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Loss of Muscarinic M1 Receptors With Aging in the Cerebral Cortex of Fisher 344 Rats

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SCHWARZ, R. D., A. A. BERNABEI, C. J. SPENCER AND T. A. PUGSLEY. *Loss of muscarinic M1 receptors with aging in the cerebral cortex of Fisher 344 rats*. PHARMACOL BIOCHEM BEHAV **35**(3) 589–593, 1990.—Age-related changes in central cholinergic muscarinic receptors were measured in young (3–6 month), middle-aged (15–17 month), and aged (22–26 month) male Fisher 344 rats by receptor binding techniques. Using [^{3}H]-quinuclidinyl benzilate as the ligand, a significant decrease (14–19%) in the number of muscarinic cortical receptors was measured in aged rats compared to both young and middle-aged rats. With the selective M1 antagonist, [^{3}H]-pirenzepine, a 17% decrease in receptor density was observed in the cortex of aged animals compared to young rats. For both ligands no differences were observed in the striatum or hippocampus between any age group and there was no change in affinity (K_d) in any of the three brain regions for the three age groups. Additionally, there was no difference in choline acetyltransferase activity measured in cortex, hippocampus, or striatum of young and aged rats. Thus, there is a loss of M1 muscarinic receptors in the cerebral cortex of aged male Fisher 344 rats.

Aging Cortex Hippocampus Striatum Muscarinic receptors Pirenzepine Quinuclidinyl benzilate (QNB) Choline acetyltransferase (ChAT)

MORPHOLOGICAL and neurochemical changes have been shown to occur as a result of aging in the CNS of both humans and experimental animals (1,7). The activity of a variety of central neurotransmitter systems, including the cholinergic system, appears to be significantly decreased due to the process of aging and also in response to certain age-related neurodegenerative disorders, such as Alzheimer's disease (AD) or senile dementia of the Alzheimer type (SDAT) (8, 24, 32). For clarity, AD is used to define those persons with early onset of the disorder, while SDAT is used for individuals with late onset. AD/SDAT is characterized in part by a marked loss of short-term memory and it is this cognitive deficit which has been associated with a loss of cholinergic neurons (2).

Muscarinic receptors have been classified into two putative subtypes, M1 and M2, on the basis of receptor binding studies. M1 binding sites show high affinity and pharmacological sensitivity for the atypical antagonist pirenzepine (PZ), while M2 sites show lower affinity for PZ (16, 36, 39). Recent cloning, sequencing, and genetic expression of muscarinic receptors has confirmed the heterogeneity of muscarinic receptors and clearly demonstrate the existence of five distinct receptors (5, 18, 19, 31). On the basis of [^{3}H]-acetylcholine (ACh) release studies, it has been suggested that M1 receptors may be postsynaptic in location, while M2 may correspond to presynaptic autoreceptors (33). However, it has been suggested that the precise localization of M1 and M2 receptors with regard to pre- and postsynaptic receptor sites has not been clearly defined (37). Further, M1 receptors may be positively coupled to phosphoinositide hydrolysis and M2 receptors negatively coupled to adenylate cyclase through guanine (G) regulatory proteins, although it has been suggested that both membrane transductions systems may be linked to either of the two receptors (4, 13, 14, 39).

A number of studies which have examined the effect of aging on muscarinic receptors in the brain measured only the total population of receptors. Some of these studies report a decrease in muscarinic receptor density with age in the hippocampus (15, 20, 21), while others report no change (6, 9, 10, 22, 26, 29, 34). Recently, it was observed that the number of cortical muscarinic M2 receptors were decreased in AD/SDAT patients compared to age-matched controls (25). Since there may also be alterations in muscarinic receptor subtypes with aging, the present study was undertaken to examine possible changes of brain M1/M2 receptors in rats during aging using receptor binding techniques. The following results demonstrate that the number of cortical M1 muscarinic receptors are decreased in aged Fisher 344 rats.

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METHOD

Animals and Brain Dissection

Male Fisher 344 rats, 3–6 (young), 15–17 (middle-aged), and 22–26 (aged) months old, were obtained from Harlan Sprague-Dawley Inc., Indianapolis, IN. Animals were individually housed under a 12-hr light/dark cycle with food and water available ad lib. Following sacrifice by decapitation, the brains were quickly removed and placed on ice. The whole cerebral cortex (minus olfactory bulbs), hippocampus, or corpus striatum were then dissected free and weighed.

Receptor Binding

[^{3}H]-Quinuclidinyl benzilate ([^{3}H]-QNB) binding was performed according to the method of Yamamura and Synder (40) as modified by Pedigo *et al.* (29). Tissue from a specific brain region was homogenized in 50 mM Na^+/K^+ phosphate buffer (1:800 w/v) using a Kinematica polytron (setting = 5, 20 sec) and centrifuged at 40,000 × g for 10 min. The resulting pellet was then rehomogenized and the process repeated twice to yield a crude membrane preparation. Saturation isotherms were obtained by adding aliquots of tissue (1–2 mg wet weight) and [^{3}H]-QNB (final concentrations, 18–300 pM), with or without 2.0 μM atropine, to glass tubes containing phosphate buffer in a final volume of 2 ml. Samples were then incubated to equilibrium (60 min at 37°C) and the binding reaction terminated by filtration under reduced pressure through Whatman GF/B filters. Each filter was rinsed 3 times with 4 ml of ice-cold buffer. Radioactivity was extracted overnight in 10 ml Beckman HP scintillation cocktail with dpms measured by a Beckman LS2800 liquid scintillation counter. All assays were carried out in triplicate. Specific binding, defined by the presence of atropine, represented 90–98% of the total binding. Protein was determined by the Lowry method (23). The total number of specific sites (B_{max}) and the ligand's dissociation constant (K_d) were estimated by Scatchard analysis. Analysis of variance (ANOVA) and Student's *t*-test were employed to establish statistical significance.

[^{3}H]-PZ binding was performed according to the method of Watson *et al.* (35). The tissue was prepared as in the [^{3}H]-QNB experiments except an initial 1% tissue homogenate was made in 10 mM Na^+/K^+ phosphate buffer. Tissue (4–5 mg wet wt.) and [^{3}H]-PZ (final concentrations, 0.5–75 nM), with or without 2.0 μM atropine, were incubated to equilibrium in a 2 ml volume for 60 min at 25°C. The reaction was terminated and samples counted as described above. Filters were soaked in 0.1% polyethylenimine for 30–60 min prior to use in order to prevent filter binding of the polar [^{3}H]-PZ. All assays were carried out in triplicate. Specific binding, defined as that which could be inhibited by 2.0 μM atropine, was greater than 90%. Data analysis was performed in a manner similar to the [^{3}H]-QNB studies.

Measurement of Choline Acetyltransferase

Choline acetyltransferase (ChAT) activity was measured according to the method of Fonnum (11,12).

RESULTS

Receptor Binding Experiments

[^{3}H]-QNB binding. Specific binding of [^{3}H]-QNB in the three brain regions examined from young, middle-aged, and aged rats was saturable over the range of concentrations tested with the Scatchard plots of the binding data being linear in all cases. This indicated that in the tissues studied, the labelled ligand bound to a homogenous population of sites. Scatchard plots for [^{3}H]-QNB binding are shown on the right side of Fig. 1 with binding characteristics obtained from the plots found in Table 1. A statistically significant decrease in the number of receptors (B_{max}) was found in the cerebral cortex of aged animals compared to both young (14% difference) and middle-aged animals (19% difference). No changes in B_{max} were measured in the hippocampus or striatum between any of the three age groups and there was no difference in receptor affinity (K_d) for all three brain areas among the age groups.

[^{3}H]-PZ binding. As with the [^{3}H]-QNB studies, receptor binding with [^{3}H]-PZ was saturable and Scatchard plots linear for all brain areas and age groups. Scatchard plots (left side of Fig. 1) and binding characteristics obtained from these plots (Table 1) revealed a significant decrease in receptor density (−17%) in the cerebral cortex of aged rats as compared to young rats. No alterations in B_{max} were seen in striatal or hippocampal regions. Additionally, no differences in K_d were observed among brain regions for the three age groups.

Measurement of Choline Acetyltransferase (ChAT) Activity

In young rats (4 months), ChAT activity (mean ± S.E.M.) in cortex, hippocampus, and striatum was found to be 44.2 ± 1.9, 48.5 ± 1.7, and 140.3 ± 4.5 nmoles/hour/mg protein respectively. There were no significant differences between young and old rats (N = 5–6 for both age groups). ChAT activity in the same brain regions of aged rats (26 months) was 48.2 ± 1.2, 52.6 ± 2.0 and 123.2 ± 5.1 nmoles/hour/mg protein, respectively.

DISCUSSION

Using [^{3}H]-QNB to measure the total population of muscarinic receptors in three brain areas which have considerable cholinergic innervation, a significant decrease (14–19%) in cortical muscarinic receptor density was found in aged male Fisher 344 rats compared to either young or middle-aged rats. Since [^{3}H]-QNB recognizes both M1 and M2 receptor subtypes equally, a second series of receptor binding studies were performed using [^{3}H]-PZ which selectively labels only M1 muscarinic receptors (16, 35, 36, 38, 39). With this ligand, a significant reduction (−17%) was also measured in the cerebral cortex of aged animals compared to young ones. The combined results demonstrate that the number of M1 muscarinic receptors are decreased in the cerebral cortex of aged rats. Since the cortex possesses both M1 and M2 receptors (38), small but significant changes in M2 receptors cannot be ruled out. M2 receptors can be indirectly measured by subtracting the number of M1 sites (PZ-labelled sites) from the total number of receptors (QNB-labelled sites). However, the almost identical decrease in the absolute number of [^{3}H]-QNB and [^{3}H]-PZ binding sites indicates a loss of the M1 subtype binding site.

Measurement of ChAT activity in young and aged rats revealed no significant differences in the three brain regions examined. Since ChAT is a marker for intact cholinergic neurons, it is possible that the decrease in M1 muscarinic receptors is occurring without a concurrent loss of cholinergic neurons. Thus, as has been previously hypothesized (33), M1 receptors may reside on nerve cells postsynaptic to cholinergic neurons.

A decrease in the total number of muscarinic receptors in the cerebral cortex of aged rodents has been consistently reported (10, 15, 17, 28–30, 34). However, two studies using female rats were exceptions where no change in receptor density was found (6,26). In addition to measuring total binding, age-related changes in the conformational state of the muscarinic receptor have also been

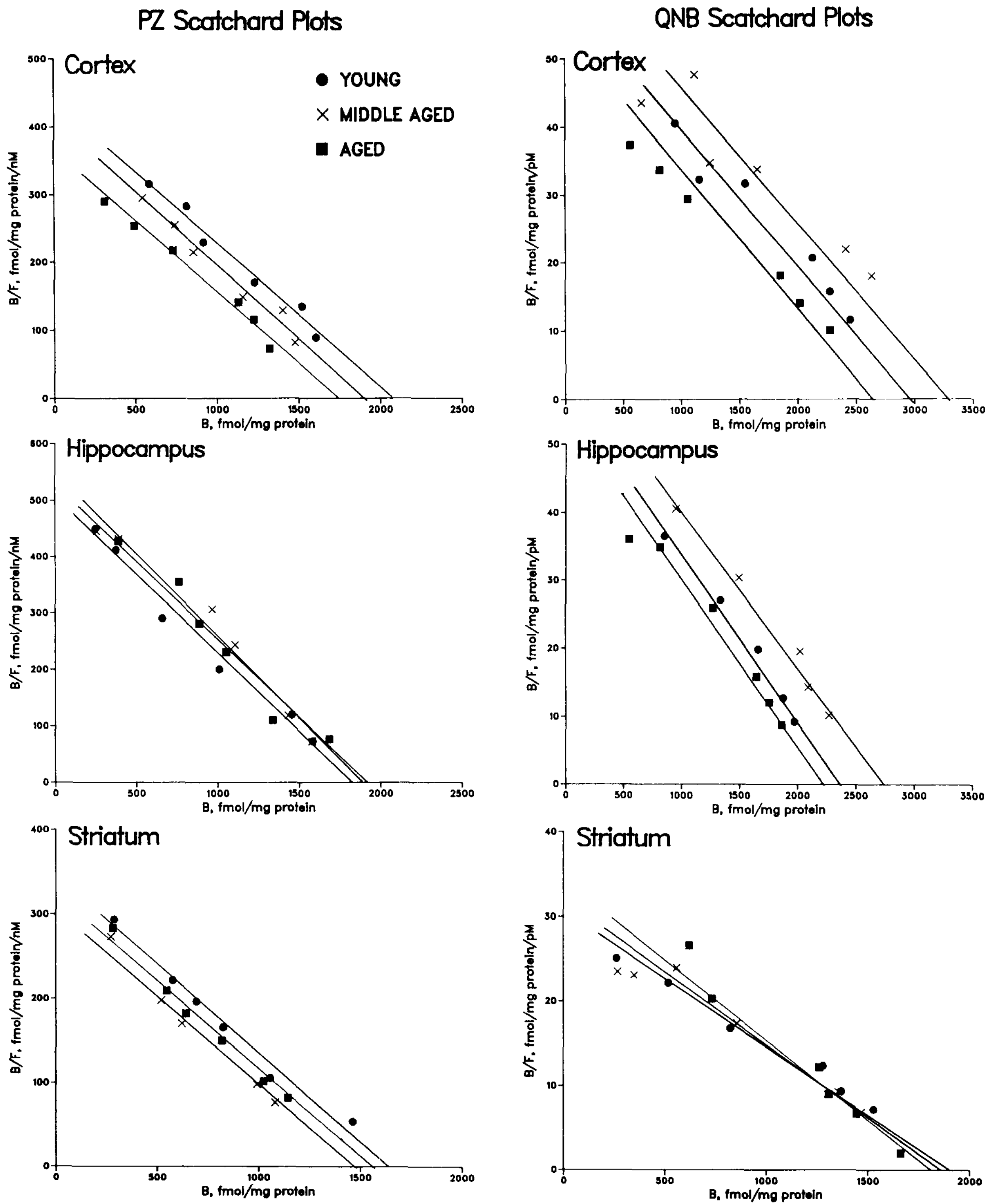

FIG. 1. Scatchard Plots for [^{3}H]-PZ and [^{3}H]-QNB receptor binding obtained using cortex, hippocampus, and striatum of young (3–6 month), middle-aged (15–17 month), and aged (22–26 month) male Fisher 344 rats.

TABLE 1

^{3}H-QNB AND ^{3}H-PIRENZEPINE BINDING IN FISHER 344 RATS

Brain Area	^{3}H-QNB Binding K_D (pM)	^{3}H-QNB Binding B_{max} (fmol/mg protein)	^{3}H-Pirenzepine Binding K_D (pM)	^{3}H-Pirenzepine Binding B_{max} (fmol/mg protein)
Cortex				
3–6 months	56 ± 4	3000 ± 90	4400 ± 400	2100 ± 90
15–17 months	54 ± 3	3200 ± 70	4600 ± 300	1900 ± 90
22–26 months	50 ± 2	2600 ± 50*†	4900 ± 300	1700 ± 50*
Hippocampus				
3–6 months	46 ± 7	2200 ± 50	3500 ± 100	1800 ± 40
15–17 months	47 ± 6	2600 ± 240	3200 ± 200	1900 ± 40
22–26 months	48 ± 10	2300 ± 140	3200 ± 100	1800 ± 90
Striatum				
3–6 months	64 ± 7	1900 ± 120	4600 ± 400	1600 ± 130
15–17 months	62 ± 6	1800 ± 120	4600 ± 500	1500 ± 160
22–26 months	59 ± 7	1800 ± 160	4700 ± 100	1500 ± 120

*$p<0.01$ for young vs. aged.
†$p<0.01$ for middle aged vs. aged.
N = 4–6 in each brain area for the three age groups.

examined. In one study, carbachol displacement of [^{3}H]-QNB binding showed no change in the proportion of high- and low-affinity states of the receptor in young versus aged rats (3). A second study (22), also using [^{3}H]-QNB binding, observed an age-related change in the IC_{50} value and Hill coefficient for oxotremorine, but not for carbachol. This indicated the possible existence of two or more receptor states with differential affinity for oxotremorine in old but not young rats. Agonist, as opposed to antagonist, binding sites were found to be reduced in the dorsal hippocampus of aged Fisher 344 rats as measured by the agonist [^{3}H]-cis methyldioxolane (21). Comparing agonist ([^{3}H]-ACh) and antagonist ([^{3}H]-N-methyl-4-piperidyl benzilate) binding, Gurwitz *et al.* (15) demonstrated a loss of low-affinity agonist sites in all brain areas of aged females, but a mixed response in aged males (a decrease in hypothalamus and an increase in brain stem of high affinity agonist sites). This suggested sexual dimorphism of the aging process in central cholinergic mechanisms. A recent study addressed M1/M2 receptor changes in senescence using [^{3}H]-QNB and [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) binding (28). Similar to the present results, an 18% decrease in cortical B_{max} was measured in aged rats using [^{3}H]-QNB with no change in cortical ChAT activity. [^{3}H]-NMS binding was also decreased by 20% in the cerebral cortex of aged rats, but there were no significant changes in the proportions or affinities of high and low PZ and (−)-NMS sites as measured by their displacement of [^{3}H]-QNB. The interpretation of these findings was that the proportions of M1 and M2 receptors had not been altered with aging. The reasons for discrepency between that study and the present results are not clear. However, the age of the rats used in the Norman study (28) are not noted and marked variation in animal age between studies could account for the difference. Alternatively, [^{3}H]-PZ binding may represent a more quantitative measurement of M1 receptors than displacement by PZ of [^{3}H]-QNB.

Memory impairment, as a result of central cholinergic dysfunction, appears to occur both in aging and in neurodegenerative disorders such as AD/SDAT. Therapeutic treatment of this cognitive decline may be achieved by enhancing or replacing cholinergic neurotransmission (2). However, this may be difficult to achieve if receptors involved in signal transduction are significantly altered. While the effect of aging on the total number of muscarinic receptors within regions of the human brain has been reported (27), measurement of muscarinic receptor subtypes has not been examined in normal aged humans. However, loss of M2 receptors have been reported in persons suffering from AD/SDAT (25). Thus, the present results showing a loss of M1 receptors in rat cerebral cortex, if translatable to humans, may be relevant for designing novel agents in the treatment of age-related cognitive decline.

REFERENCES

1. Allen, S. J.; Benton, J. S.; Goodhardt, M. J.; Haan, E. A.; Sims, N. R.; Smith, C. C.; Spillane, J. A.; Bowen, D. M.; Davison, A. N. Biochemical evidence of selective nerve cell changes in the normal ageing human and rat brain. J. Neurochem. 41:256–265; 1983.
2. Bartus, R. T.; Dean, R. L.; Beer, B.; Lippa, A. S. The cholinergic hypothesis of geriatric memory dysfunction. Science 217:408–417; 1982.
3. Briggs, R. S.; Peterson, M. M.; Cook, P. J. Muscarinic agonist receptor subtypes in aging rat brain. Neurobiol. Aging 3:259–261; 1982.
4. Brown, J. H.; Brown, S. L. Agonists differentiate muscarinic receptors that inhibit cyclic AMP formation from those that stimulate phosphoinositide metabolism. J. Biol. Chem. 259:3777–3781; 1984.
5. Buckley, N. J.; Bonner, T. I.; Brann, M. R. Localization of a family of muscarinic receptor mRNAs in rat brain. J. Neurosci. 8:4646–4652; 1988.
6. Consolo, S.; Wang, J.; Fiornetini, F.; Vezzani, A.; Ladinsky, H. In vivo and in vitro studies on the regulation of cholinergic neurotransmission in striatum, hippocampus, and cortex of aged rats. Brain Res. 374:212–218; 1986.
7. Creasey, H.; Rapoport, S. I. The aging human brain. Ann. Neurol. 17:2–10; 1985.
8. Coyle, J. T.; Price, D. L.; Darling, M. R. Alzheimer's disease: A disorder of cortical cholinergic innervation. Science 219:1184–1190; 1983.
9. Dravid, A. R. Deficits in cholinergic enzymes and muscarinic receptors in the hippocampus and striatum of senescent rats: effect of chronic Hydergine treatment. Arch. Int. Pharmacodyn. 264:195–

202; 1983.
10. Enna, S. J.; Strong, R. Age-related alterations in central nervous system neurotransmitter receptor binding. Aging 17:133–142; 1981.
11. Fonnum, F. Radiochemical micro assays for the determination of choline acetyltransferase and acetylcholinesterase activities. Biochem. J. 115:465–472; 1969.
12. Fonnum, F. A rapid radiochemical method for the determination of choline acetyltransferase. J. Neurochem. 24:407–409; 1975.
13. Gill, D. W.; Wolfe, B. B. Pirenzepine distinguishes between muscarinic receptor-mediated phosphoinositide breakdown and inhibition of adenylate cyclase. J. Pharmacol. Exp. Ther. 232:608–616; 1985.
14. Gonzales, R. A.; Crews, F. T. Characterization of the cholinergic stimulation of phosphoinositide hydrolysis in rat brain slices. J. Neurosci. 4:3120–3127; 1984.
15. Gurwitz, D.; Egozi, Y.; Henis, Y. I.; Kloog, Y.; Sokolovsky, M. Agonist and antagonist binding to rat brain muscarinic receptors: Influence of aging. Neurobiol. Aging 8:115–122; 1987.
16. Hammer, R.; Berrie, C. P.; Birdsall, N. J.; Burgen, A. S.; Hulme, E. C. Pirenzepine distinguishes between different subclasses of muscarinic receptors. Nature 283:90–92; 1980.
17. James, T. C.; Kanungo, M. S. Alterations in atropine sites of the brain of rats as a function of age. Biochem. Biophys. Res. Commun. 72:170–175; 1976.
18. Kerlavage, A. R.; Fraser, C. M.; Venter, J. C. Muscarinic cholinergic receptor structure: molecular biological support for subtypes. Trends Pharmacol. Sci. 8:426–437; 1987.
19. Kubo, T.; Fukuda, K.; Mikami, A.; Maeda, A.; Takahashi, M.; Mishina, M.; Haga, T.; Haga, K.; Ichiyama, A.; Kangawa, K.; Matsuo, H.; Hirose, T.; Numa, S. Cloning, sequencing, and expression of complementary DNA encoding the muscarinic acetylcholine receptor. Nature 323:411–416; 1986.
20. Lippa, A. S.; Pelham, R. W.; Beer, B.; Critchett, D. J.; Dean, R. L.; Bartus, R. T. Brain cholinergic dysfunction and memory in aged rats. Neurobiol. Aging 1:13–19; 1980.
21. Lippa, A. S.; Critchett, D. J.; Ehlert, F.; Yamamura, H. I.; Enna, S. J.; Bartus, R. T. Age-related alterations in neurotransmitter receptors: an electrophysiological and biochemical analysis. Neurobiol. Aging 2:3–8; 1981.
22. Lippa, A. S.; Loullis, C. C.; Rotrosen, J.; Cordasco, D. M.; Critchett, D. J.; Joseph, J. A. Conformational changes in muscarinic receptors may produce diminished cholinergic neurotransmission and memory deficits in aged rats. Neurobiol. Aging 6:317–323; 1985.
23. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with folin phenol reagent. J. Biol. Chem. 193:265–275; 1951.
24. Mann, D. M.; Yates, P. O. Neurotransmitter deficits in Alzheimer's disease and in other dementing disorders. Hum. Neurobiol. 5: 147–158; 1986.
25. Mash, D. C.; Flynn, D. D.; Potter, L. T. Loss of M2 muscarinic receptors in the cerebral cortex in Alzheimer's disease and experimental cholinergic denervation. Science 228:1115–1117; 1985.
26. Morin, A. M.; Wasterlain, C. G. Aging and rat brain muscarinic receptors as measured by quinuclidinyl benzilate binding. Neurochem. Res. 5:301–308; 1980.
27. Nordberg, A.; Winblad, B. Brain nicotinic and muscarinic receptors in normal aging and dementia. In: Fisher, A.; Hanin, I.; Lachman, C., eds. Alzheimer's and Parkinson's disease: Strategies in research and development. New York: Plenum Press; 1986:95–107.
28. Norman, A. B.; Blaker, S. N.; Thal, L.; Creese, I. Effects of aging and cholinergic deafferentation on putative muscarinic cholinergic receptor subtypes in rat cerebral cortex Neurosci. Lett. 70:289–294; 1986.
29. Pedigo, N. W.; Minor, L. D.; Krumrei, T. N. Cholinergic drug effects and brain muscarinic receptor binding in aged rats. Neurobiol. Aging 5:227–233; 1984.
30. Pedigo, N. W.; Polk, D. M. Reduced muscarinic receptor plasticity in frontal cortex of aged rats after chronic administration of cholinergic drugs. Life Sci. 37:1443–1449; 1985.
31. Peralta, E. G.; Ashkenazi, A.; Winslow, J. W.; Smith, D. H.; Ramachandran, J.; Capon, D. J. Distinct primary structures, ligand-binding properties and tissue-specific expression of four human muscarinic acetylcholine receptors. EMBO J. 6:3923–3929; 1987.
32. Perry, E. K. The cholinergic hypothesis—ten years on. Br. Med. Bull. 42:147–158; 1986.
33. Raiteri, M.; Leardi, R.; Marchi, M. Heterogeneity of presynaptic muscarinic receptors regulating neurotransmitter release in the rat brain. J. Pharmacol. Exp. Ther. 228:209–214; 1984.
34. Strong, R.; Hicks, P.; Bartus, R. T.; Enna, S. J. Age-related alterations in the rodent brain cholinergic system and behavior. Neurobiol. Aging 1:59–63; 1980.
35. Watson, M.; Roeske, W. R.; Yamamura, H. I. [^{3}H]pirenzepine selectively identifies a high affinity population of muscarinic cholinergic receptors. Life Sci. 31:2019–2023; 1982.
36. Watson, M.; Vickroy, T. W.; Roeske, W. R.; Yamamura, H. I. Subclassification of muscarinic receptors based upon the selective antagonist pirenzepine. Trends Pharmacol. Sci. (Suppl.) 1:9–11; 1984.
37. Watson, M.; Vickroy, T. W.; Fibiger, H. C.; Roeske, W. R.; Yamamura, H. I. Effects of bilateral ibotenate-induced lesions of the nucleus basalis magnocellularis upon selective cholinergic biochemical markers in the rat anterior cerebral cortex. Brain Res. 346: 387–391; 1985.
38. Watson, M.; Roeske, W. R.; Vickroy, T. W.; Smith, T. L.; Akiyama, K.; Gulya, K.; Duckles, S. P.; Serra, M.; Adem, A.; Nordberg, A.; Gehlert, D. R.; Wamsley, J. K.; Yamamura, H. I. Biochemical and functional basis of putative muscarinic receptor subtypes and its implications. Trends Pharmacol. Sci. (Suppl.) 2:46–55; 1986.
39. Watson, M.; Roeske, W. R.; Yamamura, H. I. Cholinergic receptor heterogeneity. In: Meltzer, H. Y., ed. Psychopharmacology: The third generation of progress. New York: Raven Press; 1987:241–248
40. Yamamura H. I.; Synder, S. H. Muscarinic cholinergic binding in rat brain. Proc. Natl. Acad. Sci. USA 71:1725–1729; 1974.