^3$H]OXO-M.** To determine whether chronic blockade of muscarinic receptors resulted in a change in the density of binding sites for the agonist [$^3$H]OXO-M, rats were treated with *l*-atropine for 14 days and allowed 1 day for drug clearance before the collection of tissues. The density of [$^3$H]OXO-M binding sites, unlike that of [$^3$H]QNB binding sites, did not show an increase in the treated tissues (Table 4).

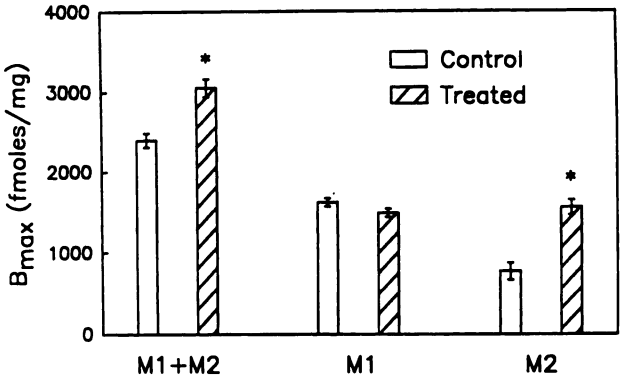


**Fig. 3.** Effects of chronic *l*-atropine infusion on the densities of M1 and M2 binding sites. Rats were treated with 5 mg of *l*-atropine/day for 14 days and allowed 1 day for drug clearance. The densities of binding sites for [$^3$H]QNB (M1 plus M2) and [$^3$H]PZ (M1) were obtained by Scatchard (28) analysis of specific binding in saturation experiments. Results shown are the mean ± standard error of duplicate determinations from several individual experiments. There were 17 control and 14 treated rats. Control and *l*-atropine-treated results are represented by *open* and *hatched bars*, respectively. The data for the *rightmost pair of bars* (M2) were obtained by taking the difference between the densities of [$^3$H]QNB binding sites and [$^3$H]PZ binding sites for each rat and then calculating the mean ± standard error. *$p < 0.01$ by unpaired *t* test.

TABLE 3

**Stability of receptor changes in CTX+DH**

Rats were treated with 5 mg of *l*-atropine/day for 14 days. The densities of binding sites for [$^3$H]QNB and [$^3$H]PZ were measured as described under Experimental Procedures. Data were pooled from several individual experiments. $B_{max}$ values were obtained from Scatchard (28) analysis of saturation curves. Results shown were calculated by first converting the $B_{max}$ value obtained for each rat to a percentage of the mean of the control $B_{max}$ values found in a given experiment and then combining these numbers from several experiments to obtain mean ± standard error. The Kruskal-Wallis test was used in the statistical analysis.

| Time after pump withdrawal | [$^3$H]QNB binding | [$^3$H]PZ binding | *n* |
|---|---|---|---|
| *hr* | *% of control* | *% of control* | |
| 24 | 128 ± 4* | 95 ± 2 | 14 |
| 40 | 128 ± 5* | 97 ± 2 | 12 |
| 64 | 123 ± 3* | 99 ± 1 | 8 |
| No drug | 100 ± 2 | 100 ± 2 | 17 |

*$p < 0.01$ compared with the no-drug group. There were no significant differences between the 24-, 40-, and 64-hr groups for the binding of either [$^3$H]QNB or [$^3$H]PZ.

**Effects of chronic *l*-atropine administration on the densities of binding sites for [$^3$H]QNB and [$^3$H]PZ and adenylyl cyclase activity in striatum.** To determine whether selective regulation of muscarinic receptors also occurred in striatum in response to chronic blockade, the densities of binding sites for [$^3$H]QNB and [$^3$H]PZ were measured. There were no significant increases in the density of binding sites for either ligand in treated tissues (Table 5). Furthermore, treated tissues did not show detectable changes in either the maximal value for inhibition or the $IC_{50}$ value for ACH-mediated inhibition of striatal adenylyl cyclase activity (Table 5).

## Discussion

Dose-dependent increases in the density of binding sites for [$^3$H]QNB resulting from chronic blockade of muscarinic receptors have been shown in the present study (Table 1), as well as in other studies examining muscarinic receptors in cortex (16, 30, 31), heart (32), and hippocampus (15, 33). The time courses for the serum accumulation of *l*-atropine and the increase in

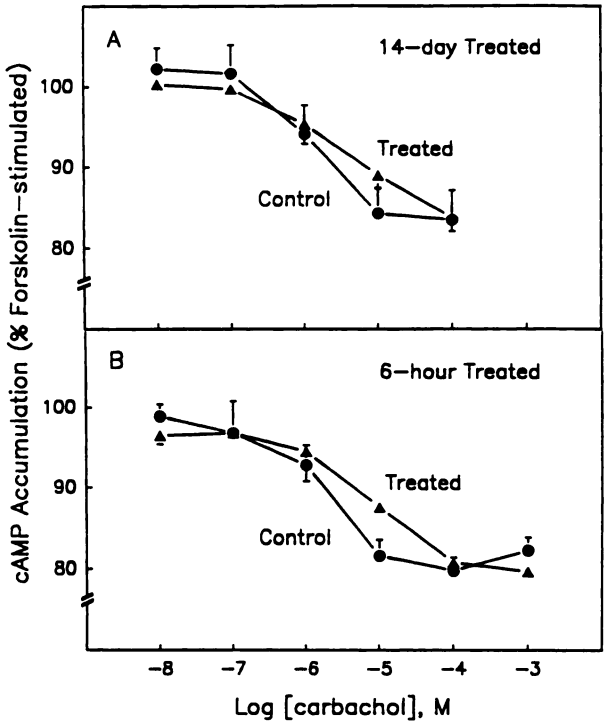


**Fig. 4.** Effects of *l*-atropine treatment on carbachol-mediated inhibition of [$^3$H]cAMP accumulation. Dose-response curves to carbachol were performed in the presence of 50 $\mu$M forskolin. Assays were performed in triplicate and data were first calculated as the percentage of conversion of total [$^3$H]ATP (assumed to be the sum of [$^3$H]ATP and [$^3$H]cAMP) to [$^3$H]cAMP. These data were then expressed as percentage of the values found in the presence of forskolin alone (ranged from 3 to 4.5%). Results shown are the mean ± standard error. A, Rats were treated with *l*-atropine (5 mg/day) for 14 days and allowed 1 day for drug clearance ($n$ = 8). B, Rats were treated similarly for 6 hr and allowed no time for drug clearance ($n$ = 4).

TABLE 4

**Effects of *l*-atropine treatment on the binding of [$^3$H]OXO-M to CTX+DH**

Rats were treated with 5 mg of *l*-atropine/day for 14 days and allowed 1 day for drug to wash out. Tissue homogenates were washed three times before the assay. Tissues were incubated at 32° for 10 min between the second and the third wash before the final resuspension for the binding assays. The density of binding sites for [$^3$H]OXO-M and $K_d$ were obtained from Scatchard (28) analyses of saturation curves. The mean $K_d$ value is shown for each group with the range of $K_d$ values in parentheses, because $K_d$ values do not follow a normal distribution. An unpaired $t$ test was used in the statistical analysis ($n$ = 8); $p > 0.05$.

| | $B_{max}$ | $K_d$ |
|---|---|---|
| | *fmol/mg* | *nM* |
| Control | 651 ± 12 | 1.43 (1.27–1.64) |
| Treated | 629 ± 15 | 1.42 (1.22–1.65) |

the density of muscarinic receptors are quite different, as shown in Fig. 1. The serum concentration of *l*-atropine reached the final concentration of 6 hr, which was the earliest time measured, after the commencement of infusion. However, the increase in the density of binding sites for [$^3$H]QNB took at least 4 days of treatment to reach the new steady state. The density of binding sites for [$^3$H]QNB remained stable during the following week of treatment, and this observation was also seen in both the cortex and hippocampus at a lower dose (3 mg/day for 7 or 14 days) (data not shown). A similar finding has been reported in the hippocampal formation in rats treated with scopolamine (33), in which the same increase in the density of

TABLE 5

**Lack of effect of chronic *l*-atropine administration on striatal muscarinic receptors**

Rats were treated with 5 mg of *l*-atropine/day for 14 days and allowed 1 day of drug clearance time. Results shown were pooled from three independent experiments. Receptor binding data shown were obtained from $B_{max}$ values in Scatchard (28) analysis of saturation curves for the binding of [$^3$H]QNB and [$^3$H]PZ and are given in fmol/mg of protein ± standard error. Adenylyl cyclase activity data were obtained from 12-point dose-response curves to ACH ($10^{-7}$ to $10^{-3}$ M, in the presence of 10 $\mu$M eserine); basal adenylyl cyclase activity (in the absence of ACH) was used as 100% activity in calculating percentage of inhibition. Determinations on each rat were carried out in duplicate for receptor binding and triplicate for adenylyl cyclase activity assays. The mean ± SE of basal adenylyl cyclase activity for control and treated rats were 53 ± 3 and 49 ± 2 pmol/mg/min, respectively. For receptor binding assays, control, $n$ = 13; treated, $n$ = 9; for adenylyl cyclase activity assays, $n$ = 15 for both control and treated. There were no significant differences ($p > 0.05$, by an unpaired $t$ test for receptor binding and the Mann-Whitney rank sum test for adenylyl cyclase activity) between control and treated tissues for any parameter.

| | Receptor binding, $B_{max}$ | |
|---|---|---|
| | Control | Treated |
| | *fmol/mg* | |
| M1 + M2 ([$^3$H]QNB) | 2181 ± 86 | 2338 ± 101 |
| M1 ([$^3$H]PZ) | 1200 ± 79 | 1176 ± 110 |
| M2 | 982 ± 112 | 1161 ± 129 |
| | Adenylyl cyclase activity | |
| | Control | Treated |
| Maximum inhibition by ACH (%) | 27 ± 1 | 25 ± 2 |
| $IC_{50}$ for ACH ($\mu$M) | 2.7 ± 1.3 | 3.0 ± 1.2 |

binding sites for [$^3$H]QNB was observed when rats were treated for either 7 or 30 days.

In contrast to the observed changes in the binding of [$^3$H]QNB, the density of binding sites for [$^3$H]PZ did not show a significant change in response to chronic *l*-atropine treatment. This lack of increase in the density of binding sites for [$^3$H]PZ could be explained by at least two possibilities. The first possibility is that most or all of the high affinity binding sites for [$^3$H]PZ (M1 sites) in the brain normally receive little or no endogenous stimulation; therefore, chronic blockade of these receptors with *l*-atropine did not cause a measurable increase in their density. The second possibility is that these sites do receive high levels of endogenous stimulation, however, for some unknown reason, do not respond to blockade with an increase in density. Supportive of the former hypothesis, Siman and Klein (34) have compared the regulation of muscarinic receptors occurring in aggregate cultures of embryonic chicken cerebrum with that occurring in cultures of N1E-115 neuroblastoma cells. The aggregate cultures form functional synapses and the N1E-115 cells do not. Treatment with different concentrations of carbachol caused both types of cells to lose binding sites for [$^3$H]QNB, whereas treatment with different concentrations of atropine resulted in increases in binding sites for [$^3$H]QNB in aggregate cell cultures only. Therefore, it seems that down-regulation of muscarinic binding sites due to receptor activation does not require the formation of functional synapses, whereas up-regulation of muscarinic binding sites as a result of receptor blockage does require functional synapses with endogenous receptor activation. The selective increase in the density of M2 binding sites in our studies is likely to indicate that these receptors normally receive endogenous stimulation and that they may have a different synaptic localization than M1 binding sites. It must also be noted that it is likely that [$^3$H]PZ binding is not confined to a single subtype of muscarinic receptor. Thus, it is entirely possible that, although the total

density of M1 sites did not change after *l*-atropine administration, there could be an increase in one of the subtypes labeled by this ligand but that the binding of [$^3$H]PZ to this subtype represents only a small fraction of the total binding sites for [$^3$H]PZ.

In a recent study by Goobar and Bartfai (17), chronic atropine treatment has been reported to cause increases in the density of binding sites for both [$^3$H]QNB and [$^3$H]PZ in rat cortex and hippocampus. The cause for the discrepancy regarding a change in the density of binding sites for [$^3$H]PZ between their study and ours is unclear. In the same study, it was shown that the maximal accumulation of [$^3$H]IP stimulated by 10 mM carbachol was the same in control and treated tissues, although carbachol at lower concentrations was less effective in stimulating the accumulation of [$^3$H]IP in treated tissues. The decreased potency of carbachol was likely to have been caused by residual atropine. A potential explanation for the difference observed between our study and that of Goobar and Barftai (17) regarding the density of binding sites for [$^3$H]PZ is the route of drug administration. In their study, rats were treated via daily injection; therefore, there was likely to be a much greater fluctuation in the serum concentration of atropine, compared with our study in which the serum concentration of atropine remained relatively constant. Thus, for a part of the time after each injection, the serum concentration of atropine would likely to have been substantially higher than that attained with osmotic minipumps. The difference in serum concentration of atropine may have differential effects on subtypes of muscarinic receptors.

The increase in the density of binding sites for [$^3$H]QNB was stable for at least 64 hr after the withdrawal of *l*-atropine treatment, because no significant differences were observed between tissues with and without 24-hr drug clearance time (Fig. 1B) or between tissues collected 24, 40, or 64 hr after the withdrawal of pumps (Table 3). This is in agreement with the study of Majocha and Baldessarini (31), who have examined the time course for the recovery of the density of muscarinic receptor from chronic treatment with scopolamine. They reported that the increase in the density of binding sites for [$^3$H]QNB was reversible, persisted for 2 to 5 days, and was lost by day 10. In the present study, the slow rate of recovery from increased density of muscarinic binding sites at a time when the serum concentration of *l*-atropine was not measurable indicates either that the brain acts as a depot for *l*-atropine after the termination of *l*-atropine infusion, thus keeping local levels of the drug high, or that the normal turnover rate of these muscarinic receptors is slow in rat brain. The presence of a muscarinic antagonist may inhibit the degradation of muscarinic receptors, as shown by Roskoski *et al.* (35), therefore prolonging the effects of chronic *l*-atropine treatment.

It has been suggested previously that, in rat forebrain, high affinity binding sites for PZ (M1) are involved in the stimulation of PI breakdown, whereas low affinity binding sites for PZ (M2) are involved with the inhibition of adenylyl cyclase activity (4). Thus, because there was no significant change in the density of binding sites for [$^3$H]PZ, it was not surprising that there was also no change in the ability of ACH to stimulate PI breakdown. However, the increase in the density of M2 sites did not result in an expected change in the ability of carbachol to inhibit the accumulation of cAMP (Fig. 4A). If there are no "spare receptors" for this response in this tissue, one may expect an increase of 2-fold in the maximal response, proportional to the increase in receptor density observed (102%). If, on the other hand, spare receptors exist in this system, one may expect a decrease of 2-fold in the $IC_{50}$ value of carbachol, proportional to the increase in receptor density (36). Although a 2-fold increase in the maximal response would have been detected easily, due to "noise" inherent in the assay a 2-fold change in the $IC_{50}$ value would be difficult to detect in these experiments. In a number of experiments, propylbenzilcholine mustard was used to irreversibly inactivate a portion of the muscarinic receptors *in vitro* in brain slices from *l*-atropine-treated rats before assaying for carbachol-mediated inhibition of cAMP accumulation. These attempts to address the possible existence of spare receptors were not successful because of the relatively small response generated by muscarinic receptors and variation in responses from tissue slices treated with propylbenzilcholine. A firm conclusion from these studies was difficult to make.

The possibility that residual atropine may have interfered with the detection of changes in the dose-response curves to carbachol was examined. Dose-response curves to carbachol were obtained in tissues that were treated either for 6 hr (Fig. 4B), a time when the serum concentration of atropine was the same as the final (14-day) concentration but no change in the density of muscarinic binding sites occurred, or for 2 weeks with no drug clearance time, to simulate the worst case in residual drug (data not shown). In neither case was there a significant change from control tissues in the dose-response curves to carbachol. Therefore, it is unlikely that residual atropine could account for the apparent lack of changes in the dose-response curves for the treated rats.

Another possibility that could explain the lack of effect on cAMP accumulation is that "induced" receptors are not coupled to G proteins. To indirectly examine this possibility, the density of binding sites for the muscarinic receptor agonist [$^3$H]OXO-M was measured. Because much (65–70%) of the binding of this ligand is eliminated by inclusion of GTP, the density of these sites may provide an estimate of the number of G protein-coupled receptors. Additionally, some investigators (37) have suggested that the binding of [$^3$H]OXO-M represents M2 sites, and data (Fig. 3) indicate that a large (102%) increase in the density of M2 sites occurred following *l*-atropine administration. The density of binding sites for [$^3$H]OXO-M, however, did not increase following the drug treatment (Table 4). This observation is consistent with the hypothesis that the "induced" receptors are not coupled to G proteins and is inconsistent with the hypothesis that [$^3$H]OXO-M binding sites are identical to those defined as M2 using PZ, as in the current study. Thus, it is possible that the induced receptors are coupled less efficiently or not coupled to the adenylyl cyclase response, accounting for the lack of change in the inhibition of cAMP accumulation in the treated tissues. A similar observation has been reported in studies involving *in ovo* treatment of chicken embryos with carbachol, followed by atropine administration to block the effects of the agonist (38), as well as in studies examining the effects of treating cultured embryonic chicken cardiac cells with carbachol and propylbenzilylcholine mustard (39). These studies showed that newly synthesized muscarinic receptors in treated cells did not differ from those in control cells in molecular weight, isoelectric point, or their affinities for both muscarinic receptor agonists or antagonists. These new receptors, however, exhibited diminished sensitivity in agonist-induced

physiological (negative chronotropic effect and $Rb^+$ efflux) and biochemical (inhibition of adenylyl cyclase activity) responses. These responses increased towards the control levels well after the density of receptors had recovered, and the recovery of responses did not require *de novo* protein synthesis.

Chronic treatment with *l*-atropine did not result in a significant change in the density of binding sites for either [$^3$H]QNB or [$^3$H]PZ in the striatum; in addition, chronic *l*-atropine treatment did not cause any significant changes in muscarinic receptor-mediated inhibition of adenylyl cyclase activity examined in dose-response curves to ACH. A potential explanation for this observation is that cholinergic neurons in striatum are normally less active than those in CTX and hippocampus and, thus, blockade of the muscarinic receptors would not result in a significant alteration in input to these receptors. Therefore, an increase in the density of the receptors may not follow chronic blockade. However, this is unlikely to be the case, because the turnover rate of ACH in the striatum has been reported to be high, compared with occipital and limbic cortex (40). If the turnover rate of ACH is used as an estimate of the activity of cholinergic neurons, the striatum would be considered to contain active cholinergic neurons. The striatum has been demonstrated to contain mRNA coding for different subtypes of muscarinic receptors from those found in cerebral CTX and hippocampus (41). Thus, levels of mRNA coding for the m4 receptor are high in the striatum and lower in cortex and hippocampus, whereas the distribution of m1 and m3 receptors is the opposite. Additionally, the innervation to the striatum is different from that to the cortex and hippocampus (42). Thus, most of the cholinergic innervation of the CTX and hippocampus comes from extrinsic sources such as nucleus basalis of Meynert for CTX and medial septum and vertical limb of diagonal band for hippocampus, whereas the cholinergic innervation of the striatum is mainly from intrinsic interneurons. Therefore, muscarinic receptors in the striatum are likely to be different from and have different properties than muscarinic receptors in the CTX and hippocampus. In a study by Boyson *et al.* (43), chronic atropine treatment caused a significant increase (17%) in the density of striatal binding sites for [$^3$H]QNB. The difference between the results of Boyson *et al.* (43) and the current study is likely to be due to daily injection versus sustained release in the delivery of drug. Alternatively, the trend toward an increase in the density of binding sites for [$^3$H]QNB in response to chronic blockade of muscarinic receptors in the current study was slightly smaller in magnitude (7 versus 17%) compared with that in the study of Boyson *et al.* (43) and, with a smaller sample size in the current study (9 versus 20), the change in the density of binding sites for [$^3$H] QNB, if there was one, may not be detected in the current study. In any case, the increase in the density of binding sites for [$^3$H]QNB in response to chronic *l*-atropine administration is much more robust in CTX and DH compared with striatum in rat brain.

One thing that seems clear from these studies is that not all muscarinic receptor subtypes in the central nervous system are regulated similarly. In fact, it appears that some subtype(s) having low affinity for PZ robustly alter their density in response to blockade, whereas at least most of the receptors having high affinity for PZ do not. The fact that PZ lacks the requisite specificity to identify a specific subtype unambiguously means that it remains to be determined exactly which subtypes(s) (m1, m2, m3, m4, m5 etc.) is (are) regulated by this paradigm. New analytical tools, pharmacological or immunological, will be required to examine this question in more detail.

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