Laminar Organization and Age-Related Loss of Cholinergic Receptors in Temporal Neocortex of Rhesus Monkey

Molly V. Wagster,^{1,4} Peter J. Whitehouse,⁵ Lary C. Walker,^{1,4} Kenneth J. Kellar,⁶ and Donald L. Price^{1–4}

Departments of ¹Pathology, ²Neurology, and ³Neuroscience, and the ⁴Neuropathology Laboratory, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21218, ⁵University Hospitals of Cleveland, Alzheimer Center, Cleveland, Ohio 44106, and the ⁶Department of Pharmacology, Georgetown University School of Medicine, Washington, D.C. 20057

Using *in vitro* receptor autoradiography, the distributions of cholinergic muscarinic [³H-N-methyl scopolamine (NMS), ³H-pirenzepine (PZ), and ³H-oxotremorine-M (OXO-M)] and nicotinic [³H-acetylcholine (ACh)] receptors were mapped in the temporal cortices of rhesus monkeys (*Macaca mulatta*) ranging from 2–22 years of age. Although high-affinity ³H-PZ, low-affinity ³H-NMS binding (M1 sites) and high-affinity ³H-OXO-M, high-affinity ³H-NMS binding (M2 sites) occurred across all layers of the temporal neocortex, the laminar distribution of M1 and M2 receptor binding sites was different. M1 muscarinic receptor binding was concentrated in layers II and III, whereas M2 muscarinic receptor binding was greatest in layers IV and V. The concentration of both muscarinic (M1 and M2) and nicotinic receptor binding sites declined with increasing age, and decrements were uniform across all cortical layers. This investigation provides evidence for a decrease in cholinergic receptor binding with age in temporal cortices of rhesus monkeys. Moreover, these changes appear to precede previously reported age-associated memory deficits and neuropathological changes that occur in this species.

Age-related declines in cognitive function occur in humans (Horn, 1975; Osborne et al., 1982; Botwinick, 1984; Rowe and Kahn, 1987), nonhuman primates (Bartus et al., 1978; Davis, 1978; Bartus and Dean, 1979; Presty et al., 1987), and rodents (Gold and McGaugh, 1975; Goodrick, 1975; Lippa et al., 1980; Strong et al., 1980; Dean et al., 1981; Ingram et al., 1981; deToledo-Morrell and Morrell, 1985). One component of such memory disorders appears to be dysfunction of cholinergic systems (Bartus et al., 1982). Some investigations of rodents have revealed significant decreases in the concentration of muscarinic receptor binding with age in the whole brain (Freund, 1980) and in the

neocortex (James and Kanungo, 1976; Waller and London, 1983; Pedigo et al., 1984; Bieganski et al., 1989). However, other studies have reported no changes in the concentration of muscarinic receptor binding in cortices of aged rodents (Morin and Wasterlain, 1980; Dravid, 1983). In humans, the pattern of age-related alterations of cholinergic receptors is uncertain. A significant decrease in the concentration of muscarinic receptor binding has been shown in frontal cortices of aged humans (White et al., 1977; Perry et al., 1980). However, a study of 19 regions of the human brain found no significant differences in the concentration of muscarinic receptor binding with age in any areas (Davies and Verth, 1978). Furthermore, significant alterations in the concentration of muscarinic receptor binding were not demonstrated in temporal cortices of humans 50–97 years of age, though a trend was detected for decreased binding with age (Allen et al., 1983). More recently, a trend was demonstrated for neocortical nicotinic receptors to decrease with age in humans (Whitehouse et al., 1988).

Continuing advances in the identification of subtypes of ACh receptors permit the clarification of the roles of these receptor subtypes in aging and in age-related disease. Three subtypes of muscarinic cholinergic receptors have been defined based on affinities for agonists (Birdsall et al., 1978). Although it was originally believed that antagonists bound to a single muscarinic receptor, the muscarinic antagonist PZ has been used to define 2 subtypes of antagonist binding sites in homogenate and autoradiographic binding studies (Hammer et al., 1980). Evidence for 5 different genes coding for muscarinic receptors in humans has been shown (Bonner et al., 1987, 1988). Following Mash et al. (1985) and others, we will use “M1” to define low-affinity agonist, high-affinity PZ sites and “M2” to define high-affinity agonist, low-affinity PZ sites.

In this study, the binding of cortical muscarinic and nicotinic receptors was examined by *in vitro* autoradiography in temporal neocortices of rhesus monkeys 2–22 years of age. This approach has several advantages: serial sections of brains of individual animals can be processed for a variety of receptor ligands, concentrations of binding sites can be assessed by semiquantitative methods, and the distribution of ligand binding sites can be related to cortical laminae.

Materials and Methods

Subjects

Animals were grouped according to the following mean ages: Group I (2 years of age, $n = 5$), Group II (6 years of age, $n = 2$), Group III (13 years of age, $n = 2$), and Group IV (20 years of age, $n = 7$). Only 5 animals in Group IV were used for studies of ³H-OXO-M autoradiog-

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Correspondence should be addressed to Molly V. Wagster, Ph.D., Neuropathology Laboratory, 509 Pathology Building, The Johns Hopkins University School of Medicine, 600 North Wolfe Street, Baltimore, MD 21205-2181.

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raphy. Animals were restrained with ketamine (10 mg/kg, i.m.) and anesthetized deeply with α -chloralose (40 mg/kg, i.v.), and the brains were removed. A 5-mm block was dissected from the caudal part of the left temporal lobe of each brain and frozen immediately on dry ice. Tissues were frozen (-80°C) until sectioned for autoradiography. Frozen tissue was serially sectioned (10 μm) on a cryostat (-20°C), thaw-mounted on chrome-alum-coated glass slides, and stored (-20°C) until used.

Autoradiography

High- and low-affinity muscarinic binding sites. For assessment of total binding to muscarinic receptors, slide-mounted tissue sections were incubated for 60 min (room temperature) in PBS (pH, 7.4) that contained 1 nM ^3H -NMS (Wamsley et al., 1980). Adjacent sections were incubated either in PBS with 1 nM ^3H -NMS with 100 μM unlabeled carbachol added to displace high-affinity agonist sites, or in PBS with 1 nM ^3H -NMS plus 1 μM unlabeled atropine to displace all muscarinic binding sites. All tissue sections were washed twice for 5 min each in ice-cold PBS to remove unbound radioactive ligand and were dipped in distilled water to remove buffer salts. Sections were dried under a stream of cool, dry air. Autoradiograms were generated by apposing sections to ^3H -sensitive film (Ultrofilm, LKB, Sweden) in the dark for 4 weeks.

High-affinity muscarinic binding sites (M2). Slide-mounted tissue sections were incubated in 20 mM HEPES-Tris buffer (pH, 7.5) plus 10 mM Mg^{2+} and either 1 nM ^3H -OXO-M alone or 1 nM ^3H -OXO-M plus 1 μM atropine sulfate (Spencer et al., 1986). After the 30-min incubation (30°C), slides were taken through 3 2-min washes in ice-cold buffer with a final dip in distilled water. Sections were dried as described previously and stored with ^3H -sensitive film for 12 weeks.

High-affinity muscarinic binding sites (M1). Slide-mounted sections were preincubated for 30 min (25°C) in Krebs phosphate buffer (pH, 7.4), then incubated for 60 min (25°C) in buffer with either 20 nM ^3H -PZ alone or 20 nM ^3H -PZ plus 1 μM atropine (Yamamura et al., 1983). Slides were then washed in ice-cold buffer for 5 min followed by a 1-min rinse in ice-cold distilled water. Sections were dried as described previously and stored adjacent to ^3H -sensitive film for 3 weeks.

Nicotinic binding sites. These receptor sites were labeled by incubation with ^3H -ACh with muscarinic receptor sites blocked by atropine (Schwartz et al., 1982). Tissues were preincubated for 10 min (room temperature) in 50 mM Tris HCl buffer with 120 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , and 1 mM MgCl_2 (pH, 7.4), followed by an additional 5 min in fresh buffer solution. This procedure was followed by a third wash in buffer for 10 min (room temperature) with the addition of the cholinesterase inhibitor diisopropyl fluorophosphate (100 μM) to prevent the hydrolysis of ACh. Slide-mounted tissue sections were then incubated (45 min) in buffer (pH, 7.4; 4°C) that contained either 6 nM ^3H -ACh plus 1.5 μM atropine to assess total nicotinic binding or 6 nM ^3H -ACh plus 100 μM carbachol and 1.5 μM atropine to assess nonspecific binding. Sections of tissue were taken through a 1-min buffer wash (0°C), followed by 2 30-sec washes (4°C) and 2 rinses in distilled water (0°C). Sections were dried as described previously and apposed to ^3H -sensitive film for 12 weeks.

Analysis of autoradiograms

Autoradiograms were quantified with a computerized image-analysis system (Loats Associates, Inc., Westminster, MD). Optical densities were converted to equivalent disintegrations/min/mg of methacrylate standard (autoradiographic ^3H microscaler, Amersham, Arlington Heights, IL; Unnerstall et al., 1982). Readings were taken from the inferior bank of the superior temporal sulcus of each animal. Binding levels in cortical laminae were assessed by using the computer for the integration of structural and functional information as follows: tissue sections processed previously for autoradiography were stained with Luxol-Fast blue/Cresyl violet for the accurate visualization of cortical cytoarchitecture; images of stained sections were placed in registration with their corresponding digitized autoradiographic images on the image-analysis system; a digitized image was made of the properly oriented, stained section; and the cortical layer of interest was delineated by a cursor on the slide image and quantitated simultaneously from the same layer on the autoradiogram. No correction was made for quenching of ^3H ligands by myelin.

Data on cortical layers were analyzed by a repeated measures analysis of variance (ANOVA) with age as the group factor and cortical layer as the within factor. Cholinergic receptor binding in the whole temporal

cortex was analyzed by a one-way ANOVA. Post hoc analyses were performed using the Newman-Keuls test for comparisons of individual age group means. Because images were too light to lend themselves to accurate registration for measurements of cortical layers, ^3H -ACh binding for nicotinic receptor sites was limited to analyses of only the whole temporal cortex. The dependent variable in all analyses was the concentration of receptor binding sites, expressed as 1×10^{-15} mol/mg standard.

Results

Distributions of receptors in cortical laminae

In all animals, the distributions of M1 and M2 receptor binding showed consistent variations in the laminae of temporal neocortex. Binding was significantly different among cortical layers for all muscarinic ligands examined: low-affinity ^3H -NMS binding ($F(5, 60) = 35.44$, $p < 0.01$), ^3H -PZ binding ($F(5, 60) = 51.11$, $p < 0.01$); high-affinity ^3H -NMS binding ($F(5, 60) = 4.57$, $p < 0.01$); ^3H -OXO-M binding ($F(5, 50) = 45.05$, $p < 0.01$). M1 binding (low-affinity ^3H -NMS, high-affinity ^3H -PZ) was highest in layers II and III and lowest in layer VI (Fig. 1). Conversely, M2 binding (high-affinity ^3H -NMS, high-affinity ^3H -OXO-M) was highest in layers IV and V and lowest in layer I (Fig. 1). ^3H -ACh binding to nicotinic receptors was not quantified for cortical layers.

Age-associated changes in receptor densities

For all ligands examined, an age-related decline in concentrations of receptor binding was observed in temporal neocortices of rhesus monkeys. Low-affinity ^3H -NMS binding for M1 receptors was reduced in the whole cortex by 32% in 20-year-old animals as compared to 2-year-old animals ($F(3, 12) = 8.38$, $p < 0.01$; Table 1, Fig. 2). ^3H -OXO-M binding for M2 receptors was significantly reduced with age in the whole temporal cortex ($F(3, 10) = 32.25$, $p < 0.01$; Table 1, Fig. 2). There was a trend for reductions in ^3H -PZ binding (for M1 receptors) and for high-affinity ^3H -NMS binding (M2 receptors) with increasing age (Table 1, Fig. 2). The ^3H -ACh binding for nicotinic receptors decreased 71% in the temporal neocortex by 6 years of age; this decrease was maintained throughout the age range studied (Table 1, Fig. 2). Significant age-by-layer interactions were not observed, except in the case of ^3H -OXO-M binding ($F(15, 50) = 5.34$, $p < 0.01$). The difference in ^3H -OXO-M binding concentration between layers V and VI was greater for 2-year-old animals than for any other age group (Fig. 1). However, in general, the phenomenon of decreased muscarinic receptor binding concentration with age occurred across the temporal neocortex rather than in discrete layers.

Numerous studies of cholinergic receptors in aged rodent and primate neocortices in which nicotinic or muscarinic binding has been examined show an age-related decline in actual number of sites (B_{max}) with no change in receptor affinity (K_D) (Strong et al., 1980; Flynn and Mash, 1986; Gurwitz et al., 1987; Kellar et al., 1987; Rinne, 1987). Although these findings lend support to the notion that the cortical cholinergic binding concentration decreases seen with old age in these rhesus monkeys are probably decreases in B_{max} , this information cannot be discerned in the current study because all receptor autoradiographic assays were performed at a single ligand concentration.

Discussion

Previous research indicates that M1 and M2 binding sites may be localized differently at the cholinergic synapse in the cerebral

Figure 1. Laminar distribution of muscarinic receptor subtypes in temporal neocortex. Concentrations are in 1×10^{-15} mol/mg standard with standard deviation indicated on the bars. A and B, The distribution of ligand binding for M1 receptor subtype. For ^3H -NMS (low affinity) and ^3H -PZ, binding predominates in cortical layers II and III and follows a similar pattern for all age groups. C and D, The distribution of ligand binding for M2 receptor subtype. For ^3H -NMS (high affinity) and ^3H -OXO-M, binding predominates in cortical layers IV and V and follows a similar pattern for all age groups.

cortex and may have different functions. M1 receptors are predominant in the neocortex (Mash et al., 1985). These receptors are not changed after lesions of the basal forebrain cholinergic system in rats (Johnston et al., 1981), nor are they reduced in

cortical tissues of patients with Alzheimer's disease (Mash et al., 1985), a disorder associated with abnormalities of the basal forebrain cholinergic system and several other transmitter systems (Price, 1986). These observations suggest that M1 receptor

Table 1. Muscarinic and nicotinic receptor binding concentration in whole temporal cortex of rhesus monkey

Group	Mean age (yr)	^3H -NMS (low affinity)	^3H -PZ	^3H -NMS (high affinity)	^3H -OXO-M	^3H -ACh
I	2	306.00 ± 42.42 (n = 5)	564.00 ± 94.22 (n = 5)	161.00 ± 37.17 (n = 5)	37.80 ± 4.55 (n = 5)	2.63 ± 1.31 (n = 5)
II	6	262.00 ± 36.77 (n = 2)	457.20 ± 72.41 (n = 2)	147.00 ± 18.38 (n = 2)	$19.50^b \pm 2.12$ (n = 2)	$0.75^a \pm 0.13$ (n = 2)
III	13	$225.50^a \pm 6.36$ (n = 2)	406.50 ± 55.86 (n = 2)	99.00 ± 35.36 (n = 2)	$10.50^b \pm 4.95$ (n = 2)	$0.79^a \pm 0.20$ (n = 2)
IV	20	$208.14^a \pm 30.06$ (n = 7)	458.67 ± 69.95 (n = 7)	113.14 ± 34.90 (n = 7)	$14.00^b \pm 4.42$ (n = 5)	$0.81^a \pm 0.22$ (n = 7)

Mean values are of specific binding expressed as 1×10^{-15} mol/mg standard $\pm$ SD.

^a $p < 0.05$, compared to 2-year-old age group.

^b $p < 0.01$, compared to 2-year-old age group.

binding is postsynaptic. M2 receptors are found in cortical laminae that have the highest choline acetyltransferase (ChAT) activity (Johnston et al., 1981); in rats, lesions of the basal forebrain, which denervate the cortex, result in reductions in ChAT activity and in high-affinity agonist M2 receptors (McKinney and Coyle, 1982; Mash et al., 1985). Furthermore, M2 receptors are decreased in cerebral cortices of individuals with Alzheimer's disease (Mash et al., 1985); however, a study from our laboratory did not replicate these findings (Kellar et al., 1987). From this evidence, M2 receptors have been viewed as presynaptic autoreceptors that modulate ACh release from cholinergic nerve terminals (Mash et al., 1985).

Although M1 and M2 receptor binding occurred across all layers of the temporal neocortex in macaques, the present study demonstrated that M1 receptor binding was predominant in layers II and III, and M2 muscarinic receptor binding was concentrated in layers IV and V. The laminar pattern in macaques is consistent with studies of the distribution of M1 and M2 receptor subtypes in the human temporal cortex (Cortés et al., 1987) and rodent neocortex (Wamsley et al., 1980; Wamsley et al., 1984; Cortés and Palacios, 1986; Mash and Potter, 1986; Spencer et al., 1986). These patterns of muscarinic receptor binding show parallels with other indices of cholinergic innervation. Immunocytochemical studies demonstrate that ChAT-positive axons, derived from the basal forebrain cholinergic system (Struble et al., 1986), are concentrated in layer V and deep layer VI of the rat cortex (Saper, 1984; Houser et al., 1985). Furthermore, ChAT-positive cell bodies, presumed to be interneurons, are concentrated in cortical layers II and III of the rat (Houser et al., 1985).

This investigation demonstrates age-related reductions in concentrations of muscarinic and nicotinic receptor binding sites in the primate neocortex. Using multiple ligands to measure a single muscarinic subtype binding site, a significant age-associated decrease was shown in low-affinity ^3H -NMS binding for M1 neocortical receptors; a reduction in high-affinity ^3H -PZ binding, labeling the same putative M1 site, did not reach statistical significance. Similarly, high-affinity ^3H -OXO-M binding for M2 receptors significantly decreased with age, but a change in high-affinity ^3H -NMS binding to the same site did not reach significance. Significant age-related decreases in receptor binding and their concomitant trends are interpreted as indicative of a true age-related decrease in concentrations of cholinergic muscarinic receptor subtypes. Using ligands specific for M1, M2, and nicotinic cholinergic receptor subtypes, significant declines in concentrations of binding sites for both muscarinic receptor subtypes (M1 and M2) were uniform across all cortical layers, and changes in cortical nicotinic receptor binding appeared to parallel this pattern.

ChAT, the synthetic enzyme for ACh and a presynaptic marker for cholinergic neurons, has been examined in aged individuals of several species. Although several researchers have reported no change in ChAT activity in whole brains and cortices of aged rodents (Meek et al., 1977; Reis et al., 1977; Ingram et al., 1981), other investigators have described a significant de-

crease in ChAT activity with age in the rodent cortex (Strong et al., 1980; Unsworth et al., 1980). Similar conflicting data concerning ChAT activity have been reported in humans. In some studies, ChAT activity declines with age in the cortex (McGeer and McGeer, 1975; Perry et al., 1977; Davies, 1978; Perry, 1980), whereas other studies have shown no change in enzyme activity (Bowen et al., 1976; Spillane et al., 1977; White et al., 1977; Carlsson et al., 1980; Yates et al., 1980). In the present study, age-related changes found in the putative presynaptic M2 receptor site and in the postsynaptic M1 receptor site correspond to reports of decreases in both cholinergic enzymes in neocortices of aged rodents and humans. Moreover, ChAT activity declines as a function of age in the rhesus monkey cortex, with statistically significant decreases occurring in the frontal pole and precentral gyrus (Wenk et al., 1989).

Although the number of animals sampled at intermediate ages was small, data suggest that there is a substantial decline in cortical muscarinic and nicotinic receptor binding concentrations between 2 and 6 years of age, with lesser reductions thereafter. Comparable information on developmental changes in muscarinic and nicotinic receptors in the human brain is not known. However, data on cholinergic enzymes is available (McGeer, 1978). Extensive studies of human postmortem tissue reveal a 78% decrease in ChAT activity in the midtemporal gyrus between 5–20 years of age. A further decrease of 71% was seen between 20–50 years of age. From the same sample, AChE levels decreased 73% between 5–20 years of age, with an additional 54% decrease between 20–50 years of age (McGeer, 1978). For comparison with rhesus monkey age in the present study, animal ages can be transformed into human years by multiplying by a factor of 3. Therefore, a 2-year-old rhesus monkey would approximate, developmentally, a 6-year-old human; a 6-year-old rhesus monkey would approximate an 18-year-old human, etc. Thus, the loss of ^3H -cholinergic ligand binding concentration between 2- to 6-year-old rhesus monkeys in this study parallels the significant drop in cholinergic enzymes in humans of comparable age. This process may represent a normal developmental paring of receptors and certainly merits further investigation, particularly to examine the idea of a "critical" number or affinity of cholinergic receptors needed to maintain normal brain function.

Because tritiated ligands were employed in this study, one issue to be resolved is that of differential quenching of tritium by white and gray matter (Rainbow et al., 1984; Kuhar and Unnerstall, 1985). Fatty or lipid-containing tissue self-absorbs tritium, resulting in less autoradiographic optical density in white matter relative to gray matter. Are the decreases in cholinergic ligand binding with age, shown in this study, actually artifactual, due to a greater density of white matter with age? At least one report (Horrocks et al., 1975) shows that, though lipid content of the brain rises during development in humans, it stays relatively stable throughout maturity and then falls during aging. Because the neocortex is a predominantly gray matter structure, tritium quenching should have little effect on this region (Rainbow et al., 1984). Furthermore, if lipid content rises during

Figure 2. Autoradiograms of caudal temporal cortex (superior temporal sulcus) from young and old rhesus monkeys. Note the decrease with age in ^3H cholinergic ligand binding. Superficial layers of the temporal cortex contain high levels of ^3H -PZ binding (M1 sites), whereas deep layers contain high levels of ^3H -OXO-M binding (M2 sites). Scale bar, 2 mm. *A* and *B*, The distribution of ^3H -PZ binding sites in a 2-year-old animal (*A*) and a 22-year-old animal (*B*). *C* and *D*, The distribution of ^3H -OXO-M binding sites in a 2-year-old animal (*C*) and a 22-year-old animal (*D*). *E* and *F*, The distribution of ^3H -ACh (nicotinic) binding sites in a 2-year-old animal (*E*) and a 22-year-old animal (*F*).

development, stabilizes, and then falls during aging, artifactually high optical densities would be predicted in very young animals, lower optical densities in animals of early to middle adulthood, and artifactually high optical densities in old animals. In the present study, optical densities, transformed into binding concentrations, decreased with age, providing evidence against a major role of tritium quenching in this region of primate brain.

The present study provides the first evidence for age-related changes in cholinergic receptors in the CNS of the nonhuman primate. Aged rhesus monkeys and other nonhuman primates develop some of the behavioral impairments and pathological changes that occur in aged humans, for example, senile plaques and amyloid deposits in plaques and blood vessels (Struble et al., 1982; Kitt et al., 1985; Selkoe et al., 1987; Walker et al., 1987). Studies of monkeys may provide valuable information concerning roles of the cholinergic system in cognition and memory processing in primates.

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