Pergamon

PII: S0306-4522(98)00374-1

Neuroscience Vol. 89, No. 3, pp. 771–780, 1999

0306–4522/99 $19.00+0.00

PRESYNAPTIC MARKERS OF CHOLINERGIC FUNCTION IN THE RAT BRAIN: RELATIONSHIP WITH AGE AND COGNITIVE STATUS

M. G. BAXTER,*¶ K. M. FRICK,† D. L. PRICE,‡ S. J. BRECKLER,† A. L. MARKOWSKA† and L. K. GORMAN§

*Curriculum in Neurobiology, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, U.S.A.

†Department of Psychology, The Johns Hopkins University, Baltimore, MD 21218, U.S.A.

‡Departments of Pathology, Neurology, Neuroscience, and the Neuropathology Laboratory and §Department of Anesthesiology and Critical Care Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD 21287, U.S.A.

Abstract—The nature of age-related changes in cholinergic function and their relationship to age-related behavioral decline were examined in the present study. Male Fischer-344 rats of four ages (four, 11, 17 and 23 months) were tested in a battery of cognitive tasks. Discrete microdissections of brain areas involved in cognitive function were performed, and activity of choline acetyltransferase and levels of hemicholinium-3 binding were determined to assess the integrity of cholinergic innervation. Age-related changes in cholinergic markers occurred predominantly in the medial septal area and its target areas (hippocampus and cingulate cortex), and were also present in the posterior caudate. However, most of the age-related changes in cholinergic markers were already present at ages at which behavioral impairment was not yet maximal. There were some consistent correlations between behavioral and neurochemical measures, independent of age, but these accounted for relatively small proportions of variance in behavioral performance. For most of these correlations, lower levels of presynaptic cholinergic markers were related to better behavioral performance. In brain areas in which correlations changed with age, lower levels of presynaptic cholinergic markers were associated with better performance in young rats, whereas higher levels were associated with better performance in aged rats.

Recent lesion studies using a toxin selective for basal forebrain cholinergic neurons have suggested that these neurons do not play as central a role in learning and memory in young and aged animals as was previously thought. When considered in this context, the present results suggest that preserved cholinergic function in old age might act indirectly to sustain cognitive ability. Changes in cholinergic function may represent one of a number of age-related neurobiological events that underlie behavioral impairments, or may be a permissive factor for other age-related processes that are more directly responsible for cognitive impairments. © 1999 IBRO. Published by Elsevier Science Ltd.

Key words: ageing, acetylcholine, spatial learning, memory, septohippocampal system, basal forebrain.

The cholinergic hypothesis of geriatric memory dysfunction[4] has predominated in the study of the neurobiology of age-related changes in cognitive function for over 15 years. The basal forebrain cholinergic system, which supplies the majority of the cholinergic input to the neocortex, hippocampus and amygdala,[26,36] undergoes alterations with age. Although age-related changes or decreases in cholinergic function are associated with impairments in learning and memory (for review see Refs 23 and 25), the precise nature of these changes, or their particular locus within the system, is still unclear. Numerous investigations have reported different patterns of change within the basal forebrain itself or in the innervation of particular target areas. Most studies have reported changes in more rostral parts of the basal forebrain (the medial septum and vertical limb of the diagonal band) and in its primary target, the hippocampal formation.[2,3,14,15,22,27,30,32]

In contrast to the correlations between age-related changes in cholinergic function and age-related declines in cognition, recent studies have questioned the importance of the cholinergic system in learning and memory. The recent development of a selective immunotoxin for basal forebrain cholinergic neurons (192 immunoglobulin G–saporin) has made it possible to determine the behavioral consequences of selectively removing these neurons. Surprisingly, little or no effect of selective removal of these neurons on learning and memory has been found, when the lesions are restricted to basal forebrain cholinergic neurons and do not include cerebellar

¶To whom correspondence should be addressed at his present address: Department of Psychology, Harvard University, 984 William James Hall, 33 Kirkland Street, Cambridge, MA 02138, U.S.A.

Abbreviations: ChAT, choline acetyltransferase; EDTA, ethylenediaminetetra-acetate; HC-3, hemicholinium-3.

damage.[5,6,7,10,39] Even removal of the septal cholinergic input in aged rats with relatively intact cognitive function does not produce a learning impairment.[8] These results pose a significant challenge for the hypothesis that age-related alterations in cholinergic function play a significant role in producing age-related cognitive impairments.[20,24]

The present study was designed to examine comprehensively the relationship between changes in one domain of cognitive ability that undergoes age-related decline (spatial learning) and changes in cholinergic function that occur with age. Male Fischer-344 rats of four ages (four, 11, 17 and 23 months) were tested in a battery of cognitive tasks.[17] These brains were then microdissected into discrete anatomical regions for determination of two markers of cholinergic function: choline acetyltransferase (ChAT) activity and binding of hemicholinium-3 (HC-3). ChAT is the synthetic enzyme for acetylcholine and is present in high abundance in cholinergic cell bodies and terminals.[40] HC-3 binds to the sodium-dependent high-affinity choline uptake site and is a marker of activity of cholinergic terminals.[28,31,38] These markers were chosen to index the functional integrity of cholinergic innervation of target areas of the basal forebrain. Several questions were addressed through this research design. First, does the cholinergic system change in a similar manner across different anatomical regions or are age-related changes regionally independent? Second, do the changes in the cholinergic system parallel behavioral declines? Third, does a unique relationship between cholinergic function and behavior exist when common effects of age are controlled for? And fourth, do relationships between the cholinergic system and behavior change with age?

EXPERIMENTAL PROCEDURES

Subjects

Male Fischer-344 rats were obtained from the National Institute of Aging colony at Harlan. At the beginning of behavioral testing, the rats were four, 11, 17 or 23 months old ($n=18$ per group) and were housed two to three per cage in a temperature- and humidity-controlled colony room on a 12-h/12-h light/dark cycle. Behavioral testing was performed during the light portion of the cycle. Food and water were available *ad libitum*. Behavioral testing began approximately one week after the rats arrived in the laboratory and required approximately three weeks to complete. Rats were killed within a week of the completion of behavioral testing. Hence, the rats were five, 12, 18 or 24 months of age at the time of death; however, their ages during behavioral testing are used to label the four groups throughout this paper. All efforts were made to minimize animal suffering and to reduce the number of animals used; alternatives to *in vivo* procedures were not available for this study. All procedures conformed to the standards set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Animal Care and Use Committee of the Johns Hopkins University.

Behavioral testing

The behavioral data from these rats were presented in a previous paper.[17] The behavioral test procedures are summarized briefly here. Spatial reference and working memory were tested in the Morris water maze. In this task, an escape platform is located just beneath the surface of a pool of tepid opaque water. The rat is placed into the tank at various points around the rim of the tank and is allowed to search for the platform, on to which it can climb to escape from the water. After an initial shaping phase to accustom the rats to the demands of the task, the rats were tested in "place discrimination" for five days, in which the escape platform was located in a constant location in the tank on each day of testing. Six trials were given each day; the final trial of each day was a variable-interval probe trial,[34] in which the platform was lowered for a variable amount of time and the rat's search pattern recorded. The primary measure from the probe trials was annulus-40 time, the time during the probe trial in which the rat was swimming within a 40-cm-diameter circle centered on the platform location. Higher scores on this measure indicated more accurate spatial memory performance. The pattern of improvement in performance across days of testing provided a measure of spatial learning ability (see section on "Selection of behavioral indices" below).

Two days after the end of place discrimination training, "repeated acquisition" testing took place, for five days. This was identical to place discrimination except that, each day, the platform was moved to a new location in the maze. Hence, performance on the second trial of each day of repeated acquisition provided an index of spatial working memory ability. We used time to locate the platform on Trial 2, averaged across days of testing, as a measure of performance on this trial. Performance on the probe trials at the end of each day provided an index of spatial reference memory ability, as determined by a factor analysis of these data.[17]

Microdissection procedure

Four to five days after the completion of behavioral testing, each rat was decapitated and the brain quickly removed and placed in a Kopf brain blocker. The brain was cut into three blocks and then bisected sagittally at the midline; the right hemisphere of each block was frozen on dry ice for use in another experiment, the results of which will not be reported here. The left hemisphere from each block was microdissected on a chilled glass plate. The first block was made by cutting the brain coronally at or immediately anterior to the optic chiasm; the posterior surface of this block was approximately at the level of the brain 0.20 mm anterior to bregma, according to the Paxinos and Watson brain atlas,[37] and was anterior to the crossing of the anterior commissure. The cingulate cortex, frontal cortex, anterior parietal cortex, medial septal area and anterior caudate samples were taken from this block. The second block was made by cutting coronally at the median eminence; the posterior surface of this block corresponded roughly to the level of the brain 2.80 mm posterior to bregma. The somatosensory cortex, posterior parietal cortex and posterior caudate samples were taken from this block. From the remaining tissue, occipital cortex, temporal cortex and entorhinal cortex samples were taken. The hippocampus was removed, flattened and separated into its subfields (CA1, CA2/3 and dentate gyrus, for both dorsal and ventral regions) based on visual landmarks. Finally, the cerebellum was removed. The designations of all these anatomical areas, and the locations from which they were taken, corresponded to those in the Paxinos and Watson atlas,[37] with the exception of the anterior/posterior caudate and anterior/posterior parietal cortex, which simply refer to the tissue block from which the samples were taken.

All samples were placed in plastic storage tubes, frozen on dry ice and stored in a freezer at −70°C until neurochemical assays were performed.

Choline acetyltransferase assay

ChAT activity was measured by the formation of [^{14}C]acetylcholine from [^{14}C]acetylcoenzyme-A (55.7 mCi/mmol; New England Nuclear, Boston, MA) and choline.[16] The product was separated from the labeled substrate by an extraction which yields a low-blank, high-extraction efficiency, and ensured measurement of only [^{14}C]acetylcholine. Tissue was homogenized in cold 0.32 M sucrose, and a 40-μl aliquot of homogenized tissue was combined with 10 μl of a solution containing 2% Triton X-100 and 50 mM EDTA (pH 7.4). Incubation was carried out for 15 min at 37°C. The incubation mixture (final volume, 100 μl) contained 200 mM sodium chloride, 50 mM sodium phosphate (pH 7.0), 0.075 mM eserine, 6 mM choline chloride, 0.1 mM [^{14}C]acetylcoenzyme-A and 0.5 mg/ml bovine serum albumin. The newly formed [^{14}C]acetylcholine was extracted into a hydrophobic mixture containing sodium tetraphenylboron. Protein content of the homogenates was assayed using the method of Bradford,[12] with bovine serum albumin as a protein standard.

Hemicholinium-3 binding

The method used was that of Swann and Hewitt.[38] Brain homogenates were centrifuged at $1000 \times g$. The resulting supernatant was recentrifuged to obtain a crude mitochondrial pellet. This pellet was resuspended in 10 mM sodium potassium phosphate buffer for the HC-3 binding assay. The assay combined 200 μl of the mitochondrial homogenate with various amounts of buffer, [^{3}H]HC-3 (140.3 Ci/mmol) and non-radiolabeled HC-3 to a uniform final volume. After 25 min of incubation, the membranes were collected by vacuum filtration using a cell harvester on to Whatman GF/B filters. The filters were put into scintillation vials containing 5 ml of Aquasol II. A Packard Tricarb scintillation spectrometer measured the radioactivity. Protein content of the homogenates was assayed using the method of Bradford,[12] with bovine serum albumin as a protein standard.

Selection of behavioral indices

Based on the results of the original behavioral study,[17] only a subset of behavioral measures was chosen for correlation with the neurochemical results, to reduce the overall number of correlations considered. As mentioned, a probe trial measure, annulus-40 time, was selected as the primary measure for correlation in order to avoid possible confounds present in platform trial measures (e.g., differential swim speed or non-spatial search strategies). Annulus-40 time was selected rather than quadrant time or platform crossings because it demonstrated the same pattern of age effects with a greater proportion of between-group variance. For place discrimination, a learning index was constructed from annulus-40 time, in a manner similar to that described by Gallagher *et al.*[21] This technique was used in an attempt to capture aspects of each individual rat's learning ability, rather than asymptotic levels of performance. The learning index weights good performance early in training more heavily than good performance later in training, so rapid learning results in higher scores on this measure. Mean annulus-40 time during probe trials on days 2–5 of place discrimination was calculated from the original sample of 31 four-month-old rats in the study of Frick *et al.*[17] (The scores on day 1 were not considered in calculating the learning index, because for many rats this value was 0, and no age effects were evident in performance on day 1.) The ratio of performance on day 2 to performance on days 3, 4 and 5 was determined to produce a set of weight coefficients, which were 1.00, 0.636, 0.605 and 0.559 for days 2, 3, 4 and 5, respectively. Hence, better performance on earlier days of testing resulted in higher scores on the learning index, as the same performance later on was multiplied by smaller weights. This weighted sum of the annulus-40 time scores on days 2–5 produced the learning index. A "spatial reference memory index" was calculated from the mean annulus-40 time score across all five days of repeated acquisition testing (as a learning curve was not apparent across the test sessions). A "spatial working memory index" was obtained from the mean swim time during Trial 2 of repeated acquisition (across all five days of testing). Because lower scores on swim time indicated better working memory, this value was subtracted from 60 (the total length of the trial) to produce a measure for which higher scores indicated better performance. As mentioned, these operational definitions of spatial reference and working memory are based on our previous analysis of measures of performance in the water maze for this group of rats.[17]

Data analysis

Three series of analyses were conducted to determine the effects of age on neurochemical markers and the relationship of the neurochemical markers to behavioral function. The structure of the analysis was designed to minimize the total number of significance tests computed. The first analysis examined overall patterns of age-related change in presynaptic cholinergic markers, in order to determine whether these patterns corresponded with age-related changes in behavior. A one-way ANOVA with age as a factor was performed on each neurochemical measure in each brain area to determine whether there were effects of age on neurochemistry. Non-orthogonal linear contrasts on each pair of age groups were calculated for measures with significant overall effects of age, to determine differences between individual age groups. The second analysis determined whether significant correlations between presynaptic cholinergic markers and behavior existed, independent of possible common effects of age that could obscure the true nature of the relationship.[9,13] Partial correlations between each neurochemical marker and the behavioral measures, partialling age, were calculated to determine relationships between behavior and neurochemistry. The third analysis determined whether relationships between behavioral and neurochemical measures existed only in certain age groups, or differed between age groups. A regression analysis for each behavioral measure used age and the behavioral measure as predictors of each neurochemical marker. The statistical significance of the interaction term between age and the behavioral measure was tested as an indicator of relationships between behavior and neurochemistry that changed with age. All statistical analysis was performed in SAS 6.04 for the IBM PC (SAS Institute, Cary, NC).

RESULTS

Low protein levels precluded determination of both ChAT activity and HC-3 binding in some samples. These missing points were relatively homogeneously distributed with respect to age and behavioral status. Because the microdissection procedure used resulted in small pieces of tissue, a substantial amount of variability was present in some neurochemical measures. In order to reduce overall variability, an initial screening removed data-points falling more than two standard deviations from the mean level of the marker in each brain area (HC-3 binding) or more than two standard deviations from the mean level of the marker in each brain area determined separately for each age group (ChAT activity).

Table 1. Summary of behavioral measures

Behavioral measure	Age (months)			
	4	11	17	23
Learning index	160.4 ± 10.11[a]	120.3 ± 6.42[b]	109.6 ± 7.49[b]	62.7 ± 6.38[c]
Reference memory index	59.6 ± 2.65[a]	47.6 ± 2.55[b]	42.6 ± 1.86[b]	31.7 ± 2.49[c]
Working memory index	45.7 ± 1.71[a]	44.3 ± 2.04[a]	40.0 ± 2.29[a]	31.2 ± 2.70[b]

Values are mean ± S.E.M. Higher values indicate better performance (learning or memory). Main effects of age were significant for each measure. Values with different superscript letters (a, b, c) are significantly different from each other (contrast analysis following the one-way ANOVA on each measure): e.g., the learning index for four-month-old rats is significantly different from all other age groups, but 11- and 17-month-old rats do not significantly differ from each other.

Table 2. Choline acetyltransferase activity (nmol/h/mg protein) in various brain regions in the four age groups of rats

Brain area	Age (months)			
	4	11	17	23
Cingulate cortex	70.51 ± 3.49	76.32 ± 5.55	77.45 ± 3.35	86.79 ± 6.51
Frontal cortex	76.84 ± 5.40	85.14 ± 7.17	82.35 ± 6.03	82.56 ± 4.21
Anterior parietal cortex	91.11 ± 6.92	95.07 ± 8.91	95.29 ± 12.49	99.50 ± 9.85
Posterior parietal cortex	58.14 ± 4.91	54.36 ± 3.76	55.45 ± 4.24	49.92 ± 7.84
Somatosensory cortex	38.69 ± 2.29	41.58 ± 2.25	44.84 ± 4.51	42.72 ± 3.80
Occipital cortex	65.74 ± 5.64	79.88 ± 7.29	83.91 ± 5.37	77.71 ± 7.89
Temporal cortex	61.98 ± 4.47	92.16 ± 17.68	84.72 ± 13.86	68.97 ± 6.86
Entorhinal cortex	100.43 ± 8.46	90.11 ± 8.04	85.68 ± 5.83	78.31 ± 5.50
Anterior caudate	209.06 ± 13.84	205.17 ± 16.61	190.43 ± 12.97	183.65 ± 22.39
Posterior caudate†	99.47 ± 10.85*	103.39 ± 10.79*	150.13 ± 11.51	158.88 ± 13.98
Medial septal area†	201.33 ± 22.36*	171.92 ± 19.34	133.31 ± 14.07	134.29 ± 19.10
Dorsal hippocampus				
CA1†	66.46 ± 6.56	76.94 ± 3.76*	61.99 ± 3.87	55.10 ± 2.24
CA2/3	53.38 ± 3.84	56.21 ± 4.26	62.91 ± 4.72	53.98 ± 3.88
Dentate gyrus	64.43 ± 5.15	78.29 ± 6.16	65.67 ± 6.65	64.43 ± 4.21
Ventral hippocampus				
CA1	36.14 ± 10.07	33.53 ± 9.20	27.73 ± 5.13	25.88 ± 3.76
CA2/3	78.10 ± 4.00	77.13 ± 3.54	64.64 ± 4.98	71.03 ± 4.17
Dentate gyrus	79.61 ± 3.63	93.14 ± 6.12	85.14 ± 4.34	96.59 ± 4.43
Cerebellum	7.11 ± 0.56	6.59 ± 0.56	7.37 ± 0.56	7.19 ± 0.50

*Significantly different from 17-month and 23-month groups, $P<0.05$.
†Significant main effect of age, $P<0.05$.

Age effects on behavioral indices

Main effects of age were apparent on the subset of behavioral measures selected for correlation with the neurobiological data (learning index, $F_{3,68}=26.87$, $P<0.01$; spatial reference memory index, $F_{3,68}=23.16$, $P<0.01$; spatial working memory index, $F_{3,68}=8.59$, $P<0.01$). Mean values (± S.E.M.) of each age group for these measures are presented in Table 1. A more complete description of the behavioral results is presented in our previous report.[17] The data in Table 1 indicate that behavior declines linearly with age: 23-month-old rats are most impaired relative to four-month-old rats, with 11- and 17-month-old rats performing at intermediate levels.

Age effects on neurochemistry

Main effects of age were significant on five of the 36 neurochemical measures: HC-3 binding in the cingulate cortex ($F_{3,49}=2.77$, $P=0.05$) and dorsal hippocampus area CA2/3 ($F_{3,34}=4.50$, $P=0.01$), ChAT activity in the medial septal area ($F_{3,55}=2.94$, $P=0.04$), dorsal hippocampus area CA1 ($F_{3,53}=3.54$, $P=0.02$) and posterior caudate ($F_{3,51}=6.70$, $P<0.01$). Means and standard errors of the neurochemical measures for all four age groups in the 18 brain areas assayed are presented in Tables 2 and 3. Significant differences between individual age groups (determined by the contrast analysis) are also indicated in the tables.

Table 3. Hemicholinium-3 binding (fmol/mg protein) in various brain regions in the four age groups of rats

Brain area	Age (months)			
	4	11	17	23
Cingulate cortex†	86.75 ± 21.94	76.32 ± 18.20	170.01 ± 38.08*	84.24 ± 19.03
Frontal cortex	70.41 ± 16.81	76.56 ± 17.94	140.18 ± 38.00	103.71 ± 29.00
Anterior parietal cortex	97.68 ± 29.74	130.09 ± 27.81	188.23 ± 43.29	170.93 ± 61.54
Posterior parietal cortex	92.37 ± 15.91	89.99 ± 20.44	111.81 ± 23.51	56.96 ± 19.59
Somatosensory cortex	58.12 ± 14.58	102.88 ± 18.81	90.70 ± 17.31	128.07 ± 26.75
Occipital cortex	102.31 ± 28.33	110.95 ± 23.63	62.65 ± 10.83	116.26 ± 29.64
Temporal cortex	130.29 ± 33.04	81.33 ± 15.05	118.35 ± 31.06	110.32 ± 21.85
Entorhinal cortex	158.80 ± 31.51	124.83 ± 29.42	197.71 ± 39.79	167.00 ± 40.31
Anterior caudate	165.73 ± 31.30	209.05 ± 41.96	236.26 ± 35.57	231.67 ± 35.34
Posterior caudate	263.22 ± 49.09	194.68 ± 42.18	263.86 ± 58.56	181.34 ± 53.52
Medial septal area	186.45 ± 32.83	307.08 ± 76.88	168.52 ± 27.58	153.47 ± 37.00
Dorsal hippocampus				
CA1	70.14 ± 21.57	309.28 ± 107.34	242.55 ± 89.62	261.12 ± 66.13
CA2/3†	334.99 ± 67.62*	158.17 ± 29.19	100.15 ± 27.08	156.74 ± 60.84
Dentate gyrus	190.90 ± 49.86	165.79 ± 37.75	187.65 ± 91.19	186.16 ± 79.14
Ventral hippocampus				
CA1	152.18 ± 34.73	80.09 ± 23.52	189.16 ± 50.53	122.55 ± 47.31
CA2/3	189.86 ± 43.12	155.46 ± 29.54	120.85 ± 33.01	137.47 ± 40.37
Dentate gyrus	115.85 ± 24.19	122.59 ± 21.02	137.40 ± 33.28	96.90 ± 25.89
Cerebellum	6.80 ± 1.15	6.47 ± 1.56	4.97 ± 1.06	5.55 ± 1.19

*Significantly different from all other age groups, $P<0.05$.
†Significant main effect of age, $P<0.05$.

Table 4. Partial correlations between behavioral and neurochemical measures (partialling age)

Behavioral measure	HC-3, cingulate cortex	HC-3, entorhinal cortex	HC-3, medial septal area	ChAT, entorhinal cortex	ChAT, hippocampus, dorsal CA1
Learning index	0.29*			−0.31**	−0.49***
Reference memory index			−0.44**	−0.27*	−0.29*
Working memory index	0.31*	0.30*		−0.28*	

*$P<0.05$; **$P<0.02$; ***$P<0.001$.
HC-3, hemicholinium-3 binding; ChAT, choline acetyltransferase activity.

Note that for only two of these five measures (ChAT in the medial septal area and HC-3 binding in the hippocampus) is the age effect actually a decrease with age. In these two measures, the levels are lowest by 17 months of age. For the other three measures, non-linear patterns of change are seen. These age effects on neurochemistry do not parallel the linear decline seen in the behavioral measures.

Correlations between behavior and neurochemistry, partialling age

This analysis determined unique relationships between behavioral measures and neurochemical measures, removing variance in both that was uniquely attributable to age and thereby preventing the generation of spurious correlations due to common main effects of age on behavior or neurochemistry.[9,13]

Partial correlations between neurochemical measures and learning index were significant in a number of different brain areas and are presented in Table 4. Positive correlations indicate that higher levels of cholinergic markers are associated with better behavioral performance. It is interesting that higher levels of HC-3 binding in the cingulate cortex are associated with better learning and with better working memory, and higher levels of HC-3 binding in the entorhinal cortex are associated with better working memory. However, lower levels of HC-3 binding in the medial septal area are associated with better reference memory, lower levels of ChAT activity in the entorhinal cortex are associated with better performance on all three measures, and lower levels of ChAT activity in dorsal area CA1 of the hippocampus are associated with better learning and reference memory. It is important to note that these partial correlations represent proportions of shared

Table 5. Univariate correlations between behavioral and neurochemical measures in each age group for measures with significant interaction terms in the hierarchical regression analysis

Behavioral measure	Neurochemical measure	Brain area	*P* value of interaction	Age (months) 4	11	17	23
Learning index	HC-3 binding	Dorsal hippocampus area CA2/3	0.005	−0.54	0.41	0.22	0.79*
	ChAT activity	Dorsal hippocampus area CA1	0.049	−0.74*	−0.04	−0.47	−0.27
		Dorsal hippocampus dentate gyrus	0.027	−0.52†	−0.06	0.59*	0.04
Reference memory index	HC-3 binding	Occipital cortex	0.044	0.31	−0.35	0.14	0.63*
Working memory index	ChAT activity	Frontal cortex	0.010	0.16	−0.60*	0.22	0.63*
		Parietal cortex	0.036	−0.41	−0.30	−0.27	0.43

*$P<0.05$, †$P<0.06$ for univariate correlations.

variance between the behavioral and neurochemical variables after unique variance attributable to age alone has been removed. Thus, they can be interpreted as relationships between behavioral measures and presynaptic cholinergic markers that are common to each of the four age groups. These relationships would not necessarily be apparent if age effects were not partialled out. For example, the simple correlation (not partialling age) between learning index and ChAT activity in dorsal area CA1 of the hippocampus is −0.14 ($P=0.28$), rather than −0.49.

Interactions between age, neurochemistry and behavior

A significant interaction term in the regression analysis indicated the presence of relationships between behavior and neurochemistry that varied between age groups. Table 5 presents univariate correlations (and interaction terms) between behavioral and neurochemical measures within each age group for measures with significant interaction terms. The univariate correlations are provided as a descriptive way to indicate the relative strength and direction of the linear relationships between variables as a function of age. *P* values for the univariate correlations are presented as a guide to the statistical reliability of the individual correlations: the significant interaction term indicates that at least two of the four correlations are significantly different from each other. Positive correlations indicate that higher levels of cholinergic function (HC-3 binding or ChAT activity) are associated with better behavioral performance; negative correlations indicate that higher levels of cholinergic function are associated with worse behavioral performance. There are several interesting aspects of the data presented in Table 5. First, of the areas with relationships to behavior that change with age, areas in the hippocampus are related to learning, whereas cortical areas are related to reference and working memory. Second, lower levels of presynaptic cholinergic markers are associated with better behavioral performance in the younger rats, whereas higher levels are associated with better behavioral performance in the older rats. Most striking is the correlation between HC-3 binding in the dorsal CA2/3 area of the hippocampus and the learning index, in which the correlation is large and negative in the youngest rats, but large and positive in the oldest rats.

DISCUSSION

The results of this study demonstrated significant effects of age on presynaptic markers of cholinergic function as measured by ChAT activity and HC-3 binding. These changes were found predominantly in areas containing cholinergic cell bodies (the medial septal area and posterior caudate) and in target areas of the medial septal area (the hippocampus and cingulate cortex). Markers of presynaptic cholinergic function in some anatomical areas were reliably correlated with behavioral performance, independent of age. In addition, there were significant interactions between cholinergic function, behavior and age, indicating that the relationship between markers of cholinergic function and spatial learning ability changed with age in some brain areas.

Patterns of age-related change in cholinergic function

Age-related changes in both presynaptic markers could be separated into four patterns (Tables 2, 3): an increase with age, a decrease with age, an increase followed by a decrease (a peak at an intermediate age) and no change. The brain areas with unaltered levels across the four age groups were primarily those that receive their projections from the nucleus basalis/substantia innominata (most cortical areas).

In the hippocampus, all four patterns were observed, with the most dramatic changes taking place between four and 11 months of age: HC-3 binding was elevated in dorsal CA1 at 11 months and remained elevated, HC-3 binding was decreased in dorsal CA2/3 at 11 months of age and remained decreased, whereas ChAT activity in dorsal CA1 was elevated at 11 months of age but returned to lower levels at 17 and 23 months of age. The substantial regional variation in patterns of age-related change illustrates the value of the discrete microdissection of the hippocampus for this analysis. Without the division into hippocampal subfields, these patterns of change would have been obscured. These dramatic neurochemical changes at 11 months of age are in contrast to the preserved cognitive ability in 11-month-old rats.[17] We note that the pattern of age-related changes between four- and 23-month-old rats is similar to that observed in earlier studies, i.e. significant decreases in septohippocampal areas.[2,14,15,22,27,30,32] Other studies that have examined changes in basal forebrain cholinergic neurons across different age groups have noted a fairly linear decline in cell number and cell size with age;[14] although we see a similar pattern of linear age-related decline in the rostral part of the basal forebrain (the medial septal area), the present data suggest that cholinergic function in the terminal fields of the basal forebrain (hippocampal and neocortical regions) does not necessarily undergo a linear decline with advancing age.

Relationship of age-related changes in cholinergic function to spatial learning ability

The significant patterns of age effects on cholinergic markers observed in this study do not necessarily parallel the pattern of age-related declines in behavior.[29] In the present study, all three learning and memory measures decrease consistently with increasing age (Table 1). As described above, the age-related changes in cholinergic markers we observed could be described by several different patterns. Neurochemical measures in most brain regions did not consistently decrease with age. Even in regions in which a consistent age-related decrease occurred, the lowest functional levels were reached before the behavioral impairment was maximal. For instance, levels of ChAT activity in the medial septal area were lowest by 17 months of age, even though behavioral performance was worst at 23 months of age. This descriptive comparison indicates that there is no gross correspondence between changes in cholinergic function and the pattern of age-related behavioral impairment.

Despite the absence of an overall pattern of correspondence between age-related changes, there were reliable relationships between cholinergic markers and behavior when main effects of age were partialled out (see partial correlations in Table 4). It is interesting that these significant correlations were present only in areas associated with the septohippocampal system. All of these correlations represent relatively small proportions of shared variance (squared correlations ranging from 9% to 24%) between behavioral and neurochemical measures. This suggests that levels of cholinergic activity are not a pivotal factor in the behavioral functions of these brain regions. Cholinergic markers may be indicators of neural activity within regions, and may therefore be indirectly linked to behavior via this intervening variable; it would be inappropriate to conclude a direct causal relationship between levels of cholinergic markers and behavioral performance based on correlational data. The pattern of regional differences may indicate some differential reliance on different structures for different aspects of cognitive ability (e.g., the cingulate cortex may be critical for rapid processing or short-term retention of spatial information, based on the correlation between cholinergic markers in this region and the measures of learning and working memory). The finding that many significant correlations were negative (meaning higher levels of cholinergic activity were related to poorer behavioral performance) is difficult to interpret. It is of note that manipulations that enhance cholinergic activity do not always enhance behavioral performance in young rats and can result in impairments (e.g., Refs 33 and 35).

The partial correlation analysis also suggests that, despite the fact that linear decreases are seen in both behavioral performance and cholinergic markers (at least in some brain areas), these declines are not coupled together; many studies that observe parallel age-related declines in behavior and neurochemical markers conclude incorrectly that the two processes are linked.[9]

Finally, some relationships between indices of behavioral performance and presynaptic cholinergic markers varied between age groups. Presynaptic cholinergic markers in the hippocampus were associated with the learning index, whereas presynaptic cholinergic markers in neocortical areas (frontal, parietal and occipital cortices) were associated with measures of reference and working memory. Interestingly, in the youngest rats, most of the significant correlations indicated that higher levels of cholinergic activity were associated with worse behavioral performance. The significance of this finding is unclear; it is possible that optimal levels of cholinergic function are relatively lower in young rats, so that rats with higher levels of cholinergic markers perform more poorly as a consequence of levels of cholinergic activity that are relatively high. Such a pattern of results can be observed with drugs that act to increase acetylcholine levels: monkeys treated with low doses of physostigmine (an inhibitor of acetylcholinesterase) perform better on a recognition memory task at low doses of the drug, but are impaired at higher doses.[1] Note that these changes in correlations can occur even in brain regions where age-related changes

in levels of cholinergic markers are not evident (e.g., dorsal dentate gyrus).

However, in the oldest rats, higher levels of cholinergic activity in several brain areas were associated with better behavioral performance. It is tempting to speculate that the reversal of some behavioral–neurobiological correlations with age from negative to positive indicates that individual aged rats that maintain high levels of cholinergic function throughout the ageing process may be less impaired in old age. In the absence of overall changes in levels of cholinergic function with age, perhaps maintenance of high levels of cholinergic function exerts some neurotrophic or neuroprotective effect. For example, although cholinergic function may not be directly related to cognitive performance at 17 months of age, rats with lower levels of cholinergic markers at that age may be predisposed to developing cognitive impairments later on. The fact that behaviorally unimpaired aged rats with acute removal of septo-hippocampal cholinergic input do not develop behavioral impairments[8] suggests that the cholinergic system does not act directly to preserve cognitive function in ageing, arguing against the possibility that aged rats are simply more sensitive to the effects of basal forebrain damage or that there is a direct relationship between loss of cholinergic function and age-related cognitive impairment.

Implications for the neurobiological basis of age-related cognitive decline

As mentioned, studies with selective lesions of the basal forebrain cholinergic system have demonstrated that loss of this system alone is insufficient to impair learning and memory.[7] Hence, it is unlikely that even large age-related changes in cholinergic function in brain areas associated with learning and memory would be sufficient to account for age-related behavioral deficits. So what is the significance of the correlations between presynaptic markers of cholinergic function and cognitive ability, and what is the nature of age-related neurobiological changes that actually cause cognitive impairment?

It is likely that age-related changes in cholinergic function do not directly cause cognitive impairment, based on the pattern of changes observed across different age groups in this study, and the lack of effect of selective damage to the basal forebrain cholinergic system on learning and memory function. Perhaps these changes are coupled to other age-related changes that do produce impairment, e.g., impaired glucose metabolism or energy utilization.[19] It is of note that manipulations which affect cholinergic function can be effective in improving age-related cognitive deficits. For instance, intraseptal infusion of the cholinergic agonist oxotremorine in 17- or 22-month-old rats improves spatial learning ability and spatial working memory.[18,35] Interestingly, these infusions also reversed age-related increases in hippocampal ChAT activity in 17-month-old rats.[18] Hence, once again, changes in cholinergic function were coupled to changes in behavior. This apparent conflict might suggest that, although enhancement of cholinergic function may be effective in producing improvements in cognitive ability, the underlying cause of cognitive deficits may not be the loss of cholinergic function (for related discussion see Refs 8 and 11).

CONCLUSIONS

Although changes in the cholinergic system are most likely not the direct cause of age-related impairments in behavior, they may play a permissive role in the development of other neurobiological changes that are more directly linked to behavioral impairment. Although this study was conducted in a cross-sectional rather than a longitudinal fashion, the data suggest that alterations in cholinergic function precede the development of cognitive impairment. Loss of cholinergic function earlier in life may permit more deleterious neurobiological processes to proceed in old age. Studies that measure cholinergic markers in conjunction with other neurobiological measures, or that examine cholinergic function longitudinally in conjunction with behavior, would be useful in elucidating this question.

Acknowledgements—We thank Dr Molly V. Wagster for her contributions to this research, and Karena P. Joung and Joyce A. Kotzuk for technical assistance. This research was supported by NIH Grant P50-NS20471 to D.L.P. Dr Linda K. Gorman would like to respectfully remember the contributions of Dr David Olton to this work.

REFERENCES

1. Aigner T. G. and Mishkin M. (1986) The effects of physostigmine and scopolamine on recognition memory in primates. *Behav. neural Biol.* **45,** 81–87.
2. Altavista M. C., Rossi P., Bentivoglio A. R., Crociani P. and Albanese A. (1990) Aging is associated with a diffuse impairment of forebrain cholinergic neurons. *Brain Res.* **508,** 51–59.
3. Aubert I., Rowe W., Meaney M. J., Gauthier S. and Quirion R. (1995) Cholinergic markers in aged cognitively impaired Long–Evans rats. *Neuroscience* **67,** 277–292.
4. Bartus R. T., Dean R. L. III, Beer B. and Lippa A. S. (1982) The cholinergic hypothesis of geriatric memory dysfunction. *Science* **217,** 408–417.
5. Baxter M. G., Bucci D. J., Gorman L. K., Wiley R. G. and Gallagher M. (1995) Selective immunotoxic lesions of basal forebrain cholinergic cells: effects on learning and memory in rats. *Behav. Neurosci.* **109,** 714–722.
6. Baxter M. G., Bucci D. J., Sobel T. J., Williams M. J., Gorman L. K. and Gallagher M. (1996) Intact spatial learning following lesions of basal forebrain cholinergic neurons. *NeuroReport* **7,** 1417–1420.

7. Baxter M. G. and Gallagher M. (1997) Cognitive effects of selective loss of basal forebrain cholinergic neurons: implications for cholinergic therapies of Alzheimer's disease. In *Pharmacological Treatment of Alzheimer's Disease: Molecular and Neurobiological Foundations* (eds Brioni J. D. and Decker M. W.), pp. 87–103. Wiley, New York.
8. Baxter M. G. and Gallagher M. (1996) Intact spatial learning in both young and aged rats following selective removal of hippocampal cholinergic input. *Behav. Neurosci.* **110,** 460–467.
9. Baxter M. G. and Gallagher M. (1996) Neurobiological substrates of behavioral decline: models and data analytic strategies for individual differences in aging. *Neurobiol. Aging* **17,** 491–495.
10. Berger-Sweeney J., Heckers S., Mesulam M.-M., Wiley R. G., Lappi D. A. and Sharma M. (1994) Differential effects on spatial navigation of immunotoxin-induced cholinergic lesions of the medial septal area and nucleus basalis magnocellularis. *J. Neurosci.* **14,** 4507–4519.
11. Björklund A. and Dunnett S. B. (1995) Acetylcholine revisited. *Nature* **375,** 446.
12. Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analyt. Biochem.* **72,** 248–254.
13. Breckler S. J. (1993) Age-related behavioral and neurochemical deficits: new data analytic strategies. *Neurobiol. Aging* **14,** 695–697.
14. Fischer W., Chen K. S., Gage F. H. and Björklund A. (1991) Progressive decline in spatial learning and integrity of forebrain cholinergic neurons in rats during aging. *Neurobiol. Aging* **13,** 9–23.
15. Fischer W., Gage F. H. and Björklund A. (1989) Degenerative changes in forebrain cholinergic nuclei correlate with cognitive impairments in aged rats. *Eur. J. Neurosci.* **1,** 34–45.
16. Fonnum F. (1969) Radiochemical micro assays for the determination of choline acetyltransferase and acetylcholinesterase activities. *Biochem. J.* **115,** 465–472.
17. Frick K. M., Baxter M. G., Markowska A. L., Olton D. S. and Price D. L. (1995) Age-related spatial reference and working memory deficits assessed in the water maze. *Neurobiol. Aging* **16,** 149–160.
18. Frick K. M., Gorman L. K. and Markowska A. L. (1996) Oxotremorine infusions into the medial septal area of middle-aged rats affect spatial reference memory and ChAT activity. *Behav. Brain Res.* **80,** 99–109.
19. Gage F. H., Kelly P. A. T. and Björklund A. (1984) Regional changes in brain glucose metabolism reflect cognitive impairments in aged rats. *J. Neurosci.* **4,** 2856–2865.
20. Gallagher M. (1997) Animal models of memory impairment. *Phil. Trans. R. Soc. Lond. B* **352,** 1711–1717.
21. Gallagher M., Burwell R. and Burchinal M. (1993) Severity of spatial learning impairment in aging: development of a learning index for performance in the Morris water maze. *Behav. Neurosci.* **107,** 618–626.
22. Gallagher M., Burwell R. D., Kodsi M. H., McKinney M., Southerland S., Vella-Rountree L. and Lewis M. H. (1990) Markers for biogenic amines in the aged rat brain: relationship to decline in spatial learning ability. *Neurobiol. Aging* **11,** 507–514.
23. Gallagher M. and Colombo P. J. (1995) Ageing: the cholinergic hypothesis of cognitive decline. *Curr. Opin. Neurobiol.* **5,** 161–168.
24. Gallagher M., Gill T. M., Baxter M. G. and Bucci D. J. (1994) The development of neurobiological models for cognitive decline in aging. *Semin. Neurosci.* **6,** 351–358.
25. Gallagher M., Nagahara A. H. and Burwell R. D. (1995) Cognition and hippocampal systems in aging: animal models. In *Brain and Memory: Modulation and Mediation of Neuroplasticity* (eds McGaugh J. L., Weinberger N. M. and Lynch G.), pp. 103–126. Oxford University Press, New York.
26. Gaykema R. P. A., Luiten P. G. M., Nyakas C. and Traber J. (1990) Cortical projection patterns of the medial septum–diagonal band complex. *J. comp. Neurol.* **293,** 103–124.
27. Gill T. M. and Gallagher M. (1998) Evaluation of muscarinic M2 receptor sites in basal forebrain and brainstem cholinergic systems of behaviorally characterized young and aged Long Evans rats. *Neurobiol. Aging* **19,** 217–225.
28. Happe H. K. and Murrin L. C. (1993) High-affinity choline transport sites: use of [^{3}H]hemicholinium-3 as a quantitative marker. *J. Neurochem.* **60,** 1191–1201.
29. Ingram D. K. (1996) Commentary. Brain–behavior linkages in aged rodent models: strategies for examining individual differences. *Neurobiol. Aging* **17,** 497–499.
30. Ingram D. K., London E. D. and Goodrick C. L. (1981) Age and neurochemical correlates of radial maze performance in rats. *Neurobiol. Aging* **2,** 41–47.
31. Lowenstein P. R. and Coyle J. T. (1986) Rapid regulation of [^{3}H]hemicholinium-3 binding sites in the rat brain. *Brain Res.* **381,** 191–194.
32. Luine V. and Hearns M. (1990) Spatial memory deficits in aged rats: contributions of the cholinergic system assessed by ChAT. *Brain Res.* **523,** 321–324.
33. Markowska A. L., Koliatsos V. E., Breckler S. J., Price D. L. and Olton D. S. (1994) Human nerve growth factor improves spatial memory in aged but not in young rats. *J. Neurosci.* **14,** 4815–4824.
34. Markowska A. L., Long J. M., Johnson C. T. and Olton D. S. (1993) Variable-interval probe test as a tool for repeated measurements of spatial memory in the water maze. *Behav. Neurosci.* **107,** 627–632.
35. Markowska A. L., Olton D. S. and Givens B. (1995) Cholinergic manipulations in the medial septal area: age-related effects on working memory and hippocampal electrophysiology. *J. Neurosci.* **15,** 2063–2073.
36. Mesulam M.-M., Mufson E. J., Wainer B. H. and Levey A. I. (1983) Central cholinergic pathways in the rat: an overview based on an alternative nomenclature (Ch1–Ch6). *Neuroscience* **10,** 1185–1201.
37. Paxinos G. and Watson C. (1986) *The Rat Brain in Stereotaxic Coordinates*, 2nd edn. Academic, Sydney.
38. Swann A. C. and Hewitt L. O. (1988) Hemicholinium-3 binding: correlation with high-affinity choline uptake during changes in cholinergic activity. *Neuropharmacology* **27,** 611–615.
39. Torres E. M., Perry T. A., Blokland A., Wilkinson L. S., Wiley R. G., Lappi D. A. and Dunnett S. B. (1994) Behavioural, histochemical and biochemical consequences of selective immunolesions in discrete regions of the basal forebrain cholinergic system. *Neuroscience* **63,** 95–122.
40. Wu D. and Hersh L. B. (1994) Choline acetyltransferase: celebrating its fiftieth year. *J. Neurochem.* **62,** 1653–1663.

(*Accepted* 3 *July* 1998)