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# Age-dependent decrease in the affinity of muscarinic M1 receptors in neocortex of rhesus monkeys

(aging/cerebral cortex/pirenzepine/carbachol/primates)

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ABSTRACT **_In vitro_ autoradiography on tissue sections and receptor assay in cortical membrane homogenates revealed that pirenzepine high-affinity muscarinic sites (M1) decrease in affinity in the prefrontal cortex and in other cortical areas of aged rhesus monkey (*Macaca mulatta*). Carbachol competition experiments detected only a single, low-affinity class of sites in old monkeys, while two classes of sites (low and high affinity) were observed in young adults. The change in affinity in the aged monkeys is not accompanied by a decrease in the density of these sites and, further, the age-related decline in the affinity of the M1 site is reversible. In the presence of $Mg^{2+}$, the M1 muscarinic receptors in the aged monkeys were capable of forming carbachol high-affinity sites. These results provide evidence for age-dependent functional changes in receptor activity in cerebral cortex and indicate that these receptors maintain a degree of plasticity that could be a strategic target for research aimed at treatment of memory disorders in aged humans.**

Memory decline during aging has been extensively documented in rodents (1, 2), nonhuman primates (3, 4), and humans (5). Although the aging process seems to impinge on several neurotransmitter systems, special emphasis has been placed on the role of the cortical cholinergic innervation in geriatric memory dysfunction (6). Studies in rodents have shown a consistent decline in cortical acetylcholine (ACh) synthesis and release during aging (7–9), although little or no change occurs in cortical choline acetyltransferase activity (1, 8, 6, 10). Further, the cholinergic basal forebrain neurons that project to the cortex undergo an age-related decrease in size though their number is apparently not diminished (11).

In the present study we have examined the status of the M1 muscarinic cholinergic receptor in aged rhesus monkey, a species that exhibits behavioral impairment and cortical pathological changes remarkably similar to those observed in elderly humans (4, 6, 12). M1 sites are present in high concentration in the primate neocortex (13, 14) and are involved in cortical cholinergic excitation (15). However, little is known about changes in these cortical receptors with age. In aged rats, there have been conflicting reports of decrease (16) or no change (17) in binding-site density. Only two recent studies have examined the status of ACh receptors in monkey cortex during aging, and both found a decrease of nicotinic and muscarinic receptors, mainly in temporal (18, 19) and parahippocampal (18) cortex. Neither study found any significant decreases in the affinity of binding sites in aged monkey cortex.

In the present study we confirm that the number of M1 sites does not change with age but we provide evidence for changes in the molecular structure of the cortical receptor or of the coupled effector systems able to alter the affinity of these sites. To obtain this evidence, we studied the binding of the highly selective compound pirenzepine (PZ) (20) to the M1 sites in nine distinct areas of monkey cortex both in membrane homogenates and in tissue sections examined by *in vitro* autoradiography. Our experimental design allowed determination of several parameters of [$^3$H]PZ binding, including the distribution, number of sites, and affinity.

## METHODS

**Tissue Preparation.** The cerebral hemispheres of four adult (two males and two females, 3–7 years of age) and four aged (three males and one female, 20–30 years of age) rhesus monkeys (*Macaca mulatta*) were used. Brains were prepared by published procedures (21). Briefly, the animals were deeply anesthetized and perfused with ice-cold phosphate-buffered saline followed by a weak (0.1%) paraformaldehyde perfusate containing increasing concentrations of sucrose. The brains were rapidly removed, blocked, and immersed in isopentane at −80°C for 4 min before storage at −80°C.

**Binding Assay.** The PZ high-affinity binding sites (M1) were labeled with [$^3$H]PZ (specific activity, 87 Ci/mmol; 1 Ci = 37 GBq) in 10 mM phosphate buffer (pH 7.4) (22). The ability of [$^3$H]PZ to bind to one site (M1) was evaluated by saturation and competition experiments. Samples taken from an adult monkey brain were homogenized in ice-cold 10 mM phosphate buffer/10 mM EDTA (pH 7.4) with a Brinkmann Polytron PT10. Homogenates were centrifuged twice at 20,000 × *g* for 10 min with an intermediate rehomogenization in fresh buffer. Aliquots of 100 μl (1:50, wt/vol) of homogenates were incubated at 25°C for 60 min in the presence of 12 concentrations (range 0.05–30 nM) of [$^3$H]PZ in the same buffer (total volume, 500 μl) plus 1 mM EDTA (23). The specific binding was assessed as the excess over blanks containing 10 μM atropine sulfate. Each point was measured in triplicate. The inhibition of [$^3$H]PZ (3 nM) specific binding was examined in the presence of 14 concentrations (50 pM to 1 mM) of methoctramine (R.B.I., Natick, MA), *p*-fluorohexahydrosiladiphenidol [HHSiD, 1-cyclohexyl-1-(*p*-fluorophenyl)-4-piperidino-1-silabutan-1-ol; gift from H. Ladinsky, Institute De Angeli, Milan, Italy], and PZ (Sigma). The incubation was terminated by rapid filtration and three washings with 3 ml of ice-cold 10 mM phosphate buffer (pH 7.4) through Whatman GF/B filters in a Brandell M-242 cell harvester. The radioactivity trapped in the filters was measured in a Packard 3320 liquid scintillation counter with Opti-Fluor (Packard) as the scintillator. The competition experiments with the agonist carbachol (R.B.I.) were performed separately in each animal. Samples of prefrontal cortical membranes taken from the same areas used in the autoradiographic investigations were processed as described

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Abbreviations: ACh, acetylcholine; ANOVA, analysis of variance; HHSiD, *p*-fluorohexahydrosiladiphenidol; PZ, pirenzepine.

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above and incubated in 10 mM phosphate buffer/1 mM EDTA with 3 nM [$^3$H]PZ and 14 different concentrations of carbachol (100 mM to 100 nM). Some experiments were carried out in the presence of $MgCl_2$ (10 mM). At the end of the incubation, the samples were filtered for scintillation counting as described above. Protein concentration was determined by the Pierce BCA assay.

**Receptor Autoradiography.** Once the binding parameters for [$^3$H]PZ were established, quantitative autoradiography of the M1 receptors in the prefrontal cortex and in other cortical areas of adult and aged animals was carried out. Brains were sectioned (20 $\mu$M thick) on a Bright cryostat and mounted on acid-cleaned, chrom alum-soaked slides. For the assay, sections were preincubated in 10 mM phosphate buffer/10 mM EDTA, pH 7.4 for 20 min on ice. The labeling with [$^3$H]PZ was done at room temperature in 10 mM phosphate/1 mM EDTA, pH 7.4 using seven to nine concentrations of the labeled compound (0.3–36 nM). Each point was labeled in triplicate. Nonspecific binding was determined in the presence of 10 $\mu$M atropine sulfate. After 1 hr of incubation the sections were rinsed, dried, and exposed to $^3$H-sensitive Ultrofilm for 1 week. Following film exposure, the sections were stained with cresyl violet for identification of cortical layers on the corresponding autoradiographic images. The intensity of receptor binding in tissue areas and layers was analyzed with a computer imaging system (21).

**Statistical Analysis.** The results were analyzed by a nonlinear curve-fitting computer program (EBDA/LIGAND, Elsevier Biosoft, Cambridge, U.K.); one- or two-site model fitting was used. Statistical analysis of the data was performed by one-way analysis of variance (ANOVA) and Student $t$ test.

## RESULTS

**[$^3$H]PZ Binds to a Class of High-Affinity Sites (M1).** The saturation experiments showed that [$^3$H]PZ binds to the muscarinic receptors with high affinity (Fig. 1). The Scatchard plot for five experiments carried out in triplicate gave a $K_d$ of 3.4 ± 0.2 nM and $B_{max}$ of 780 ± 71 fmol/mg of protein. The Hill coefficient, 1.0 ± 0.1, indicates that only one class of site was detected. Competition studies with nonradioactive PZ, HHSiD, and methoctramine confirmed these results, since the Hill coefficient did not differ from 1 for any of the competitors (Table 1). When nonradioactive PZ was used, the apparent $K_i$ had almost the same value (3.3 ± 0.1 nM) as

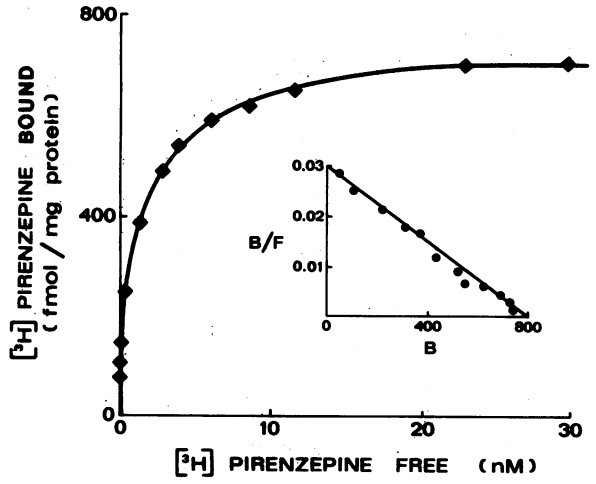


FIG. 1. Specific binding saturation curve of [$^3$H]PZ in prefrontal cortex of adult monkey. Data are expressed as the average of triplicate determinations in a representative experiment. (*Inset*) Scatchard replot of the binding data. B = fmol of [$^3$H]PZ bound; F = free [$^3$H]PZ concentration (nM).

Table 1. Competition of nonradioactive drugs for [$^3$H]PZ specific binding sites in monkey cortex

| Competitor | $IC_{50}$, nM | Apparent $K_i$, nM | Pseudo Hill coefficient |
|---|---|---|---|
| PZ | 28 ± 2.0 | 3.3 ± 0.1 | 1.0 ± 0.2 |
| HHSiD | 157 ± 23 | 18.7 ± 2.4 | 1.1 ± 0.2 |
| Methoctramine | 129 ± 10 | 15.2 ± 1.3 | 1.0 ± 0.1 |

Cortical membranes were incubated with 22 nM [$^3$H]PZ in the presence of various concentrations (total number, 14) of competitor. Data are the average ± SE of two experiments for each competitor carried out in triplicate.

the $K_d$ found in the saturation experiments (3.4 nM), indicating a selective interaction with high-affinity putative M1 sites (22).

**Distribution of M1 Muscarinic Receptors in Cortical Laminae by Quantitative Autoradiography.** The distribution of M1 receptors in a variety of prefrontal areas (Brodmann's areas 46, 9, 12, and 25) displayed a characteristic pattern (Fig. 2). Although all the layers were rich in M1 receptors, the highest concentration was in layers II, IIIa, IIIb, and IV, while the lowest density was in layers I, V, and VI. A similar pattern was observed in the posterior association cortex, area 18. In primary sensorimotor cortex, the M1 receptors showed a different pattern, with a preferential distribution in the upper layers I, II, and IIIa and a progressive decrease in receptor density in the deepest strata. The repeated-measures ANOVA in association cortex and in primary sensorimotor cortex showed a highly significant $B_{max}$-by-layers as well as $K_d$-by-layers interaction. In association areas, polynomial contrasts showed a significant quadratic component. In sensorimotor cortex, the test for trend showed a significant linear component rather than a quadratic component (see legend to Fig. 2 for statistical data). The distribution of the values among the layers was complex and two populations of affinity could be identified: one population with higher affinity in the three upper layers and one with lower affinity in the deepest layers. Taken together, these results show a positive correlation between receptor number and affinity in adult monkeys in both association and primary cortices, so that the layers with the highest receptor number correspond to the layers with the highest affinity and vice versa. The aged monkeys showed a similar pattern of receptor distribution as well as a significant $B_{max}$-by-layers (but not $K_d$-by-layers) interaction in the prefrontal cortex, in the two occipital areas, and in sensorimotor cortex (data not shown).

**Effect of Aging on [$^3$H]PZ Binding in Prefrontal Cortex and Other Cortical Areas Measured by Quantitative Autoradiography.** The density and the affinity of [$^3$H]PZ binding to the M1 receptors in the prefrontal areas are shown in Table 2. Statistical analysis showed a significant decrease in PZ affinity in prefrontal cortex of aged monkeys when the ANOVA and Student $t$ test were applied to the logarithms (24) of the $K_d$ values of the two groups of animals. Fig. 3 shows the [$^3$H]PZ specific binding in layer IIIb of area 46 in young adult and old animals in the presence of a saturating concentration of the ligand. The difference in binding course between the two groups indicates a decrease in affinity rather than a change in receptor number with aging, since the same level of maximal binding was reached by the two groups of animals. The distribution of PZ affinity in aged monkeys was similar among the cortical layers and among the four prefrontal areas examined. To determine whether the age-relevant changes in affinity were specific to prefrontal association cortex, [$^3$H]PZ binding was investigated in striate (area 17) and extrastriate (area 18) cortices, in the motor cortex (area 4), and in the somatosensory cortex (areas 1–3) of some adult (three animals) and some aged (two animals) monkeys. Interestingly, the age-related change in PZ affinity

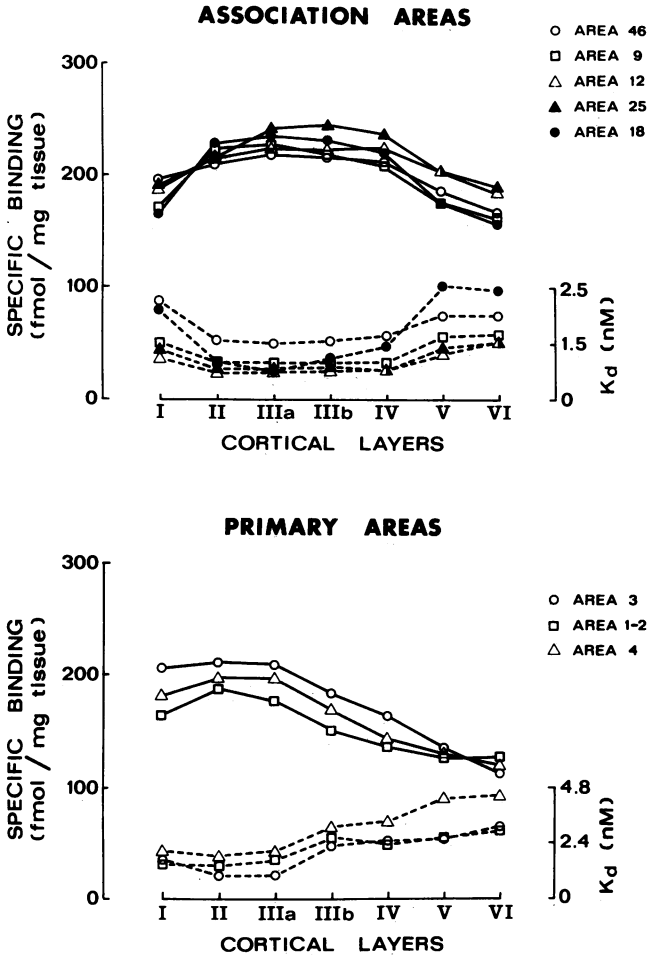


FIG. 2. Laminar distribution of [$^3$H]PZ specific binding and affinity ($K_d$) in association (*Upper*) and primary (*Lower*) cortices in adult monkeys. The M1 receptor pattern in the association areas shows the highest specific binding in cortical layers II, IIIa, IIIb, and IV. In the primary areas, instead, PZ specific binding predominates in cortical layers I, II, and IIIa. A complementary pattern was observed for the $K_d$ value distribution along the laminae, in both groups of area. The standard errors, ranging between 2% and 8%, have been omitted for clarity. One-way ANOVA showed significant quadratic components in association areas. Area 46: $B_{max}$, $F(1,3) = 51.8$, $P < 0.006$; $K_d$, $F(1,3) = 11.3$, $P < 0.05$. Area 9: $B_{max}$, $F(1,3) = 17.4$, $P < 0.03$; $K_d$, $F(1,3) = 17.4$, $P < 0.03$. Area 12: $B_{max}$, $F(1,3) = 23.7$, $P < 0.02$; $K_d$, $F(1,3) = 115.6$, $P < 0.002$. Area 25: $B_{max}$, $F(1,3) = 234.4$, $P < 0.001$; $K_d$, $F(1,3) = 28.1$, $P < 0.02$. In primary areas the one-way ANOVA showed significant linear components. Area 4: $B_{max}$, $F(1,2) = 14.8$, $P < 0.06$; $K_d$, $F(1,2) = 20.4$, $P < 0.05$. Area 3: $B_{max}$, $F(1,2) = 18.6$, $P = 0.05$; $K_d$, $F(1,2) = 78.1$, $P < 0.02$. Areas 1 and 2: $B_{max}$, $F(1,2) = 15.6$, $P < 0.06$.

was also seen in some of these other neocortical areas. Motor and somatosensory cortices, in fact, showed a significant decrease in PZ affinity. In striate and extrastriate areas the difference in affinity was not significant. Further, one of the two old monkeys studied showed a constant decrease in receptor number in somatosensory and visual cortex (but not in prefrontal and motor areas). This monkey was the oldest (about 30 years) among the aged animals investigated.

**Loss and Reversibility of High-Affinity Carbachol Binding Sites in Aged Monkeys: Effect of Magnesium.** Because of the homogenous decrease in PZ affinity in prefrontal cortex of aged monkeys, we decided to study agonist binding in membrane homogenates prepared from this cortex. Inhibition of [$^3$H]PZ specific binding to the M1 receptors by carbachol in adult monkeys confirmed the existence of two different affinity states for the agonist (25). The percentage of

Table 2. Maximal binding ($B_{max}$) and dissociation constants ($K_d$) of the high-affinity PZ site (M1) in several areas of prefrontal monkey cortex

| | Adult | | Aged | |
|---|---|---|---|---|
| Area(s) | $K_d$ | $B_{max}$ | $K_d$ | $B_{max}$ |
| 9 | 1.3 ± 0.3 | 199 ± 6.5 | 5.6 ± 1.3‡ | 194 ± 19 |
| 12 | 1.2 ± 0.1 | 206 ± 2.4 | 5.8 ± 0.7† | 200 ± 12 |
| 25 | 1.2 ± 0.2 | 213 ± 14 | 5.0 ± 0.4* | 209 ± 11 |
| 46 | 1.7 ± 0.1 | 228 ± 5.8 | 7.2 ± 0.7* | 212 ± 12 |
| 4 | 3.0 ± 0.6 | 145 ± 19 | 12.9 ± 4.3§ | 172 ± 23 |
| 3 | 1.9 ± 0.4 | 179 ± 19 | 7.1 ± 0.05§ | 133 ± 15 |
| 1 and 2 | 2.0 ± 0.6 | 157 ± 22 | 11.8 ± 0.4§ | 126 ± 10 |
| 17 | 1.9 ± 0.6 | 196 ± 3.5 | 5.8 ± 2.4 | 168 ± 33 |
| 18 | 1.6 ± 0.3 | 199 ± 3.5 | 5.3 ± 2.1 | 167 ± 11 |

$K_d$ and $B_{max}$ are the average of single values from each layer. The decrease in affinity observed in aged monkey cortex was statistically significant in all four prefrontal areas (*, $P < 0.001$; †, $P < 0.002$; ‡, $P < 0.04$) and in the areas 4, 3, 1 and 2 (§, $P < 0.05$). The difference in the occipital cortex did not reach significance. No significant difference in number of sites was observed between the two groups of animals in any prefrontal areas and in motor cortex. The decrease in receptor sites in visual and somatosensory cortices was due to one of the two aged monkeys that showed a constant decrease in site number in these areas (though not in the frontal lobe).

high-affinity sites (24 ± 1.8%, Fig. 4) compares well with reported values (23, 25). In the aged monkey, however, only one class of low-affinity site was seen in the presence of the agonist (in two aged monkeys, the two-site model slightly improved the fit compared with the one-site model, but only 5% of the total sites showed high affinity for carbachol).

When $MgCl_2$ (10 mM) was added to the incubation buffer, two states of affinity, low and high, were detectable in tissue homogenates of both the young adult and the aged animals. In the young adults, the percentage of high-affinity sites for the agonist (27 ± 2.5%) did not change significantly. However, the $IC_{50}$ for both sites was significantly decreased. In the aged animals, in addition to the evidence for the high-affinity class of sites, which represented 24 ± 0.9% of the total sites, the $IC_{50}$ for the low-affinity sites was also significantly decreased.

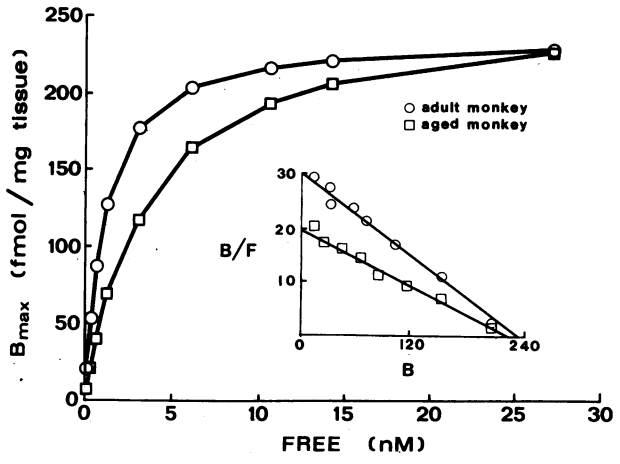


FIG. 3. Specific binding of [$^3$H]PZ in layer IIIb of area 46 of adult and aged monkeys. Each point represents the average of the binding values obtained at each concentration in any single animal. The binding curve of the adults is steeper and the specific binding sites are saturated at lower ligand concentrations. In the old monkeys, a similar level of saturation is reached at higher concentrations of [$^3$H]PZ. This result implies a difference in affinity of these sites for the ligand between the two age groups. (*Inset*) Scatchard replots of the binding data. SE for each point was ≤10%.

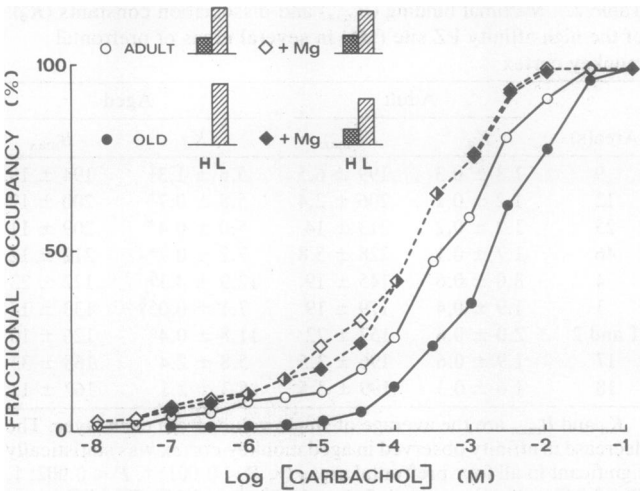


FIG. 4. Distribution of high- and low-affinity binding sites in EDTA buffer, in adult (○) and aged (●) monkeys. The ordinate measures the percent inhibition of [$^3$H]PZ binding by carbachol. Each point is the mean of the values obtained in every animal (SE was <10%). In the younger animals the carbachol competition curve did not follow a normal bimolecular interaction and the best fit was observed when a two-site model was applied. The $IC_{50}$ values for the two theoretical isotherms were 1.1 ± 0.03 mM and 11 ± 2.5 μM for low- and high-affinity site, respectively. In the old monkeys, the two-site model did not improve the fitting compared with the one-site model, and all of the receptors were considered to be in a low-affinity state with $IC_{50}$ = 1.2 ± 0.6 mM. Thus, the high-affinity binding sites for the agonist were absent from the old animals. In the presence of $Mg^{2+}$ (10 mM) (dashed lines), two (low and high) affinity sites were detected in both young adult (◇) and old (◆) monkeys. The $IC_{50}$ values for the two theoretical isotherms were 114 ± 64 μM and 2.2 ± 0.85 μM in the young adult monkeys and 196 ± 58 μM and 4.0 ± 2.1 μM in the old animals respectively. The $IC_{50}$ values for low-affinity sites in young adult and old monkeys and the $IC_{50}$ for the high-affinity site in the young adults were significantly decreased in the presence of $Mg^{2+}$ ($P < 0.02$ and $P < 0.05$, respectively). (*Inset*) Estimated proportions of high (H)- and low (L)-affinity sites.

## DISCUSSION

**Laminar Distribution and Area Specificity of M1 Receptors.** *In vitro* receptor autoradiography in monkey neocortex demonstrates a distinct laminar organization of M1 receptors with a prevalence of these sites in the upper layers both in association and primary cortices. This distribution has been observed in rat (26), monkey (27), and human (14) and is shared by many other neurotransmitter receptors (28), appearing to be correlated with a possible modulatory role in the cortex. In fact, the upper layers are rich in dendritic processes of pyramidal cells and are the site for the majority of cell–cell interactions within the cortex (see ref. 29 for review). Moreover, the highest M1 receptor concentration is found in the prefrontal cortex with a peak in area 46 and in areas 17 and 18, the primary and secondary visual areas, of the occipital lobe (see Tables 1 and 2). A lower concentration of receptors is present in sensorimotor cortex. The relatively low M1 receptor density in these areas reported in a previous study (13) may be explained by the different methodology used to identify this class of receptors. In this latter study the M1 receptors were labeled with [$^3$H]quinuclidinyl benzilate (QNB) in the presence of carbachol to occupy the M2 sites.

**Effect of Aging on PZ High-Affinity Binding Sites.** A major finding of the present study is that aging decreases the affinity of the M1 receptor subtype for the highly selective antagonist PZ in the neocortex of rhesus monkey. Further, in agreement with previous reports (18, 19), aging does not necessarily significantly affect the M1 cortical receptor density in cortex. Of course, alterations in affinity may presage later loss of sites as suggested by the decrease in M1 sites in the oldest monkey of the present study.

A recent study of PZ binding in the cortex of aged rats showed a modest decrease in M1 sites without changes in affinity (8). The discrepancy between those data and our results could depend on several factors. Among them, the use of a high-ionic-strength buffer containing $Mg^{2+}$ could have affected the receptor conformation and masked a potential difference in affinity between young and old rats. Studies in primates and humans have reported an age-related decline in the density of nicotinic receptors in human cortex (30) and a decrease in oxotremorine M high-affinity binding sites and nicotinic sites in temporal cortex of aged rhesus monkey (18, 19).

Taken together the results of previous studies seem to indicate that aging primarily affects receptor number ($B_{max}$) rather than receptor affinity ($K_d$). On the other hand, in these prior investigations, the use of nonselective ligands (1, 16, 17) and the prevalent use of a single concentration of ligand (18, 19) make it difficult to differentiate between changes in affinity and maximal binding. The importance of a full saturation analysis of the binding sites is shown in Fig. 3. The use of only a single concentration, corresponding to one of the points in the saturation curve shown, could lead to the erroneous conclusion that there was a change in $B_{max}$.

In the present study we found a positive correlation in adult monkeys between M1 receptor number and PZ affinity so that the upper cortical layers, richer in receptors, show higher affinity. Indeed the M1 receptor concentration in these layers (245 ± 35 fmol/g of tissue) is among the most concentrated compared with monoaminergic receptors, at least in primate prefrontal cortex (28). Further, the distribution of choline acetyltransferase immunohistochemistry in monkey neocortex, particularly in the primary cortices, corresponds closely to the distribution reported here in M1 receptor density (31). The majority of cortical–cortical connections originate specifically in the more superficial layers II and III (32). Our data suggest that the upper layers are the main target of the cholinergic input to the cortex, and a greater quantity of ACh could be synthesized and released in these strata. Possibly the higher levels of the neurotransmitter in these layers even in the young adult monkey may be responsible for a different affinity state of the receptor. That muscarinic agonists are capable of inducing different conformational changes in receptors and in the distribution of the high- and low-affinity forms is well established (33). We observed that the positive correlation between receptor number and affinity present in young monkeys disappeared in the aged animals, with a relatively greater impairment in the high-affinity receptors located in the upper layers. Reduced levels of the endogenous agonist in the intersynaptic space could be responsible for changes of conformation/orientation of the receptor and its affinity. Impairment in the ability to synthesize and release ACh has been consistently reported in aged rodent cortex (e.g., refs. 7–9). Electrophysiological experiments in the hippocampus of old rats showed impairment in neuronal response to local application of ACh (but not of glutamate) without consistent changes in receptor number (1). Chronic treatment with the cholinergic agonist or antagonist induced changes in receptor number (down- and up-regulation, respectively) in the frontal cortex of young but not aged mice (34). On the other hand, modifications in receptor structure could be secondary to the changes in membrane properties reported in aging. Increases in cholesterol/phospholipid ratio and sphingomyelin content in the membrane as well as increases in membrane viscosity have been observed in the brains of aged rodents (35). Therefore, variations in membrane structure and/or synaptic deficit of ACh concentration due to the aging process could induce alteration in the local microenvironment and, in turn, a

change in receptor conformation and mobility that would modify the antagonist affinity.

**Plasticity of Muscarinic Receptor to Form the High Affinity Agonist Binding Site During Aging.** Competition experiments of [$^3$H]PZ specific binding with carbachol showed that aging selectively impairs the ability to form the high-affinity agonist binding site in primate cerebral cortex. A selective involvement of the high-affinity agonist binding site of receptors during aging has also been described for cardiac muscarinic receptors in rat (36) and adrenergic receptors in human circulating leukocytes (37). The affinity change suggests a decrease in the coupling efficiency between the receptor and the guanine nucleotide-binding protein (G protein). At least two states of affinity have been described for the agonist (23, 25). The percentage of the different affinity states (high and low) depends on the proportion of receptors coupled to the G protein (38). When the system is coupled, the affinity for the agonist increases and it represents the functionally active state of the receptor (23). GTP and GTP analogues decrease agonist affinity, uncoupling the receptor from the G protein. $Mg^{2+}$ (and other divalent cations) increases agonist affinity, thereby promoting receptor–G protein coupling (38). When $Mg^{2+}$ (10 mM) was added to the EDTA buffer, carbachol detected two sites in the cortex of both adult and old monkeys. Further, the $IC_{50}$ values for high- and low-affinity sites were consistently decreased in both groups as well. The presence of EDTA in our experiments removes a considerable proportion of the endogenous divalent cations bound to the membranes that are thought to modulate agonist affinity, particularly of the high-affinity site, through a cation binding site (39). In the heart, $Mg^{2+}$ induces an increase of the high-affinity site only in EDTA pretreated membranes. In the brain, the cation's effect is seen independently of EDTA treatment (39). Moreover, in the brain, but not in the heart, the effect of the GTP nucleotide is observed only in the presence of $Mg^{2+}$ and is much smaller than in the heart. These data indicate that the affinity of the cation binding site is lower in the brain than in the heart. Nevertheless, the receptor complex is very sensitive to the presence of divalent cations in the central nervous system. It has been suggested that, in physiological conditions, $Mg^{2+}$ may occupy the sites involved in the coupling process (39).

The loss of the high-affinity component of the M1 receptor in old monkeys could depend on decreased affinity of the cation binding site and/or inability to access the site by the cation. In turn, it could be that the coupling process does not depend on the divalent cation in the adult but, following conformational age-dependent changes, it becomes dependent on the divalent cation during aging.

The effect of $Mg^{2+}$ in our experiments shows that the receptor impairment induced by aging is reversible, and the possibility that $Mg^{2+}$ could improve receptor function *in vivo* needs to be explored. Increasing concentrations of $Mg^{2+}$ in the bathing medium have been shown to reverse the decrease in magnitude of the "frequency potentiation" phenomenon in hippocampal slices of aged rats to the level recorded in young rats (40). Furthermore, although no change in $Mg^{2+}$ concentration in cerebrospinal fluid has been found during aging, and the $Mg^{2+}$ levels in the brain's extracellular space is tightly regulated by barrier and transport mechanisms, a $Mg^{2+}$-enriched diet, able to increase the blood level of this cation about 2%, prevented an impairment in the retention of an active avoidance test observed in old rats (40) and significantly improved the frequence potentiation response in urethane-anesthetized old rats.

These findings suggest that the plasticity of the high-affinity M1 site may be a target for therapeutic strategies in the treatment of age-related memory decline and possibly in senile dementia of the Alzheimer type.

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