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Memory and Postsynaptic Cholinergic Receptors in Aging Mice

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KUBANIS, P., S. F. ZORNETZER AND G. FREUND. *Memory and postsynaptic cholinergic receptors in aging mice.* PHARMAC. BIOCHEM. BEHAV. 17(2) 313–322, 1982.—Significant retention deficits were observed on passive avoidance tasks (step-down and step-through) in 15-, 20-, and 25-month-old male C57BL/6 mice compared with 4- and 8-month-old mice. In contrast, cholinergic muscarinic receptor binding ([^{3}H]quinuclidinyl benzilate) in cerebral cortex, striatum, hippocampus, and cerebellum in these same animals revealed no difference in this 4- to 25-month age range. In a separate comparison of 4- and 29-month-old female mice, [^{3}H]QNB binding was significantly decreased in the older group in cerebral cortex, hippocampus, and striatum. Environmental enrichment, compared with an impoverished environment, significantly improved retention in mice on 24-hr step-down performance but affected QNB binding only minimally (6–7% decrease of QNB binding in cerebral cortex and hippocampus). Benzodiazepine ([^{3}H]flunitrazepam) receptor binding was significantly (12–15%) decreased in 29-month-old mice compared with 4-month-old mice in the cerebral cortex, hippocampus, cerebellum, and brain stem.

Aging Cholinergic receptors Avoidance learning Benzodiazepine Enviromental enrichment

CHOLINERGIC transmitter systems appear important for the formation of memory because selective pharmacological suppression of these systems causes impairment of memory [5, 8, 9] and anticholinesterase drugs that potentiate these systems can result in facilitation of memory [8]. Both CNS cholinergic function and memory decline with aging in humans and animals [6, 11, 13, 15, 16, 18, 19, 22], but it is not firmly established that these biochemical changes are causes or even effects of the age-related behavioral changes. The purpose of this investigation was to test the "cholinergic hypothesis" further by determining whether age-related decrements in memory are correlated with a similar decrease of postsynaptic cholinergic receptors in various regions of mouse brain. Radioautographic studies have shown that degeneration of neurons causes a loss of the synapses on their surface including their postsynaptic receptors [25]. Acute presynaptic lesions may result in denervation supersensitivity as a result of a compensatory increase in postsynaptic receptor molecule numbers (upregulation) in the remaining synapses. In either case all postsynaptic events depend on the chemical interaction between the transmitter and the receptor molecule. In this context, the determination of synaptic receptor number per volume of tissue (by measuring the radioactivity of specifically bound cholinergic antagonists) is a chemical equivalent of counting the total maximal capacity of cholinergic synapses. This measure represents the maximal, rate-limiting number of postsynaptic receptor molecules available for interaction with their respective transmitter—that is, the maximal capacity for interaction between these neurons. A loss of neurons or synapses results in a loss of the associated receptors and their subsequent coupling events [7, 10, 25]. However, synapses appearing to be morphologically normal could well be functionally impaired [4] and contain receptors decreased in numbers or affinity.

Beyond such correlations between age, performance, and receptors, we attempted to actively manipulate behavior and receptors simultaneously by enriching and impoverishing the environment [1]. If environmentally-induced alterations of both learning and receptors were synchronous, quantitatively related in time and direction, then the cholinergic hypothesis would be greatly strengthened. In contrast, the lack of such relationships between behavior and receptors would not disprove the cholinergic hypothesis, but instead could be a result of suboptimal behavioral or biochemical conditions, which could guide future experiments.

To determine the effects of age and environment on behavior and receptors, we designed our experiments as follows: Five age groups of male mice were submitted to three behavioral tests: spontaneous alternation, step-through passive avoidance, and step-down passive avoidance. Both acquisition and retention of passive avoidance were tested. To determine the effect of altering the environment on performance, we subdivided each age group into mice raised in

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enriched (EC) and impoverished (IC) environments. After completion of the behavioral experiments, brains were removed, dissected into various regions, and density and affinity of cholinergic receptors were determined in homogenates of these regions. A third group of mice, housed under standard conditions (SC) and not submitted to behavioral testing, was included in the receptor binding studies. The effect of very old age on receptor binding was determined in a separate comparison of 4- and 29-month-old female mice.

Benzodiazepine receptor binding was assayed in these animals in addition to cholinergic receptor binding. Fear and conflict situations induce the release of specific endogenous ("anxiolytic") synaptic transmitters in the brain [28] analogous to the release of endorphins in response to painful stimuli. These transmitters interact with the same receptors that are also occupied by the benzodiazepines (BZ), a group of anxiolytic drugs (in analogy to opiates occupying endorphin receptors). These anxiolytic receptors are present in lower concentrations in animals genetically more susceptible to fear (e.g., Maudsley reactive rats [24]). The decrease of BZ receptors in the brain with chronic alcohol consumption [12] and aging also probably predisposes to a greater sensitivity to fear-inducing stimuli. This would be expected to enhance performance of avoidance behaviors because of enhanced motivation. But, in fact, the opposite happens—namely, a decreased performance of avoidance behavior with increasing age. This decrease is probably attributable to an impairment of associative processes of learning that cannot be counterbalanced by enhanced motivation.

METHOD

Behavioral Experiments

Animals. The male C57BL/6 mice were 3, 7, 14, 19, and 24 months of age at the beginning of the behavioral experiments and were purchased from Charles River breeders. All female C57BL/6 mice, used for receptor studies only, were purchased from Jackson Laboratories when they were 8 weeks old and were housed in the animal colony (supervised by G. F.) under identical condition 6 mice/cage until ages 4 and 29 months. They received Purina Lab Chow and water ad lib.

Housing conditions and environmental manipulations. The male animals were housed for 1 month before and during testing in either an EC or an IC environment:

Twenty animals in each age group were placed in the EC environment and were housed 10/cage in large metal cages (60×45×26 cm). These cages contained a variety of objects—ladders, sandboxes, ramps, tunnels, running wheels, mirrors, and colored movable toys of different sizes—to provide perceptual and sensorimotor stimulation. A unique enriched environment was set up in each of the 10 cages. Every 2 days the environments were rotated from cage to cage to expose all animals to each environment and provide continually changing environments.

The remaining 20 animals in each age group were placed in the IC environment. These animals were housed individually in metal cages (24×18×17 cm). No exposure to other mice was allowed during this period, and the cages contained no play objects. Racks of cages were arranged so that animals could see only a wall or divider separating them from an adjacent rack of cages.

All animals (EC and IC) were handled only during cage cleaning once a week. Food and water were provided ad lib, room temperature was approximately 23°C, and a 7 a.m. to 7 p.m. light-dark cycle was maintained. Cage floors were covered with wood shavings. Although animals in both conditions were housed in the same room, IC animals were confined to a far corner and separated by a divider to prevent unintentional environmental stimulation.

Spontaneous Alternation

Apparatus. The apparatus consisted of a T-maze constructed of white Plexiglas. The start alley measured 51 cm long × 7 cm wide × 10 cm high. Perpendicular to the start alley, the left- and right-side arms measured 15 cm long × 7 cm wide × 10 cm high.

Procedure. Mice were tested by a single-trial method considered preferable to multi-trial methods [23]. In a dimly lit testing room each mouse was first placed in the start alley and allowed to enter whichever side arm it preferred. A Plexiglas divider was then placed behind the mouse, confining it to the chosen arm for 5 sec. The mouse was then removed and placed in a holding cage for 45 sec before retesting. On the basis of directional choice on the second trial, the behavior of each animal was classified as alternating (choosing the opposite arm) or not alternating (choosing the same arm). The apparatus was cleaned with 50% ethyl alcohol between animals.

Step-Through Passive Avoidance

Apparatus. The step-through apparatus was a trough-shaped chamber consisting of a smaller (7 cm long) brightly illuminated compartment and a longer (22 cm long) dark compartment constructed of Plexiglas. The width of the larger compartment at the top was 10 cm; at the floor it was 2.5 cm. A bright lamp illuminated the outside compartment. The floor of the inside compartment consisted of two metal plates through which DC footshock was delivered from a Scientific Products shock source.

Procedure. During training each mouse was placed in the illuminated compartment of the chamber, facing away from the entrance to the dark compartment. Initial step-through latency (STL) was recorded. After the animal completely entered the dark compartment, a footshock (300 μA) was delivered to the floor of the apparatus and continued until the animal escaped to the illuminated compartment. The mouse was then returned to its home cage. Any animal that did not enter the dark compartment in 300 sec was assigned an initial STL of 300 and was eliminated from further testing.

Animals were randomly divided into two test groups. Approximately two-thirds of the animals from each age group were tested for retention 5 days after training. The remaining one-third were tested 2 hr after training. The 2-hr group was included to examine the effects of age and environment on acquisition of this task. Thus, poor initial acquisition would presumably be reflected in poor performance scores 2-hr after training. Good performance measured 2 hr after training, but poor performance measured 5 days after training, would indicate a long-term retention impairment.

Testing was identical to training except that footshock was not delivered. Step-through latency difference scores were calculated by subtracting each animal's initial STL from its testing STL. An arbitrary ceiling of 600 sec was imposed on the STL difference scores.

Step-Down Passive Avoidance

Apparatus. The step-down apparatus consisted of a box

18 cm long × 13 cm wide × 16 cm high. The long walls were constructed of clear Plexiglas, and the end walls and top were black-painted aluminum. An aluminum platform 6 × 6 cm was attached to one of the end walls approximately 2.5 cm above the floor of the apparatus. Footshock was delivered to the grid floor from a Lafayette AC shock source by activating a foot pedal.

Procedure. A single-trial procedure was used in these experiments because reliable age-related deficits in Swiss mice have been found by the use of this procedure [17]. During training each mouse was placed on the platform, and initial step-down latency (SDL) was recorded. After all four paws contacted the grid floor, footshock was delivered until the mouse escaped to the platform. The animal then was removed from the apparatus and returned to its home cage.

Animals were tested either 24 hr or 2 hr after training. Approximately two-thirds of the animals were tested for retention after 24 hr on the basis of deficits we have found in aged Swiss mice at this training-test interval [17]. The remaining one-third were tested after 2 hr to evaluate acquisition, as in the step-through experiment.

Testing was identical to training except for the absence of footshock. Step-down latency difference scores were calculated by subtracting initial SDL from testing SDL. A 600-sec ceiling was imposed on the difference scores.

Receptor Binding

After the behavioral testing, we investigated the effects of age and differential environments on muscarinic cholinergic binding of quinuclidinyl benzilate [^{3}H]QNB (New England Nuclear Corp.) in the cerebral cortex, striatum, hippocampus, and cerebellum. In the comparison of environment, a third housing condition the standard condition (SC) was included. Mice in the SC environment were group housed (six per cage) in clear plastic cages, 40×24×15 cm. No behavioral testing was performed on these animals. Therefore, SC mice served as controls for possible effects of training and testing procedures on receptor binding.

Because behavioral data were available on individual EC and IC animals, an additional variable, performance, was included in the binding studies. Animals were classified as either good or poor performers on the basis of their STL and SDL difference scores. The best and worst performers in each age group were selected for this dichotomous classification.

To determine the effect of very old age on cholinergic receptors, we assayed [^{3}H]QNB binding assayed in 4- and 29-month-old mice. Benzodiazepine receptor binding was evaluated in these animals by regional assay of [^{3}H]flunitrazepam (FNP).

Subjects. Animals in the EC and IC conditions were approximately 4, 8, 15, 20, and 25 months of age by the end of the behavioral experiments. Animals in the SC condition were obtained at the same time as the EC and IC animals and therefore were the same ages. Eighty animals in total—30 EC, 30 IC, and 20 SC—were used for [^{3}H]QNB binding assays.

In the comparison of young and very old mice, subjects were female C57BL/6J mice (Jackson Laboratories), ages 4 and 29 months. These animals (eight in each group) were raised under identical conditions from the age of 2 months and were not exposed to any experimental treatment before sacrifice for binding assays.

Dissection. Immediately after decapitation, intact heads were sealed in plastic bags and placed in a −15°C freezer. After 24 hr the bags were transferred to a −60°C freezer for storage. Twenty-four hours before dissection, they were returned to the −15°C freezer and exactly 1 hr before dissection each brain was allowed to thaw to 6°C in a refrigerator. It was determined during pilot experiments that this particular combination of freezing temperatures and durations yielded brain tissue most closely resembling fresh tissue for dissecting.

After 1 hr of thawing each brain was removed from the skull and placed on a glass plate over ice. A fiber optics light provided cool illumination during dissection under a dissecting microscope. As each structure was removed, it was immediately weighed and placed into ice cold Tris (pH 7.4) approximately 5% tissue wt/vol for homogenization. The homogenates were frozen at −15°C for subsequent binding assays.

The frontoparietal cortices of both sides weighing from 950 to 1085 mg were dissected free from their underlying white matter from the longitudinal fissure to the lateral olfactory tract. The entire striatum, easily recognized by its darker color, was dissected free; both sides were pooled and weighed from 210 to 226 mg. The entire hippocampi were removed, both sides were pooled and weighed from 30.5 to 32.1 mg. The cerebellum was removed in toto and weighed from 53.4 to 55.5 mg. "Brain stem" is the medulla from the for foramen magnum to the rostral border of the cerebellum (inferior colliculi).

Receptor Binding Assays

A modified version of the [^{3}H]QNB binding method of Yamamura and Snyder [29] was used as described in detail elsewhere [11]. Pilot experiments established previously that the above freezing and thawing procedures did not affect the binding assays. On separate 100-ml aliquots of resuspended homogenate, protein was determined in triplicate according to the method of Lowry, Rosebrough, Farr and Randall [20]. The procedure for the [^{3}H]FNP binding assay was similar to that used in the [^{3}H]QNB binding assay. It is the method developed by Speth, Wastek, Johnson and Yamamura [26] modified and adapted to mouse brain regions [12]. Scatchard plots were calculated according to Bennett [2].

Statistical Analysis

For both [^{3}H]QNB and [^{3}H]FNP, maximal specific binding was calculated by subtracting the mean cpm of radioactivity of the three nonspecific samples from the mean cpm of the three total binding samples. These data were expressed as fmol/mg protein as determined by the Lowry assay [20].

The effects of age and environment on [^{3}H]QNB binding were analyzed by two-way analysis of variance procedures (ANOVA) for each brain region: cortex, striatum, hippocampus, and cerebellum. Twelve to 16 animals in each age group were included in the assays.

For analyzing the relationship between performance and receptor binding, one-way ANOVA comparisons of [^{3}H]QNB binding in good performers vs poor performers were calculated for each brain region. Because only the EC and IC animals were behaviorally tested, SC animals were not included in these analyses. Spearman rank correlation coefficients were also calculated to determine the degree of association in individual animals between difference scores on the passive avoidance tasks and regional binding of [^{3}H]QNB.

Maximal binding of [^{3}H]FNP in the 4- vs 29-month comparison was calculated as described above. The effect of age on binding of each ligand was analyzed using one-way ANOVA procedures.

Autopsies

To be certain that possible differences in behavior or receptor binding between age groups were not due to the presence of disease conditions in older animals, we performed postmortem examinations. Twenty of the 25- and 20-month-old animals and 10 of the 4-, 8-, and 15-month-old animals were autopsied grossly. Autopsies were performed on all of the animals in the 4- vs 29-month comparison. After the animals were killed by cervical dislocation and decapitation, the peritoneal cavity was opened, and all internal organs were examined carefully. Each organ system was inspected and then dissected with a scalpel to look for tumors, signs of inflammation, infection, enlargement, atrophy, or other abnormalities.

RESULTS

Behavioral Comparisons

Spontaneous alternation. The overall frequency of alternation in all animals tested (n=181) was 67.4%. A one-way χ^2 test indicated that this differed significantly from a chance level of alternation, $\chi^2(1)=21.24, p<0.001$. Table 1 shows the frequency of alternation in each age group and the EC and IC comparison. Although there appeared to be a slight decrease in alternation with increasing age, a two-way χ^2 test indicated that this difference was not statistically significant, $\chi^2(4)=4.86, p>0.30$. A two-way χ^2 test indicated no difference between EC animals and IC animals in frequency of spontaneous alternation $\chi^2(1)=0.19, p>0.60$.

Step-Through Passive Avoidance

Effect of age. In the 2-hr acquisition experiment Kruskal-Wallis analysis of variance indicated a significant effect of age on STL difference scores, $H(4)=10.16, p<0.04$. Figure 1A shows the median STL difference scores for all age groups and demonstrates that STL difference scores of 25-month-old mice were significantly lower than those of 4-, 8-, or 15-month-old mice. Behavioral observations of mice placed back in the apparatus 2 hr after initial training and footshock did not indicate any incidence of freezing or abnormal autonomic arousal. In general, mice sniffed, reared, and were active while remaining in the outer compartment. A Kruskal-Wallis analysis of variance revealed no significant effect of age on initial STL in the 2-hr acquisition experiment, $H(4)=7.94, p>0.09$.

The effect of age on STL difference scores in the 5-day retention experiment was statistically significant, $H(4)=24.89, p<0.0001$. Figure 1B shows the median STL difference scores for all age groups and demonstrates that, in general, performance of the 15-, 20-, and 25-month-old groups was impaired relative to the 4- and 8-month-old groups. There was no significant effect of age on initial STL in the 5-day retention experiment, $H(4)=1.05, p>0.90$.

Effect of environment. Statistical comparison of EC and IC animals indicated no effect of environment on STL difference scores in the 2-hr acquisition experiment, $H(1)=0.84$, $p>0.35$. Median 2-hr STL difference scores for both the EC group (n=30) and the IC group (n=27) were 600 sec. There was no significant difference between initial STLs of EC and IC animals in the 2-hr experiment.

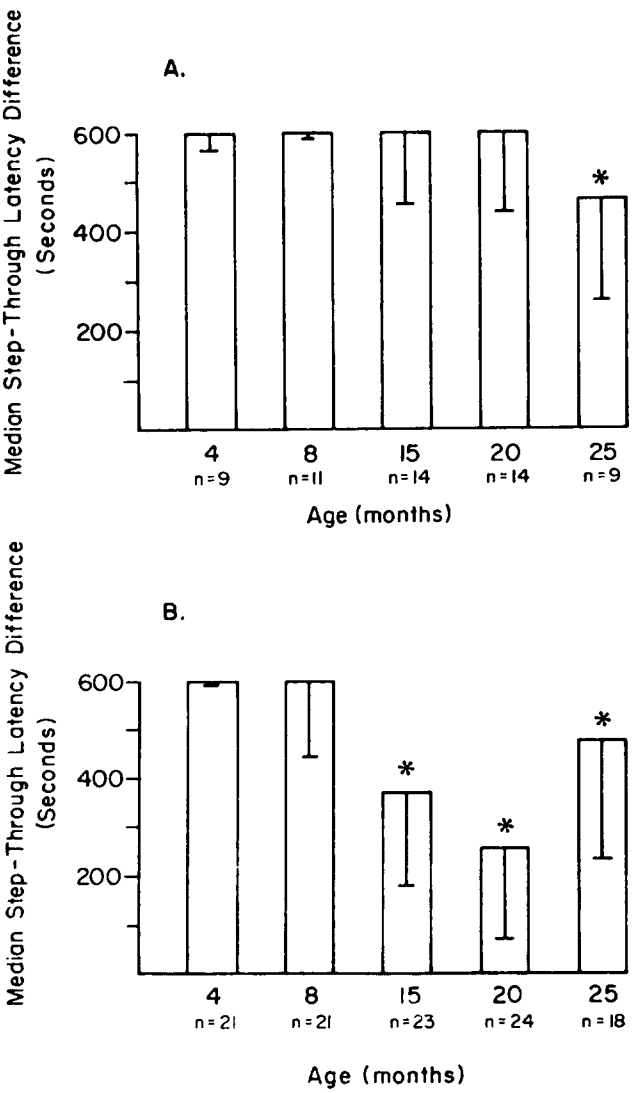

FIG. 1. Age comparison of performance on step-through passive avoidance. A. Acquisition. STL difference scores by age, using a 2-hr training-test interval. Asterisk indicates significantly different from 4-, 8-, and 15-month-old groups. B. Retention. STL difference scores by age, using a 5-day training-test interval. Asterisk indicates significantly different from 4-month-old group.

In the 5-day step-through retention experiment the effect of environment on STL difference scores was not statistically significant, $H(1)=0.40, p>0.52$. Median STL difference scores were 432 sec and 540 sec for the EC group (n=53) and IC group (n=53), respectively. The effect of environment on initial STL in this experiment was statistically significant, $H(1)=10.92, p<0.001$. Median initial STLs were 16 sec and 31 sec for the EC and IC groups, respectively.

Step-Down Passive Avoidance

Effect of age. In the 2-hr acquisition experiment the effect of age on SDL difference scores was not statistically significant, $H(4)=3.45, p>0.48$. Figure 2A shows the median SDL difference scores for all age groups. Analysis of the effect of age on initial SDL in this experiment revealed no significant difference, $H(4)=4.38, p>0.35$. As in the previous step-

TABLE 1

FREQUENCY OF SPONTANEOUS ALTERNATION COMPARED BY AGE AND ENVIRONMENT

	(n)	Frequency of Alternation
Age (months)		
4	(38)	79.0%
8	(36)	72.2%
15	(38)	60.5%
20	(36)	58.3%
25	(33)	66.7%
Environment		
EC	(87)	69.0%
IC	(94)	66.0%

through experiment, mice returned to the elevated platform 2 hr after initial training did not show any unusual freezing behavior. They explored the platform and engaged in frequent rearing and turning behavior.

In the 24-hr step-down retention experiment the effect of age on SDL difference scores was statistically significant, $H(4)=16.30$, $p<0.003$. Figure 2B shows the median SDL difference scores for all age groups and demonstrates that SDL difference scores of 15-, 20-, and 25-month-old animals were significantly lower than those of the 4-month-old animals. A comparison of the SDL difference scores in the 8- and 20-month-old animals was also significant.

In this experiment the effect of age on initial SDL was statistically significant, $H(4)=10.45$, $p<0.04$. Median initial SDLs were 30, 9, 11, 4, and 4 sec for the groups 4, 8, 15, 20, and 25 months of age, respectively. It is unlikely that this difference could account for the significant effect of age on 24-hr SDL difference scores. Since SDL difference scores are calculated by subtracting initial SDL from testing SDL, the longer initial SDLs in the 4-month-old group would tend to lower that group's SDL difference scores. Nevertheless, SDL difference scores of the 4-month-old group were significantly higher than those of the older groups.

Effect of environment. Statistical comparison of EC and IC animals indicated that environment did not significantly affect SDL difference scores in the 2-hr acquisition experiment, $H(1)=0.24$, $p>0.62$. There was no significant effect of environment on initial SDL, $H(1)=0.41$, $p>0.52$.

In the 24-hour step-down retention experiment SDL difference scores of EC animals were significantly higher than those of IC animals, $H(1)=4.19$, $p<0.04$. The median SDL difference score was 514 sec for the EC group (n=44), compared with 368 sec for the IC group (n=53). There was no significant difference between the two groups in initial SDL, $H(1)=0.30$, $p>0.58$.

Receptor Binding

The effects of age on [^{3}H]QNB binding are shown in Table 2 and effects of environmental changes in Table 3. Two-way ANOVA procedures indicated no statistically significant effects of aging, environment, or aging × environment interaction ($p>0.05$ in all regions). Table 4 shows that there was no significant relationship between the level of performance and [^{3}H]QNB binding.

To examine the relationship between [^{3}H]QNB binding values and performance of individual animals, we calculated Spearman rank correlation coefficients. Binding values of [^{3}H]QNB were correlated with difference scores on step-through and step-down passive avoidance. The results of these correlations are shown in Table 5. The only correlation that was statistically significant was between [^{3}H]QNB binding in the hippocampus and 24-hr difference scores on step-down avoidance ($\rho=0.475$, $p<0.03$).

In the comparison of 4- and 29-month-old animals, statistically significant age-related differences in binding of

TABLE 2

EFFECT OF AGE ON REGIONAL BINDING OF [^{3}H]QNB IN THE C57BL/6 MOUSE

	Age									
	4 Months		8 Months		15 Months		20 Months		25 Months	
Region	[^{3}H]QNB* (fmol/mg protein)	n	[^{3}H]QNB* (fmol/mg protein)	n	[^{3}H]QNB* (fmol/mg protein)	n	[^{3}H]QNB* (fmol/mg protein)	n	[^{3}H]QNB* (fmol/mg protein)	n
Cerebral Cortex	1096 ±20	(14)	1050 ±48	(15)	1099 ±32	(15)	1056 ±30	(16)	973 ±45	(16)
Striatum	1195 ±54	(15)	1133 ±47	(16)	1077 ±34	(16)	1061 ±39	(16)	1065 ±59	(16)
Hippocampus	710 ±41	(12)	803 ±20	(12)	792 ±18	(12)	763 ±23	(11)	710 ±22	(12)
Cerebellum	103 ±8	(12)	92 ±6	(11)	109 ±5	(12)	89 ±7	(12)	104 ±4	(12)

*Mean ± S.E.M.

TABLE 3

EFFECT OF ENVIRONMENT ON REGIONAL [^{3}H]QNB BINDING IN THE C57/BL6 MOUSE

	Environment					
	SC		EC		IC	
Region	[^{3}H]QNB* (fmol/mg protein)	n	[^{3}H]QNB* (fmol/mg protein)	n	[^{3}H]QNB* (fmol/mg protein)	n
Cerebral Cortex	1102 ±22	(20)	1052 ±26	(28)	1020 ±34	(28)
Striatum	1041 ±39	(20)	1116 ±37	(30)	1138 ±34	(29)
Hippocampus	752 ±24	(20)	777 ±15	(19)	753 ±23	(20)
Cerebellum	106 ±4	(20)	101 ±6	(20)	91 ±5	(19)

*Mean ± S.E.M.

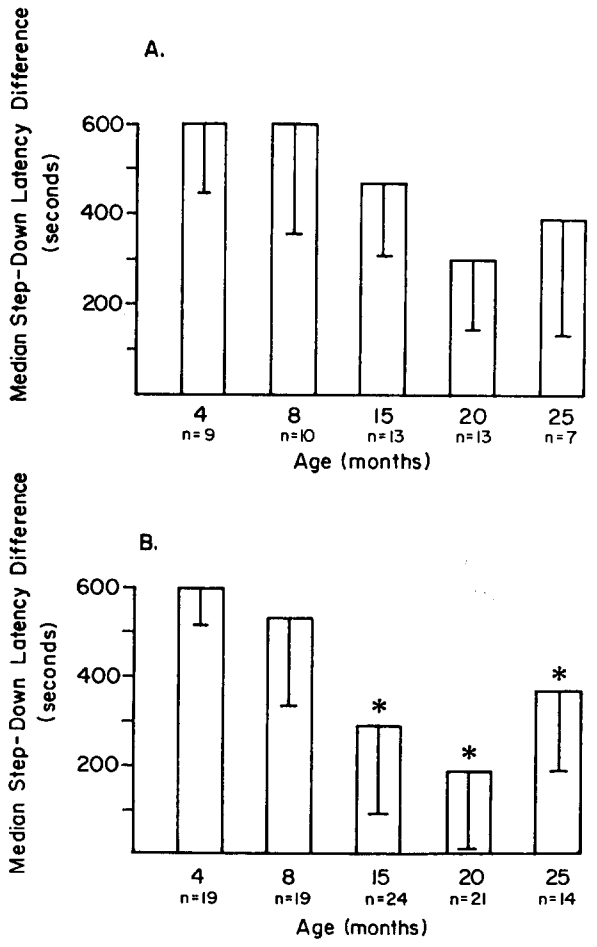

FIG. 2. Age comparison of performance on step-down passive avoidance. A. Acquisition. SDL difference scores by age, using a 2-hr training-test interval. B. Retention. SDL difference scores by age, using a 24-hr training-test interval. Asterisk indicates significantly different from 4-month-old group.

TABLE 4

COMPARISON OF REGIONAL [^{3}H]QNB BINDING IN ANIMALS DEMONSTRATING GOOD AND POOR PERFORMANCE ON PASSIVE AVOIDANCE

	Performance Level			
	Good		Poor	
Region	[^{3}H]QNB* (fmol/mg protein)	n	[^{3}H]QNB* (fmol/mg protein)	n
Cerebral Cortex	1074 ±18	(28)	998 ±38	(28)
Striatum	1148 ±35	(29)	1107 ±36	(30)
Hippocampus	788 ±16	(20)	739 ±21	(19)
Cerebellum	96 ±5	(20)	96 ±6	(19)

*Mean ± S.E.M.

TABLE 5

SPEARMAN RANK CORRELATION COEFFICIENTS BETWEEN [^{3}H]QNB BINDING AND PASSIVE AVOIDANCE RETENTION

Region	Step-Through 5 days	Step-Down 24 hr
Cerebral Cortex	ρ=0.124 p>0.42	ρ= 0.172 p>0.34
Striatum	ρ=0.112 p>0.45	ρ=−0.048 p>0.79
Hippocampus	ρ=0.022 p>0.89	ρ= 0.475 p<0.03
Cerebellum	ρ=0.055 p>0.75	ρ= 0.026 p>0.59

TABLE 6

AGE COMPARISON OF REGIONAL BINDING OF CHOLINERGIC [^{3}H]QNB AND BENZODIAZEPINE [^{3}H]FNP RECEPTORS IN THE C57BL/6J MOUSE

Region	[^{3}H]QNB Binding* (fmol/mg protein) Age			[^{3}H]FNP Binding* (fmol/mg protein) Age		
	4 Months	29 Months	Decrease %	4 Months	29 Months	Decrease %
Cerebral Cortex	1185 ±14	950† ±13	19.8	1186 ±23	1039† ±22	12.4
Striatum	1473 ±20	1067† ±14	27.6	467 ±16	426 ±18	8.8
Hippocampus	939 ±37	840† ±23	10.5	899 ±16	781† ±32	13.1
Cerebellum	121 ±7	115 ±5	5.0	538 ±7	459† ±9	14.7
Brain Stem	403 ±12	329† ±28	18.3	624 ±54	529† ±50	15.2

*Mean ± S.E.M. of 8 animals in each age group.
†Significantly different from 4-month-old group ($p<0.05$.

[^{3}H]QNB and [^{3}H]FNP were observed in several brain regions. Table 6 shows that maximal [^{3}H]QNB binding decreased significantly in the aged animals in all regions except cerebellum and maximal [^{3}H]FNP binding decreased significantly in all regions except hippocampus. Binding values of [^{3}H]QNB were somewhat higher overall in this experiment (compared with the previous experiments) because L-QNB was used after discontinuation of the D,L-form of the isotope by the manufacturer.

Because the amount of tissue available was limited, Scatchard analyses were performed on homogenates pooled from the eight animals in each age group. This quantity of tissue was sufficient for determination of 4 points on the Scatchard plots. Therefore, no statistical analysis was possible, and the results of the Scatchard analyses can only be considered suggestive. Two representative Scatchard plots are shown in Figs. 3 and 4. There appeared to be a 35% increase in affinity for [^{3}H]QNB in the striatum of the aged group. In all other regions there was no difference in affinities for either [^{3}H]QNB or [^{3}H]FNP between young and aged animals.

Autopsies

Results of postmortem examinations on the EC, IC, and SC animals revealed one skin tumor in a 20-month-old SC animal. No other gross abnormalities were observed in the sample of animals autopsied.

Autopsies on the 4- and 29-month-old animals showed no abnormalities in the eight young animals. Two of the aged mice had small pituitary adenomas. A pleural exudate was also observed in one of the two mice with pituitary tumors.

In view of the relatively high incidence of disease in the aged group, binding values in the two abnormal animals were compared with binding values in the six healthy appearing aged animals for each region. There was no significant difference between these two groups in [^{3}H]QNB binding or [^{3}H]FNP binding in any of the brain regions examined.

DISCUSSION

The results of this experiment, as a whole, do not support the cholinergic hypothesis of age-related memory loss because the age-related decrease in passive avoidance performance occurred at a relatively early age before any decline of cholinergic receptors was detectable. In the behavioral experiments significant retention deficits on passive avoidance tasks were observed in 15-, 20-, and 25-month-old male C57BL/6 mice compared with 4- and 8-month-old mice. A comparison of [^{3}H]QNB binding in the same animals from these five age groups revealed no significant effect of age on [^{3}H]QNB binding in the cerebral cortex, striatum, hippocampus, or cerebellum.

A dissociation between the temporal onset of age-related behavioral deficits and alterations in [^{3}H]QNB binding has been reported by other investigators [27], who found that 24-hr retention of step-through passive avoidance was impaired in 12- and 30-month-old C57BL/6J mice compared with 6-month-old mice, whereas [^{3}H]QNB binding was decreased only in 30-month-old animals. The results of our step-through passive avoidance studies (using a 5-day training-test interval) indicate that the lack of association found in 12-month-old animals [27] extends to include 15-, 20-, and 25-month-old animals. Additionally, the present investigation revealed similar results in regard to performance on a step-down passive avoidance task. A test of "delayed alternation" may have demonstrated the critical link between age effects on short-term memory and the cholinergic hypothesis.

These results of course do not mean that the cholinergic system is not related to memory function. Many types of transmitters are probably involved in memory storage and

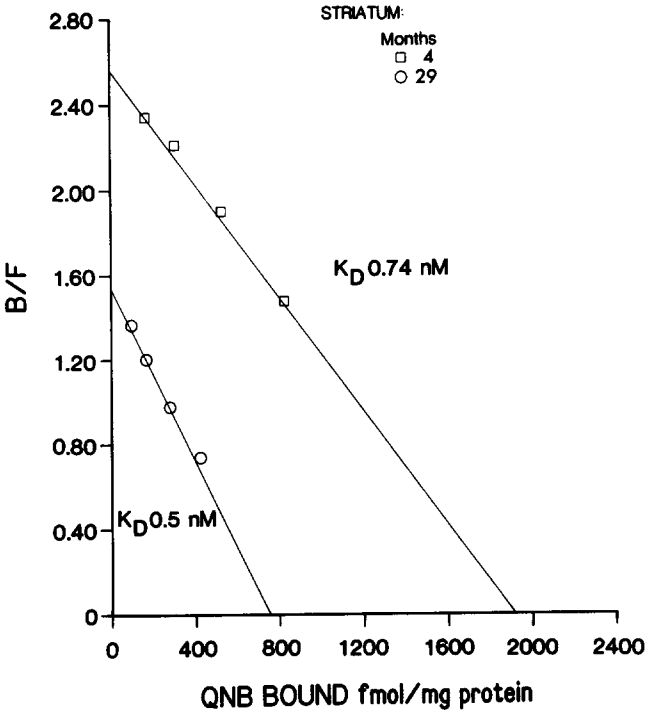

FIG. 3. Scatchard plot. Age comparison of cholinergic [^{3}H]QNB binding in the striatum. B: specifically bound. F: free, unbound.

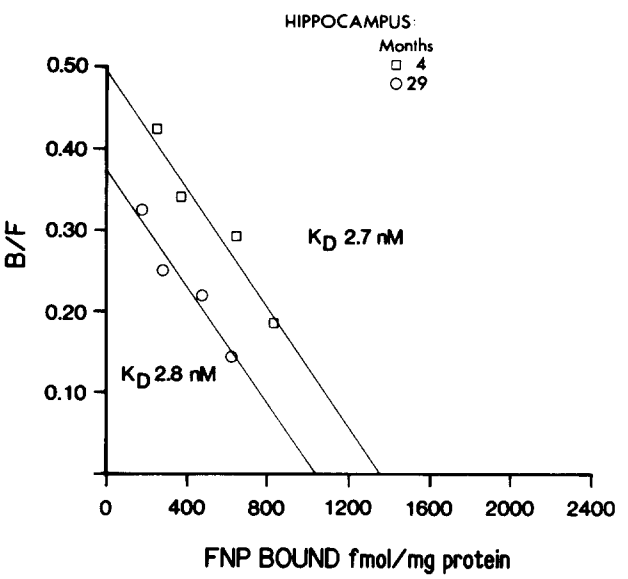

FIG. 4. Scatchard plot. Age comparison of benzodiazepine [^{3}H]FNP receptor binding in hippocampus. B: specifically bound. F: free, unbound.

retrieval and in arousal [30]. A closer temporal relationship between cholinergic receptors and performance may exist when other types of learning paradigms and longer-term retention are tested.

A significant correlation (ρ=0.475) was found between 24-hr retention of step-down passive avoidance and [^{3}H]QNB binding in the hippocampus. This finding indicates an association between hippocampal [^{3}H]QNB binding and performance on the step-down task (there was no correlation with step-through performance), but does not suggest that age-related deficits are caused by differences in [^{3}H]QNB binding. Since [^{3}H]QNB binding in the hippocampus did not decrease until the age of 29 months, this is probably a moot point. A presumed behavioral indication of hippocampal function, spontaneous alternation, was also not significantly affected by age.

One of the objectives of this investigation was to distinguish between acquisition and retention deficits by using two different training-test intervals. On the step-through task a significant 5-day retention deficit was found in 15-, 20-, and 25-month-old animals, and a 2-hr deficit was also found in the 25-month-old group, indicating a deficit in acquisition. Therefore, the 5-day effect in the 25-month-old group may be partly, or entirely, a result of poor acquisition. We did not observe this effect in the 15- and 20-month-old groups, which exhibited pronounced retention deficits at 5 days, but no deficit at 2 hr. These data indicate a specific memory impairment in these animals.

Significant age-related deficits on 24-hr retention of step-down passive avoidance were found in the 15-, 20-, and 25-month-old groups. There was no significant effect of age on 2-hr acquisition, suggesting that the 24-hr deficit was an actual retention deficit and not the result of impaired acquisition. However, 2-hr performance on this task was poorer and more variable than that on the 2-hr step-through task. This variability and weaker initial training could conceivably have obscured an aging effect on acquisition.

These data do not permit a clear-cut characterization of the age-related deficits on passive avoidance tasks as either acquisition or retention deficits. In view of previous reports of performance deficits in rodents after short training-test intervals [3,21], impaired acquisition appears to be at least one factor in age-related performance deficits. The importance of including short and long intervals in studies of this sort is reemphasized by the present findings.

Environmental enrichment significantly improved retention in mice on 24-hr step-down passive avoidance compared with retention in mice in the impoverished condition. This finding is in agreement with a reported deficit on 24-hr step-down performance in impoverished rats compared with enriched rats [14]. Because SC animals were not tested in the present investigation, it cannot be determined whether the effect was caused by facilitation in the EC animals, impairment in the IC animals, or both, relative to animals reared under standard housing conditions.

The effect of environment on step-down performance was statistically significant in an overall comparison, in which animals in all age groups were considered together. EC vs IC comparisons within individual age groups were not significant, suggesting that a large sample size was required for the effect and also that no particular age group was more affected than the others.

Performance on step-through passive avoidance was not affected by environment in this experiment. Previous reports have shown facilitation, impairment, and no difference in performance on the step-through task in rodents exposed to impoverished environments. Of course, a longer duration of

differential housing might have affected step-through performance in this experiment.

Regarding amelioration of age-related memory deficits through environmental manipulations, the step-down results can only be considered suggestive, since significant effects in individual age groups were not observed. Nevertheless, it is clear that performance of animals living in a socially and perceptually enriched environment for one month before training was superior on at least one task to that of animals housed in isolation.

No significant effect of environment on [^{3}H]QNB binding was observed in any of the brain regions examined. This suggests that the behavioral effect of environment was not mediated by an effect on [^{3}H]QNB binding.

Statistically significant differences between 4- and 29-month-old mice were observed in binding of [^{3}H]QNB and [^{3}H]FNP in several brain regions. These data add to previous reports on aged C57BL/6J mice, indicating a decrease in maximal [^{3}H]QNB binding in whole brain homogenates [11] and a regional decrease in cerebral cortex and striatum [27]. The results of the Scatchard analyses must be regarded as preliminary. Nevertheless, an age-related increase in affinity for [^{3}H]QNB in the mouse striatum, a possible compensatory effect suggested by the present investigation, has also been reported previously [27]. This increase of postsynaptic receptor affinity may be a well-recognized upregulation that compensates for a decreased presynaptic input.

The 29-month-old mice in which [^{3}H]QNB binding was decreased were females, whereas it was in males that we observed no effect of age on binding in mice as old as 25 months. However, it is unlikely that gender differences could account for the results, since significant decreases in [^{3}H]QNB binding in 30-month-old male C57BL/6J mice have also been reported [27]. On the whole, it appears that [^{3}H]QNB binding remains stable for most of the lifespan of the C57BL/6 mouse and declines relatively suddenly as animals approach 30 months of age [11].

The effect of age on [^{3}H]FNP binding had not been reported as of this writing. In the present investigation significant 12–15% decreases were observed in the cerebral cortex, hippocampus, cerebellum, and brainstem of 29-month-old mice compared with 4-month-old mice. This finding could be related to reports of increased anxiety and fear in aged organisms. Although this increased motivation by fear should enhance the performance of avoidance behavior, it is apparently not enough to counterbalance the impairment thought to be caused by diminished associative learning.

Comparison of apparently healthy 29-month-old animals and animals with pathological findings at autopsy revealed no difference between the two groups in binding of either [^{3}H]QNB or [^{3}H]FNP in any brain region. This suggests that pathological conditions associated with aging are not responsible foc the observed effects of aging on behavior and receptors. Severe weight loss and nonspecific illness do not alter binding of [^{3}H]QNB in the C57BL/6J mouse brain [11]. Nevertheless, direct effects of aging on the cholinergic system cannot, at present, be dissociated from indirect effects mediated by other age-related changes.

In conclusion, the age-related decline in passive avoidance performance under the conditions of this experiment was not related to a decrease in cholinergic receptors because the decline in behavior antedated the decline in receptors by 14 months. Similarly, a performance difference induced by manipulating the environment was not associated with changes in cholinergic receptors. However, individually good performance was associated with a modest elevation in [^{3}H]QNB binding irrespective of environmental conditions. Behavioral or biochemical conditions different from those used in this experiment may show a closer relationship between performance and cholinergic receptor binding.

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