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Preserved neuron number in the hippocampus of aged rats with spatial learning deficits

(aging/stereology/memory)

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ABSTRACT **Hippocampal neuron loss is widely viewed as a hallmark of normal aging. Moreover, neuronal degeneration is thought to contribute directly to age-related deficits in learning and memory supported by the hippocampus. By taking advantage of improved methods for quantifying neuron number, the present study reports evidence challenging these long-standing concepts. The status of hippocampal-dependent spatial learning was evaluated in young and aged Long–Evans rats using the Morris water maze, and the total number of neurons in the principal cell layers of the dentate gyrus and hippocampus was quantified according to the optical fractionator technique. For each of the hippocampal fields, neuron number was preserved in the aged subjects as a group and in aged individuals with documented learning and memory deficits indicative of hippocampal dysfunction. The findings demonstrate that hippocampal neuronal degeneration is not an inevitable consequence of normal aging and that a loss of principal neurons in the hippocampus fails to account for age-related learning and memory impairment. The observed preservation of neuron number represents an essential foundation for identifying the neurobiological effects of hippocampal aging that account for cognitive decline.**

A substantial body of research conducted over the last two decades in rats, monkeys, and humans has led to the view that the hippocampus is particularly susceptible to neuronal degeneration during normal aging (1). Based on this background it seems reasonable to suppose that neuron loss might be responsible for deficits in hippocampal-dependent learning and memory associated with advanced age (2–7). An important source of evidence consistent with this proposal derives from studies examining hippocampal neuron density in rat models of age-related learning and memory impairment. Earlier reports, for example, demonstrated that pyramidal cell density in the hippocampus decreases by more than 30% in aged Long–Evans rats with learning and memory deficits indicative of hippocampal dysfunction but that neuron density is less affected among aged subjects with preserved learning and memory (8). In addition, perinatal manipulations that prevent age-related declines in hippocampal cell density also protect against age-related spatial learning deficits (9). Thus, these observations have prompted intensive efforts to define the cell biological mechanisms responsible for age-related neuronal death in the hippocampus (2, 4–9).

Recent advances in the field of stereology have made it possible to determine with greater precision the magnitude of neuronal degeneration in the aged hippocampus and to document statistical relationships between neuron loss and age-related learning and memory impairment (10, 11). In contrast to measures of neuron density, modern stereological methods are specifically aimed at providing estimates of total neuron number in a region of interest. The primary advantage of this approach for research on aging is that neuron number represents an unequivocal parameter that is independent of cell size, shape, orientation, and volumetric differences between young and aged brains. Neuron density, in contrast, is strongly influenced by these factors and, as a consequence, density values can vary widely in the absence of any change in neuron number (10).

The present experiments capitalized on these advances in stereological methods to reevaluate the relationship between hippocampal neuron loss and cognitive aging. The status of learning and memory function was documented according to the same Morris water maze procedures that have been widely used to examine the effects of hippocampal damage in young subjects and the effects of normal aging (12–17). Subsequent stereological analysis was directed at quantifying total neuron number in each field of the hippocampus for the behaviorally characterized subjects. To allow meaningful comparison with the behavioral findings, the stereological sampling strategy was specifically designed to yield highly precise estimates of hippocampal neuron number in individual subjects (11). The prediction based on earlier research was that substantial neuron loss would be observed in the CA3 and CA1 pyramidal cell fields of the aged hippocampus and, importantly, that neuron death would be especially pronounced among aged Long–Evans rats with the most severe spatial learning impairments (8, 9).

MATERIALS AND METHODS

Behavioral Methods. The subjects were pathogen-free male Long–Evans hooded rats; the same strain examined in previous studies reporting substantial age-related decreases in hippocampal cell density (8, 9). Young (n = 9; 6 months old) and aged (n = 16; 27–28 months old) animals were trained using a standardized procedure that required the use of distal cues in the maze environment to learn the position of a camouflaged escape platform (17). Briefly, rats were tested for three trials per day, with intertrial delays of 60 sec, for a total of eight consecutive days. The location of the platform remained constant, and on each training trial, animals swam for 90 sec or until they found the platform. Across trials, the starting location varied among four equidistant points around the perimeter of the apparatus. Every sixth trial was a probe test during which the platform was retracted to the bottom of the pool for 30 sec and then made available for escape. Probe trial performance provides assessment of the search strategies used to navigate the maze (17). Nonspatial learning was subsequently assessed in a single session of six trials in which rats were allowed to swim to a visible black escape platform that varied in location across trials.

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Spatial behavior was recorded throughout training using a video tracking system designed for this purpose (HVS Imaging, Hampton, England), and the status of spatial learning was evaluated according to two performance measures (see ref. 17 for details of computation). During each trial, the distance of the rat from the escape platform was sampled 10 times per sec, and these values were averaged in 1-sec bins. Cumulative search error was then calculated as the summed 1-sec averages of this proximity measure corrected for the particular start location on each trial. A learning index score of spatial bias was also derived, based on data collected from the three interpolated probe tests in which the platform was initially unavailable for escape. This measure reflects the average proximity during probe trials relative to the training location of the escape platform; lower index scores indicate more accurate search focused on the target location. Previous research demonstrates that these measures are especially effective for distinguishing spatial learning impairments from other age-related performance deficits that are presumably independent of hippocampal function (17).

Stereological Methods. At the conclusion of behavioral testing, the total number of neurons in each of the principal cell layers of the hippocampus was estimated according to the optical fractionator technique (18). All stereological analyses were conducted blind with respect to the age and cognitive status of the donor subjects. Animals were euthanized under deep anesthesia and, after transcardial perfusion with formaldehyde fixative (10%), one side of the brain was embedded in methacrylate plastic resin (Leica, Heidelberg, Germany). Serial sections were cut on a motorized rotary microtome at 50 μm and every fifth section was stained with thionin.

The optical fractionator method was implemented using a NeuroLucida morphometry system (MicroBrightField, Colchester, VT), in strict accord with the guidelines described by West *et al.* (18). Neuron counts were derived from a minimum of 20 histological sections, spaced at 250-μm intervals, through the entire rostrocaudal extent of one hippocampus from each brain. A color video camera (Optronics, International, Chelmsford, MA), interfaced with a Leitz Medilux microscope, was used to view sections on a high-resolution monitor, and neuroanatomical borders corresponding to the classically defined principal cell fields of the hippocampus were digitized under low-power magnification. Subsequent cell counting was confined within these borders. The sections were inspected according to a systematic random sampling scheme such that counts were derived from a known and representative fraction of each hippocampal subfield (18). Specifically, the motorized stage of the microscope system was under computer control, and the pyramidal cell fields in every histological section were surveyed at evenly spaced x–y intervals of 220 μm by 220 μm. The x–y step size for the granule cell layer was 330 μm. For each x–y step, cell counts were derived from a known fraction of the total area by using an unbiased counting frame (19). The dimensions of the counting frame were 20 μm × 20 μm for the pyramidal cell fields, and 15 μm × 15 μm for the granule cell layer. Counting was further confined to 15 μm centered within the thickness (z axis) of the histological preparations, avoiding a variety of errors encountered when cells at the cut surfaces of the sections are included in the analysis. Total section thickness was determined directly for every histological section and hippocampal field by focusing through the preparation and recording z-axis movements of the microscope stage with a microcator (resolution = 0.5 μm). A dynamic counting rule was enforced, and while viewing through a ×100 oil-immersion objective, neuronal nucleoli were counted only as they first came into focus within each optical disector (19). Glia were excluded from the counts based on size and cytological characteristics. This distinction is readily apparent at high-magnification because principal hippocampal neurons are substantially larger and morphologically distinct relative to the small population of resident glial cells. Using these sampling parameters, the number of optical disectors examined (granule cell layer mean = 108; CA3/2 mean = 272; CA1 mean = 222) and the number of cells counted per hippocampus (granule cell mean = 318; CA3/2 mean = 191; CA1 mean = 319) substantially exceeded the

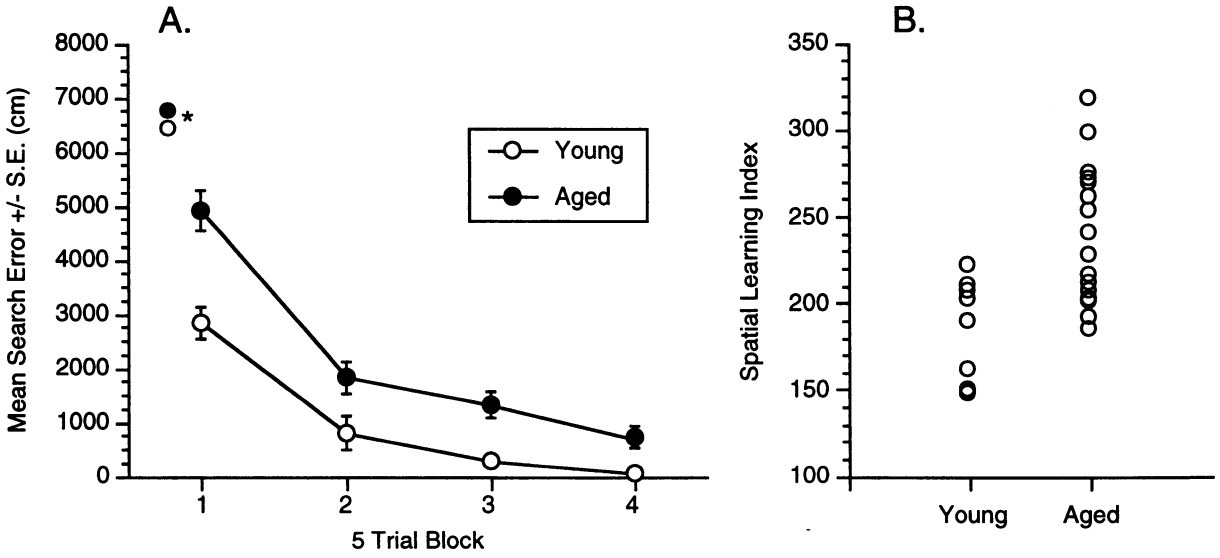

FIG. 1. Performance of young (n = 9; 6 months old) and aged (n = 16; 27–28 months old) male Long–Evans hooded rats in the spatial version of the Morris water maze. (*A*) Cumulative search error measure of spatial learning during five trial blocks of training trials. This parameter reflects the distance of the animal from the escape platform throughout its search (17). Learning was significantly impaired in the aged rats according to this measure. On the first training trial (data points indicated by asterisk), however, performance was equivalent across age groups, indicating that patterns of random search were similar in naive young and aged rats. (*B*) Learning index score of spatial bias derived from three interpolated probe tests in which the platform was initially unavailable for escape. Symbols represent the values for individual subjects. This measure reflects the average proximity during probe trials relative to the training location of the escape platform; lower index scores indicate more accurate search focused on the target location (17). As a group, aged rats exhibited significant deficits in the accuracy of spatial searching. Substantial variability was observed across the aged individuals, however; half performed substantially more poorly than the worst young rat while the remaining individuals displayed spatial learning capacities equivalent to a large database of normative results from young subjects (i.e., learning index scores < 230).

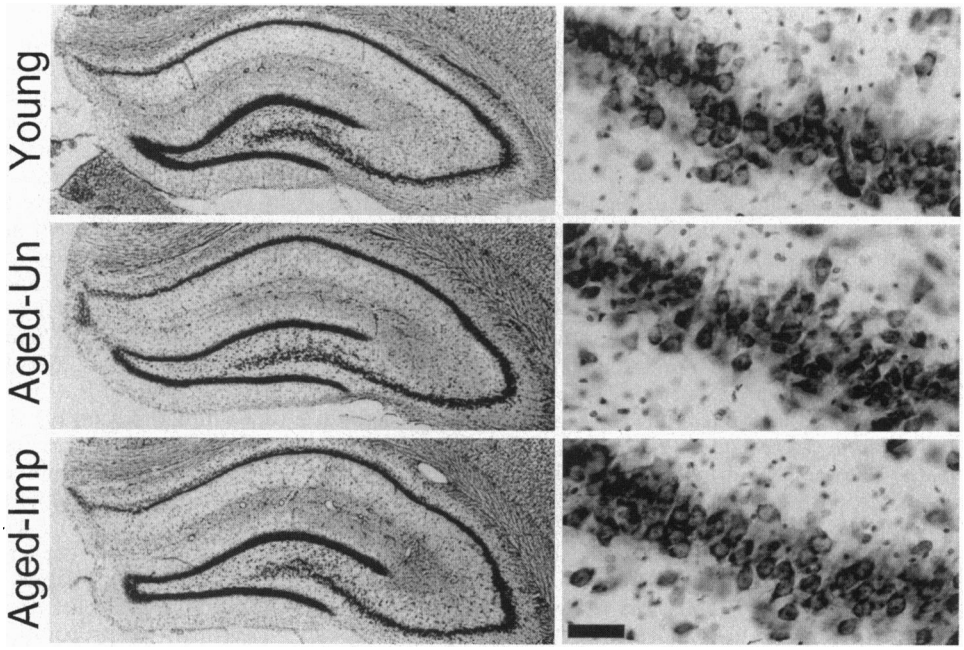

FIG. 2. Digital scanning photomicrographs of histological preparations through the dorsal hippocampus for representative young (*Top*), aged-unimpaired (*Middle*), and aged-impaired (*Bottom*) subjects. The total number of hippocampal pyramidal neurons in these individuals approximated the mean value for their corresponding groups. The higher magnification photomicrographs (*Right*) illustrate a portion of the CA3 layer located slightly distal to the supra- and infrapyramidal limbs of the dentate gyrus granule cell layer shown on the left. Note the grossly similar packing density of pyramidal cells in the young, aged-unimpaired, and aged-impaired brains. (Bar = 500 μm and 50 μm for *Left* and *Right*, respectively).

recommended standard for achieving highly precise estimates of total neuron number (10). This more intensive sampling approach was adopted specifically to facilitate comparison between the behavioral and stereological results for individual subjects (11). Finally, total neuron number was estimated by multiplying the sum of the neurons counted by the reciprocal of the fraction of the hippocampus that was sampled (i.e., the fraction of the histological sections examined, the fraction of the x–y step interval covered by the counting frame, and the fraction of the total section thickness examined). In this way, counts derived from a series of samples, uniformly distributed throughout the entire extent of the hippocampus, were converted to estimates of total neuron number for each principal cell field (18).

RESULTS AND DISCUSSION

Consistent with a substantial body of earlier research (12–15), significant age-related deficits were observed on the hippocampal-dependent "place" version of water maze testing, but no age effect was evident during nonspatial cue learning. Fig. 1 presents results for the two standardized measures of spatial learning. Although the aged animals as a group were impaired (e.g., repeated measures ANOVA of search error; $F1, 23 = 19.17, P = 0.0002$, and learning index; $F1, 23 = 13.89$, $P = 0.001$), in agreement with many other studies of this type, there was marked variability across aged individuals. Specifically, a significant proportion of aged rats scored within the range of data for young adults (Fig. 1*B*), while other aged animals performed well outside this normative range (spatial learning index score > 230). Nonspatial cue learning, measured by either pathlength (young mean [SEM] = 214.9 [32.6] cm; aged mean = 189.4 [23.6] cm) or escape latency (young mean [SEM] = 8.1 [1.52] sec; aged mean = 7.9 [0.69] sec), failed to differ across groups. These latter findings indicate that aged rats were able to swim proficiently and were motivated to escape using a local visual cue. A qualitatively similar profile of impaired spatial learning, and intact nonspatial cue-guided performance, is observed in young rats with hippocampal lesions (16). Thus, this behavioral characterization provides a window on the status hippocampal-dependent learning that is appropriate for comparison with the cell count results.[§]

The general histological appearance of the various hippocampal subfields was grossly normal in the aged brains. Fig. 2 presents low (*Left*) and higher (*Right*) magnification photomicrographs of histological preparations through the dorsal hippocampus from representative young, aged-unimpaired, and aged-impaired rats. The individuals were selected for illustration based on the outcome of the cell count analysis; total pyramidal neuron number in these subjects was close to the mean for their respective groups. The packing density of neurons appeared similar in the young and aged brains (Fig. 2 *Right*).

Quantitative results from the stereological analysis of total neuron number in the behaviorally tested young and aged subjects are illustrated in Fig. 3. Consistent with previous estimates (21, 22), there were approximately 1.2 million neurons in the granule cell layer, 225,000 neurons in CA3/2, and 390,000 neurons in the CA1 field of the hippocampus. Neuron number failed to differ, however, as a function of either chronological age or cognitive status. In CA3/2, for example, average neuron number differed by less than 3% across age groups, and this effect did not approach statistical significance (Fig. 3*B*). Moreover, neuron number was unrelated to the status of spatial learning; cell number estimates were statistically indistinguishable for the impaired and unimpaired subgroups in each of the regions examined. Fig. 3*C* illustrates the CA3/2 cell count values for individual subjects as a function of the learning score derived during probe testing on the spatial version of the water maze. The expectation based on earlier research using the same strain of rats (8, 9) was that a relationship between neuron number and age-related spatial learning impairment might be especially pronounced in the pyramidal cell fields. Although a wide range of spatial learning ability was evident across the aged animals, there was relatively little variability in the cell count values and no suggestion of a

[§]In this context, we note that a recent study (20) has examined hippocampal neuron number in small groups ($n = 5$) of behaviorally tested young and aged rats. It is not clear in this case, however, whether the pattern of performance observed among "impaired" aged rats should be considered indicative of hippocampal dysfunction. For example, this subgroup failed to exhibit deficits on two standard measures derived during probe testing that are specifically intended to assess the status of spatial learning.

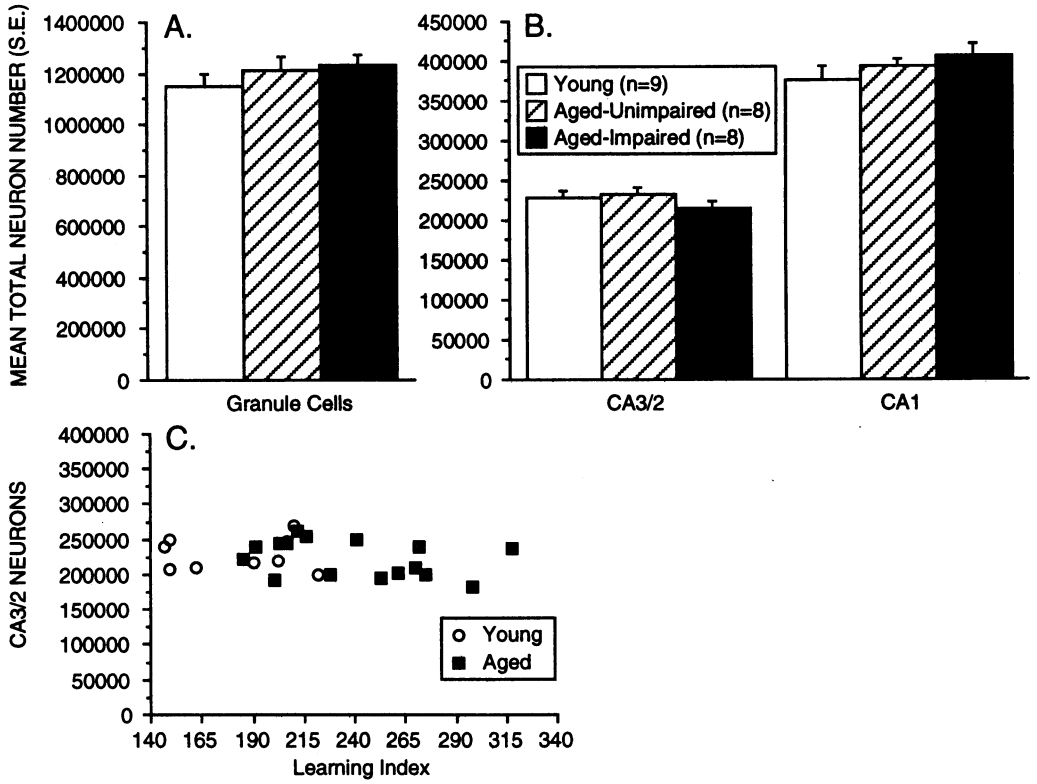

FIG. 3. Estimated total neuron number in the principal cell layers of the hippocampus for behaviorally characterized young and aged rats (all values are unilateral). To facilitate comparison with the behavioral findings, aged subjects were classified as impaired or unimpaired based on the spatial learning index derived during testing in the water maze (Fig. 1*B*). (*A*) Mean estimated total neuron number (SEM) in the granule cell layer for young, aged-unimpaired, and aged-impaired rats. Average granule cell number was statistically indistinguishable across the groups. (*B*) Mean estimated total neuron number (SEM) in the CA3/2 fields and CA1 pyramidal cell layer of the hippocampus for behaviorally characterized young and aged rats. Neuron number failed to differ as a function of age or cognitive status. (*C*) Scatter plot of total neuron number estimates in the CA3/2 fields of the hippocampus for individual rats plotted as a function of spatial learning index scores (Fig. 1*B*). The data illustrate that neuron number was stable with age and across a broad range of learning capacities.

decline in neuron number associated with age or behavioral impairment (Fig. 3*C*). A similar pattern of results was obtained for each of the principal cell layers of the hippocampus.

It is noteworthy that the quantitative techniques used here were sufficiently sensitive to detect even small age-related changes in neuron number. Coefficients of error provide a standardized statistic for evaluating the precision of neuron number estimates derived by modern stereological techniques (23). Averaged across animals in each age group, this parameter ranged from less than 4% in the CA1 pyramidal cell field to less than 6% for the granule cell layer. These results suggest that experimental error in estimating hippocampal neuron number is unlikely to account for the absence of age-related cell loss. Moreover, a power analysis revealed that, based on the number of brains available for analysis and the observed variability between young individuals, cell loss averaging only 9% below the young group mean (8.8% for the granule cell layer, 7.8% for CA3/2, and 11.3% for CA1) would reach conventional levels of statistical significance. Thus, the results count strongly against the proposal that frank neuronal degeneration is a prominent feature of aging in the hippocampus.

Recent stereological studies provide additional evidence consistent with the view that hippocampal neuron number is relatively preserved during chronological aging in humans (24) and animal models (20, 25). The important implication from early research (8, 9), however, was that a dramatic loss of pyramidal neurons might be selectively apparent among aged individuals with documented deficits in learning and memory supported by the hippocampus. The present investigation was specifically designed to test this hypothesis by taking advantage of behavioral testing procedures that are highly sensitive to age-related spatial learning impairment and using a stereological sampling strategy intended to yield very precise estimates of neuron number in individual subjects. Based on this approach, the results provide compelling evidence that degeneration among principal neurons in the hippocampus is not responsible for the behavioral impairments observed in a substantial proportion of aged individuals. Accordingly, these data may prompt a shift in the focus of accounts that view hippocampal cell death as an inevitable outcome of aging and as the final common pathway mediating age-related functional decline. Other prominent neurobiological alterations in the hippocampal system that have been associated with age-related learning and memory impairment include synapse loss, neurochemical changes, and modifications in the information encoding properties of neurons (12–15). By documenting that such effects are not merely secondary to neuronal loss, the present study provides an essential background for identifying the consequences of hippocampal aging that are responsible for functional impairment.

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