Age-Related Changes of Cholinergic Markers in the Rat Brain

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ABSTRACT—To evaluate whether any degenerative changes affect the brain cholinergic systems during natural aging, we compared various cholinergic biochemical markers (number of muscarinic receptors, mAChR; choline acetyltransferase activity, ChAT; acetylcholinesterase activity, AChE; and sodium-dependent high affinity choline uptake) in the cortical (CR) and subcortical (SS) regions of the brains of aged (24 month) and young (2 month) rats. Using [3H]-quinuclidinyl benzilate ([3H]-QNB) as the ligand of muscarinic receptor binding, the numbers of mAChR decreased about 30% in both the CR and the SS of aged rats compared with those in young rats, while a significant age-related increase in the affinity of mAChR was observed. [3H]-QNB binding in both the young and aged rat brain was displaced markedly by pirenzepine, while [3H]-QNB binding in the SS of the aged rat brain was displaced at low concentrations of atropine. The $V_{\text{max}}$ values of ChAT and AChE also decreased about 20–30% compared with those of young rats. The sodium-dependent high affinity choline uptake was lower in the crude synaptosomal fraction prepared from aged rat brain than in young brain. Hemicholinium-3 inhibited the choline uptake in young rat brain at a concentration range of 1 pM–10 nM, but choline uptake in aged brain was insensitive to hemicholinium-3. These results indicate that natural aging brings about a diffuse and multiple depletion of various biochemical markers in cholinergic neurons.

Keywords: Aged rat, Muscarinic receptor, Acetylcholinesterase, Choline acetyltransferase, Sodium-dependent choline uptake

Numerous neurochemical studies using both animals and humans have revealed age-related changes in neurotransmitter enzyme activities and receptor binding (1–4). Age-related memory disorders, including Alzheimer’s disease, show a substantial decrease in cholinergic markers, such as choline acetyltransferase (ChAT), acetylcholinesterase (AChE) and the number of muscarinic receptors (mAChR), in the hippocampal formation and other cortical and basal forebrain structures (5–9). However, there are conflicting reports from both human and animal studies. Many studies have shown that the levels of ChAT and mAChR are unchanged (10) or slightly increased with age (11), but decreased levels have also been found (12). These discrepancies could be due in part to the differences in strain or species (13, 14), sex (15), tissue sampling (16, 17), assay procedures (18) or the choice of time points during the life cycle used for comparison (19, 20).

Electrolytic or neurotoxic lesions of the nucleus basalis magnocellularis (NBM) reduce cortical ChAT activity, AChE activity, acetylcholine (ACh) release and choline uptake (21–23). Moreover, NBM-lesioned rats showed impaired acquisition of a passive avoidance response (24). These data should be useful for establishing a model of Alzheimer’s disease which could be used to study therapeutic drugs and methods. In the meantime, investigation of brain cholinergic systems in aged laboratory animals could provide useful information pertaining to age and age-related disorders in humans.

The present study examines the characteristics of certain cholinergic markers (ChAT and AChE activity, number of muscarinic binding sites and choline uptake) in the cortex and subcortical structures in aged and young rats.

MATERIALS AND METHODS

Animals
Male, Sprague-Dawley rats aged 6 weeks (young) and 24 month (aged) were used. The rats were housed in a controlled environment (25±2°C, 50±5% humidity, lit between 6:00 and 18:00 hours) with food and water available ad libitum.

Under ether anesthesia, the rats were sacrificed by decapitation. The brain was quickly removed from the
skull and dissected into two parts: the forebrain and temporal, parietal and occipital lobes (which were combined for all assays and are generically referred to below as the cortical regions, CR) and the hippocampus, globus pallidus, thalamus, nucleus basalis of Meynert, caudate nucleus, putamen and striatum (which were combined for all assays and are generically referred to below as subcortical structures, SS). To minimize individual variations, tissues from at least five rats were pooled for use in each experiment and stored at −80°C.

**Muscarinic receptor binding assay**

Brain tissues were homogenized in 5 vol. (wt./vol.) of 50 mM Tris-HCl buffer, pH 7.5. The homogenates were centrifuged at 900 × g for 10 min. The supernatants were again centrifuged at 12,000 × g for 20 min. Pellets were resuspended in 10 mM Tris-HCl buffer containing 5 mM MgSO₄, pH 7.5, to a protein concentration of 1.0 mg/ml yielding a crude synaptosomal fraction. The apparent dissociation constant (Kₐ) and the density of muscarinic receptors (Bₘₐₓ) in the crude synaptosomal fraction were assayed according to the method of Yamamura and Snyder (25) using [³H]-quinuclidinyl benzilate ([³H]-QNB) as the specific ligand. Aliquots of the crude synaptosomal fraction (100 µl, 1 mg protein/ml) were incubated in a total volume of 1.0 ml at 37°C for 30 min with various concentrations of [³H]-QNB (0.04–0.6 nM) in 10 mM Tris-HCl buffer containing 5 mM MgSO₄. After incubation, 4 ml of ice-cold 10 mM Tris-HCl buffer, containing 145 mM NaCl, pH 7.5, was added to the aliquots, and the bound ligand was separated from the free ligand using rapid filtration. The filter papers (GF/B glass filters, Whatman) were washed four times with the same ice-cold buffer. After the filters dried overnight, 10 ml of Triton X-100-toluene scintillation fluid was added to each. Samples of the extract were mixed with Triton X-100-toluene scintillation fluid and their radioactivities measured by a liquid scintillation spectrometer. The enzymic activities were calculated from the dpm values and expressed as nanomoles of acetylcholine synthesized/min per mg protein. The values of the apparent Kₐ and Vₘₐₓ were obtained from Lineweaver-Burk plots corresponding to various concentrations of choline bromide.

**Acetylcholinesterase activity assay**

Acetylcholinesterase (AChE) activity was measured according to the method of Ellman (27). Brain tissues were homogenized in 5 vol. (wt./vol.) of 50 mM Tris-HCl buffer, pH 7.5. The homogenates were centrifuged at 900 × g for 10 min. The supernatants were again centrifuged at 12,000 × g for 20 min. The resultant pellets were suspended in Tris-HCl buffer containing 5 mM MgSO₄, pH 7.5, at a protein concentration of 1.0 mg/ml, yielding the crude synaptosomal fraction. The 50-µl aliquot of synaptosomal preparation was incubated for 5 min at 37°C in 100 mM phosphate buffer, pH 8.0, containing 10 mM 5,5'-dithiobis-2-nitrobenzoic acid in a final volume of 3.5 ml. After adding 20 µl of various concentrations of acetylthiocholine (47–473 µM), the absorbance at 412 nm was measured with a Hitachi 557 spectrophotometer. The enzymic activity was expressed as nanomoles of acetylthiocholine hydrolyzed/min per mg of protein. The apparent Vₘₐₓ and Kₐ values were obtained from Lineweaver-Burk plots corresponding to various concentrations of acetylthiocholine.

**Sodium dependent high affinity [³H]-choline uptake assay**

The sodium-dependent high affinity [³H]-choline uptake was assayed according to the modified method of Sorimachi and Kataoka (28). The brain tissues were homogenized in 9 vol. of 0.32 M sucrose, pH 7.4. Crude synaptosomal fractions were obtained by differential centrifugation on conventional discontinuous sucrose density gradients. The resulting pellets were suspended in Krebs-Ringer bicarbonate buffer, pH 7.4, containing 140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgSO₄, 1 mM NaH₂PO₄, 20 mM NaHCO₃ and 11 mM glucose. The
100-μl samples of crude synaptosomes (200 μg protein/tube) were preincubated at 37°C for 5 min in the same buffer oxygenated with a 95%O₂-5%CO₂ gas mixture. [³H]-Choline chloride (0.25 - 4.0 μM) was then added, and the solution was incubated at 37°C for 4 min. Reactions were terminated by rapid filtration of the crude synaptosomes under reduced pressure through Whatman GF/B glass fiber filters. Each filter was immediately washed four times with 4 ml of ice-cold saline. The filters were then dried, and the radioactivities were measured by liquid scintillation spectrometry after adding Triton X-100-toluene scintillation fluid. The specific choline uptake was defined as the difference between measurements made with the above buffer and those made with a sodium-free buffer (Na⁺ replaced with 280 mM sucrose and 21 mM Tris-HCl). The values of apparent Vₘₐₓ and Kₘ were obtained from Lineweaver-Burk plots.

Table 1. Binding of [³H]-QNB to muscarinic ACh receptors in brains of young and aged rats

|                  | Young rat | Aged rat |
|------------------|-----------|----------|
|                  | K_d       | B_max    | K_d       | B_max    |
| Cortical regions | 0.123±0.001 | 2.35±0.15 | 0.107±0.005* | 1.63±0.03* |
| Subcortical structures | 0.087±0.008 | 1.16±0.17 | 0.042±0.006* | 0.56±0.03* |

Binding data were derived from saturation analysis. Binding for each concentration of radioligand was measured in triplicate, and nonspecific binding was defined as the binding in the presence of 1 μM atropine, also in triplicate. The maximal density of muscarinic receptors (Bₘₐₓ) and the apparent dissociation constant (K_d) for [³H]-QNB binding were determined graphically by Scatchard analysis and expressed as the mean ± S.E. of the values obtained for five rats. Bₘₐₓ: pmol/mg protein, K_d: nM, CR: cortical regions, SS: subcortical structures. *: P<0.05, compared with young rats.

Proteins

The protein content was determined by the method of Lowry et al. (29) using bovine serum albumin as the standard.

Materials

[³H]-Quinuclidinyl benzilate ([³H]-QNB) (2.22 - 3.22 TBq/mmol) and [methyl-³H]-choline chloride (2.22 - 3.33 TBq/mmol) were purchased from New England Nuclear (Boston, MA, USA). [³H]-Acetyl coenzyme A (111 - 122 GBq/mmol) was purchased from Amersham (Buckinghamshire, England). Atropine sulfate, acetyl coenzyme A, choline bromide, p-chloromercuribenzoic acid (PCMB), hemicholinium-3 and physostigmine sulfate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Pirenzepine hydrochloride was supplied by Boehringer Ingelheim, Kawanishi. All other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka).

Statistics

Results are expressed as the mean or means±S.E. for four or five rats. The significance of the mean difference was determined by Student's t-test for unpaired data.

Fig. 1. Inhibition of specific [³H]-QNB binding by pirenzepine and atropine in young and aged rat brain. Membrane preparations of each brain part were incubated with 0.04 nM [³H]-QNB and various concentrations of pirenzepine and atropine in 10 mM Tris-HCl buffer (pH 7.4) containing 5 mM MgSO₄ for 30 min at 37°C. Each point represents the mean ± S.E. of the specific [³H]-QNB binding values determined in three separate experiments. Top: cerebral cortex, bottom: subcortical structure. Pirenzepine in aged: -○-, pirenzepine in young: - -●-, atropine in aged: - ■-, atropine in young: - -□- rat brain.
Table 2. Choline acetyltransferase activities in brains of young and aged rats

|                  | Young rat          | Aged rat        |
|------------------|--------------------|-----------------|
|                  | $V_{\text{max}}$  | $K_{\text{m}}$  | $V_{\text{max}}$  | $K_{\text{m}}$  |
| Cortical regions | 1.89±0.33          | 2.82±0.15       | 1.58±0.17$^*$     | 2.61±0.58       |
| Subcortical structures | 1.29±0.18          | 9.21±0.51       | 0.92±0.09$^*$     | 8.14±2.30       |

Each value is expressed as the mean±S.E. for five rats. $V_{\text{max}}$ and $K_{\text{m}}$ values were determined from Lineweaver-Burk double reciprocal plots of values obtained from graphic representations of the kinetic data. $V_{\text{max}}$: nmol/min per mg protein, $K_{\text{m}}$: mM against choline bromide. $^*$: P<0.05, compared with young rats.

RESULTS

Muscarinic receptors

To determine whether muscarinic receptors in the central nervous system are affected during natural aging, kinetic analysis of [$^{3}$H]-QNB binding in two parts (CR and SS) of young and aged rat brain was performed. Table 1 shows a slight age-related reduction in the apparent dissociation constant for [$^{3}$H]-QNB (Kd) in the CR and SS. A significant age-related decrease was found in the density of muscarinic receptors (Bm) in the CR and SS. Changes in the values of $B_{\text{max}}$ and $K_{d}$ with age are more marked in the SS than in the CR.

The effects of the mAChR antagonists pirenzepine and atropine on [$^{3}$H]-QNB binding sites in the CR and the SS of young and aged rats were compared. Figure 1 shows that [$^{3}$H]-QNB bindings were strongly displaced with increasing concentrations of pirenzepine and atropine. Inhibition curves of pirenzepine in aged rats were similar to those in young rats. There was no difference between the atropine inhibition curve in the CR in young rats and that in aged rats. The inhibition of [$^{3}$H]-QNB binding at lower concentration of atropine (1–100 pM) was 20% lower in the SS of aged rats as compared with that of young rats.

Choline acetyltransferase (ChAT) activity

Table 2 shows that with advancing age, the value of $V_{\text{max}}$ of ChAT declined significantly in both the CR and SS. However, no significant age-related differences were observed in the $K_{\text{m}}$ values.

It is known that PCMB inhibits ChAT (30), and Fig. 2 shows a marked PCMB-induced inhibition of ChAT activity in the CR of both young and aged rats. ChAT activity in the SS of young rats was more sensitive to PCMB than that of aged rats.

Fig. 2. Inhibition by p-chloromercuribenzoic acid (PCMB) on choline acetyltransferase (ChAT) activity in young and aged rat brain. An enzyme solution of each brain part was incubated with the substrate mixture for 15 min at 37°C with various concentrations of PCMB. Symbols with bars represent the mean±S.E. of three separate experiments. Left: cerebral cortex, right: subcortical structure. Aged: –●–, young: - - ○ - - rat brain.
Table 3. Acetylcholinesterase activities in brains of young and aged rats

| AChE          | Young rat | Aged rat |
|---------------|-----------|----------|
|               | $V_{max}$ | $K_m$    | $V_{max}$ | $K_m$    |
| Cortical regions | 283.8±10.2 | 83.2±16.0 | 205.0±13.0* | 77.9±8.6 |
| Subcortical structures | 188.7±8.3  | 56.0±3.3  | 122.5±5.3*  | 58.5±5.2  |

Each value is expressed as the mean±S.E. for five rats. AChE activity was spectrophotometrically determined with acetylthiocholine as a substrate. $V_{max}$ and $K_m$ values were estimated from Lineweaver-Burk double reciprocal plots of values obtained from graphic representations of the kinetic data. $V_{max}$: nmol/min per mg protein, $K_m$: μM. *: P<0.05, compared with young rats.

Acetylcholinesterase activity

As with ChAT, the value of $V_{max}$ of AChE was 28% less in the CR of aged rats than in young rats and 35% less in the SS of aged rats than in young rats (Table 3). No significant difference was observed between the $K_m$ values of AChE in young and aged rats.

No difference was found in the inhibition of AChE activity produced by physostigmine between young and aged rats (Fig. 3).

Sodium-dependent high affinity choline uptake

The values of both $K_m$ and $V_{max}$ of sodium-dependent high affinity $[^3H]$-choline uptake were significantly lower in the aged rat brain than those of the young rat brain. Aged rats showed a 40% lower value of $V_{max}$ in the CR and a 60% lower value of $V_{max}$ in the SS (Table 4). Aged rats showed a 57% lower value of $K_m$ in the CR and a 43% lower value of $K_m$ in the SS.

Figure 4 shows the effect of hemicholinium-3 (HC-3) on sodium-dependent high affinity $[^3H]$-choline uptake in rat brain synaptosomes. In young rats, the uptake was inhibited strongly by low concentrations of HC-3. However, in aged rats, the uptake was not completely inhibited, even at high concentrations of HC-3. The residual activities at $10^{-4}$ M HC-3 were 20% in the CR and 30% in the SS.

DISCUSSION

The age-related decrease in neurochemical markers in rodents supports their use as a model of human aging. In particular, the muscarinic binding in the brain of rats is frequently used in aging research.

Waller and London (31) reported that the affinity for...
[3H]-QNB increased in the cortex with age, but that the maximal capacity of muscarinic binding decreased in the cerebral cortex, striatum and hippocampus of 24-month-old Fischer 344 rats. These findings agree with our present results from male, 24-month-old Sprague-Dawley rats.

Haba et al. (32) and Ogawa et al. (12) showed that the mAChR level was markedly reduced in all brain regions of aged Fischer rats, but found no change in $K_d$. Other reports show a decrease in muscarinic receptor binding sites in the cortex, but no change in the hippocampus of Sprague-Dawley (13), Fischer 344 (33, 34) or Wistar (35) aged rats. These differences could be due in part to different strains or species (13, 14), tissue sampling (16, 17) or assay procedures (18). Michalek et al. (14) also reported significant strain-related differences in muscarinic receptor sites in three brain areas of Fischer 344 and Wistar male rats. They also explained the need to consider genotype in the assessment of age-related cholinergic deficits in animal models. In the present study, the value of $B_{max}$ decreased significantly with age, especially in the SS, and the value of $K_d$ also decreased. This finding may show that the age-dependent decrease in $B_{max}$ of muscarinic receptors results from the increase in muscarinic receptor affinity with age.

Muscarinic binding sites can be divided into two classes: $M_1$ receptors, which have a high affinity for pirenzepine, and $M_2$ receptors, which have a low affinity for this muscarinic antagonist (36, 37). The effects of aging on these subtypes of muscarinic receptors has been studied using various radioligands. Watson et al. (38) found an age-related decline in $M_1$-receptor binding in the cortex, striatum, hippocampus and hypothalamus using [3H]-pirenzepine. Schwarz et al. (39) also found an age-related decrease in $M_1$-receptors in the cortex of rats using the same selective $M_1$-receptor antagonist. However, Sirvio et al. (40) reported that the maximal number of $M_1$ binding sites was unaltered in the cortex and hippocampus as rats aged. The present study found that pirenzepine, a muscarinic antagonist, bound with high affinity to $M_1$ receptors, inhibiting [3H]-QNB binding in both aged and young rats. No age-related differences were found in the amount atropine-inhibited [3H]-QNB binding in the CR, but in the SS of aged rats, [3H]-QNB binding was reduced by 20% in the presence of low concentrations of atropine. This indicates the existence of muscarinic receptors highly sensitive to atropine in aged rats. Although the relation-

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**Table 4. Sodium-dependent high affinity choline uptake in brains of young and aged rats**

|                    | Young rat | Aged rat |
|--------------------|-----------|----------|
|                    | $V_{max}$ | $K_m$   | $V_{max}$ | $K_m$   |
| Cortical regions   | 17.5±2.4  | 2.3±0.5  | 10.5±3.3* | 1.0±0.2* |
| Subcortical structures | 16.1±1.1  | 3.0±0.7  | 6.3±1.1*  | 1.7±0.1* |

Each value is expressed as the mean±S.E. for five rats. Uptake of [3H]-choline chloride was carried out at 37°C for 4 min at each concentration of radioligand. $V_{max}$ and $K_m$ values were determined from Lineweaver-Burk double reciprocal plots of values obtained from graphic representations of the kinetic data. $V_{max}$: pmol/min per mg protein, $K_m$: µM. *: P<0.05, compared with young rats.

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**Fig. 4.** Inhibition by hemicholinium-3 (HC-3) of sodium-dependent high affinity choline uptake in young and aged rat brain. After incubation with crude synaptosomes and various concentrations of HC-3 in oxygenated (95% O₂, 5% CO₂) Krebs-Ringer bicarbonate buffer at 37°C for 5 min, [3H]-choline chloride was added and incubated for another 4 min. Symbols with bars represent the mean±S.E. of three separate experiments. Top: cerebral cortex, bottom: subcortical structure. Aged: - - - - - - ; young: - - - - - - - - - - - - rat brain.
ship between this atropine-sensitive receptor and the M₁ receptor is unclear, it may involve some alteration at the muscarinic receptor sites of the postsynaptic membrane with aging.

Choline acetyltransferase (ChAT) is found in the brain in considerable excess and thus does not limit the rate of ACh synthesis, but it is generally agreed that the level of ChAT activity is a sensitive marker of changes in cholinergic innervation. Age-related decreases in ChAT are sometimes found in animal studies of normal aging. Some of these studies report a decrease with age in ChAT activity in the cerebral cortex and corpus striatum (13, 14, 31, 32, 41), while others report no change (39, 40). Although these age-related differences in ChAT activity from animal studies may be due to differences in strain or species (13, 14), sex (15), tissue sampling (16, 17) or assay procedures (18), these differences alone do not adequately explain the discrepancies.

An age-dependent decline of cholinergic activity is also supported by an analysis of biochemical markers in their terminal sites. Specifically, the current study has demonstrated that AChE activity is reduced with aging in all brain regions. These findings are in good agreement with previous reports (11, 14, 42). In the present study, the $V_{\text{max}}$ values of ChAT and AChE activity decreased during aging, resulting in a decreased quantity of enzymes. The $V_{\text{max}}$ value of ChAT activity decreased more during aging in the SS than in the CR. The reduced synthesis of ACh found to occur in the rodent brain during aging may be due to the decrease in ChAT activity.

Additionally, decreased levels of AChE were found in both the CR and the SS in the aged rats. Because of the decrease in ChAT activity, which is considered to be a more reliable presynaptic marker of the cholinergic system, the low levels of AChE may be due to a loss of postsynaptic enzyme activity.

It is commonly believed that sodium-dependent high affinity choline uptake is by means of Na⁺ transport (Na⁺ channel) at the surface of the synaptic membrane of the presynaptic nerve terminals and is one of the rate-limiting steps in acetylcholine synthesis. Thus, given the observation that acetylcholine synthesis is diminished during aging, it is interesting to consider the possible role of age-related changes in sodium-dependent high affinity choline uptake. An age-dependent reduction of sodium-dependent high affinity choline uptake was shown by Sherman et al. (43) and Sirvio et al. (44), but other studies did not confirm it (45, 46). In the current study, a significant, age-related decrease in choline uptake was found in both the CR and the SS resulting from the decreased values of $K_m$ and $V_{\text{max}}$. Thus, decreased sodium-dependent high affinity choline uptake may be due to a reduced number of carrier molecules for the presynaptic choline (44).

The availability of $[^{3}H]$-hemicholinium-3 ($[^{3}H]$-HC-3), a potent, reversible inhibitor of sodium-dependent high affinity choline uptake, has led to the development of the ligand-binding method to label sodium-dependent choline uptake sites (47, 48). In the current study, the in vitro effects of HC-3 were investigated in aged and young rat brain synaptosomes. In young rat brain, the uptake was markedly inhibited at low concentrations of HC-3, but in aged brain, it was not inhibited completely, even at a HC-3 concentration of $10^{-4}$ M. These results indicate that the age-related decline in sodium-dependent high affinity choline uptake is due to conformational changes or the occupation of the binding sites by lipids and unknown endogenous ligands.

In 24-month-old Sprague-Dawley rats, the cholinergic markers of ChAT and AChE activity, numbers of mAChR and sodium-dependent high affinity choline uptake all decreased nearly 30–45% with age. However, the ability of choline uptake in presynaptic membranes and function of muscarinic receptors in postsynaptic membranes was maintained by the increase in the remaining binding site affinity with age. Moreover, it has been suggested that the heterogeneity of the muscarinic receptor and sodium-dependent high affinity choline uptake sites could be studied using sulfhydryl reagents, metal ions, preferences for specific ligands and displacing agents (43, 49, 50).

Table 5 summarizes the data from studies of mAChR, ChAT, AChE and sodium-dependent high affinity choline uptake (SDHACU) of aged rat brains compared to those of young rats. The data reviewed here suggested that $B_{\text{max}}$ or $V_{\text{max}}$ values of these markers were simultaneously decreased in both pre- and postsynaptic nerve terminals of aged rat brains. However, it is considered that aged rats strive to maintain mAChR and SDHACU functions that were decreasing during natural aging by increasing these affinities, while the decreased AChE and ChAT activities were associated with only reductions of the enzyme molecule itself with advancing aging. Moreover, it appears that age-relatedly, these changes occurred to greater extents in some parts of the subcortical structures (including hippocampus, globus pallidus, thalamus, nucleus basalis of Meynert, caudate nucleus, putamen and striatum). These observations suggest that the functioning of the cholinergic system in subcortical structures is impaired predominantly during natural aging. However, it is known that some regional differences in these age-related changes are present.

To investigate these changes in the dynamic properties of cholinergic input to the hippocampus and cortex, further studies are need to clarify the regional variations within the basal forebrain.
Table 5. Comparison of cholinergic markers in aged rats and young rats

|                  | Km or Kd | Vmax or Bmax | Affinity against inhibitor |
|------------------|----------|--------------|---------------------------|
| **Cortical regions** |          |              |                           |
| mACHR           | 14%↓     | 30%↓         | no change                 |
| AChE            | ±        | 28%↓         | no change                 |
| ChAT            | ±        | 28%↓         | no change                 |
| SDHACU          | 57%↓     | 40%↓         | low sensitive to HC-3     |
| **Subcortical structures** |          |              |                           |
| mACHR           | 50%↓     | 50%↓         | high sensitive to atropine|
| AChE            | ±        | 35%↓         | no change                 |
| ChAT            | ±        | 30%↓         | low sensitive to PCMB     |
| SDHACU          | 43%↓     | 60%↓         | low sensitive to HC-3     |

mACHR: muscarinic receptor, AChE: acetylcholinesterase, ChAT: choline acetyltransferase, SDHACU: sodium-dependent high affinity choline uptake. ± or no change: did not observe the differences between aged and young rats.

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