Neuron Number in the Parahippocampal Region is Preserved in Aged Rats with Spatial Learning Deficits

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The entorhinal, perirhinal and parahippocampal cortices are anatomically positioned to mediate the bi-directional flow of information between the hippocampus and neocortex. Consistent with this organization, damage involving the parahippocampal region causes significant learning and memory impairment in young subjects. Although recent evidence indicates that neuron death in the hippocampus is not required to account for the effects of normal aging on learning and memory, other findings suggest that changes in parahippocampal interactions with the hippocampus may play a significant role. Prompted by this background, we tested the possibility that age-related deficits in hippocampal learning are coupled with neuron death in the parahippocampal region. The experiments took advantage of a well-characterized rat model of cognitive aging in combination with stereological methods for quantifying neuron number. The results demonstrate that total neuron number in the entorhinal, perirhinal and postrhinal cortices is largely preserved during normal aging. Furthermore, individual variability in hippocampal learning among the aged rats failed to correlate with neuron number in any region examined and there was no indication of selective or disproportionate loss among the aged animals with the most pronounced cognitive impairment. Taken together with earlier findings from the same study population, the results demonstrate that age-related cognitive decline can occur in the absence of significant neuron death in any major, cytoarchitectonically defined component of the hippocampal system. These findings provide an essential framework for identifying the basis of cognitive aging, suggesting that alterations in connectivity and other changes are more likely causative factors.

Introduction

Reciprocal connectivity between the neocortex and hippocampus derives principally from a collection of cortical areas adjacent to the hippocampus, collectively termed the parahippocampal region. This region comprises the entorhinal, perirhinal and parahippocampal cortices of the primate brain and, respectively, the entorhinal, perirhinal and postrhinal cortices in the rat (Witter *et al.*, 1989; Burwell, 2000). Although research on learning and memory has traditionally focused on the hippocampus, recent studies have prompted a consensus that the parahippocampal region directly participates in a variety of information processing capacities critical for normal learning (Squire and Zola, 1996; Eichenbaum, 2000). Damage involving individual or multiple components of this region in rats can produce substantial impairment as determined by a variety of assessments, including the widely used Morris water maze test of spatial learning (Nagahara *et al.*, 1995; Otto and Garruto, 1997; Liu and Bilkey, 1998a,b; Wiig and Burwell, 1998; Bohbot *et al.*, 2000; Bucci *et al.*, 2000; Gaffan *et al.*, 2000; Bussey *et al.*, 2001). Similar observations have been reported in studies of humans and monkeys (Meunier *et al.*, 1993, 1996; Suzuki *et al.*, 1993; Leonard *et al.*, 1995; Buffalo *et al.*, 1998).

Although the cognitive consequences of parahippocampal damage might be interpreted as a disconnection effect, resulting from the disruption of normal hippocampal interactions with the neocortex, available evidence suggests a different conclusion. Studies in rats and monkeys have reported a number of qualitative differences in the effects of lesions involving the hippocampus and parahippocampal region, consistent with the conclusion that these areas mediate distinct capacities in support of learning and memory (Gaffan, 1994; Vnek *et al.*, 1995; Meunier *et al.*, 1996). Neurophysiological findings support this interpretation, demonstrating that, relative to neuronal encoding in the hippocampus, firing patterns among parahippocampal neurons are less tightly coupled to conjunctions or relationships among multiple cues in a testing environment and more strongly influenced by individual visual or olfactory stimuli relevant to current reward contingencies (Young *et al.*, 1997; Burwell *et al.*, 1998). The important conclusion from these findings is that, rather than serving as passive relays in the flow of information between the hippocampus and neocortex, the parahippocampal region itself is critical for normal learning and memory.

Hippocampal learning frequently declines during normal aging and there is substantial reason to suspect that a disruption in parahippocampal circuitry might contribute to these deficits. Quantitative electron microscopic data, for example, demonstrate that synaptic density declines with age in areas of the hippocampus that receive direct input from the entorhinal cortex. In addition, the magnitude of this effect predicts the severity of spatial learning impairment among aged rats and deficits in an electrophysiological index of hippocampal cellular plasticity, i.e. kindling (Geinisman *et al.*, 1995). Recent morphometric studies extend these observations, demonstrating that age-related spatial learning impairment is coupled with other potential markers of altered parahippocampal input to the hippocampus, including volumetric change in the hippocampal targets of these projections and a corresponding decrease in immunohistochemical staining for the presynaptic vesicle marker synaptophysin (Rapp *et al.*, 1999; Smith *et al.*, 2000). A straightforward account of these findings is that they might result from neuron death in the parahippocampal region, the major source of cortical input to the hippocampus. Although a recent investigation found that spatial learning impairment in aged Fischer 344 rats can occur in the absence of detectable neuron loss in the entorhinal cortex (Merrill *et al.*, 2001), the effects of aging on neuron number throughout the parahippocampal region have not been examined previously.

The present study used stereological methods to estimate the total number of neurons in the entorhinal, perirhinal and postrhinal cortices of young and aged, behaviorally characterized Long–Evans rats. Two aspects of the experimental design are noteworthy. First, the analysis took advantage of a particularly well-characterized model of cognitive aging in which a substantial proportion of aged subjects perform below the range

of younger animals on the hippocampus-dependent, spatial version of the Morris water maze. Other, age-matched subjects perform as well as young controls. Using this model allowed us to test the possibility that, even in the absence of detectable neuron loss associated with advanced chronological age, cell death in the parahippocampal region predicts individual differences in the cognitive outcome of aging. Second, the brain material examined here came from the same animals as an earlier study of neuron number in the aged hippocampus (Rapp and Gallagher, 1996). By this design we were able to extend that earlier assessment, yielding a comprehensive study of neuron number throughout all major components of the hippocampal memory system, specifically in relation to the effects of normal aging on learning capacities supported by this system.

Materials and Methods

Subjects and Behavioral Testing

Twenty-four male Long–Evans male rats served as subjects. These were the same animals as in a previous report (Rapp and Gallagher, 1996), except that a histological artifact prevented analysis of the para-hippocampal region in the brain from one young subject. Prior to morphometric evaluation, the young (n = 8; 6 months of age) and aged (n = 16; 27–28 months of age) rats were trained on a hippocampus-dependent, 'place' version of the Morris water maze. This task measures learning for the position of a hidden escape platform on the basis of cues surrounding the test apparatus (Gallagher *et al.*, 1993). Following the same protocol as many other studies using this model (Gallagher and Rapp, 1997) and as described in detail elsewhere for animals included in the present investigation (Rapp and Gallagher, 1996), three trials were provided per day for 8 consecutive days (60 s inter-trial interval). The platform location was held constant for each subject and, across trials, animals entered the maze from one of four points around the perimeter of the apparatus, according to a predetermined sequence. Rats swam until they escaped onto the platform, or for a maximum of 90 s. The final trial on every other day was a probe test in which the platform was initially unavailable for escape. Search patterns recorded during these 30 s probe trials provided a window on the strategies subjects used to solve the task (see below). Non-spatial learning that does not require the hippocampus was subsequently examined during a single session of six trials in which rats swam to a visible platform that varied in location across trials.

The overall aim of this experiment was to test the possibility that the cognitive outcome of aging is coupled to neuron death in the para-hippocampal region. Adopting a strategy validated in earlier studies using this animal model, a learning index score was derived for each rat, calculated as an animal's average proximity from the training location of the escape platform over the course of searching during the interpolated probe trials (Gallagher *et al.*, 1993). Note that by this measure, lower scores reflect better learning and more accurate searching focused on the target location. For certain comparisons (see Results), aged rats with index scores outside the range of the young group (i.e. >230) were classified as impaired (n = 8) and the remainder were considered un-impaired (n = 8).

Histological Processing and Stereological Quantification

Rats were perfused after behavioral testing and the brains processed as described in an earlier report using the same histological material (Rapp and Gallagher, 1996). Briefly, following transcardiac perfusion with aldehyde fixatives, one hemisphere from each brain (varied across animals) was embedded in methacrylate resin and cut in the coronal plane on a motorized rotary microtome at a nominal thickness of 50 μm. Every fourth section through the rostro-caudal extent of the para-hippocampal region was stained with thionin and total neuron number in the lateral entorhinal, medial entorhinal, perirhinal and postrhinal cortices was estimated according to the optical fractionator method. This approach involves counting the number of neurons in sampling sites (i.e. disectors) distributed in a systematic random fashion throughout the structure of interest and estimating total number by multiplying the sum of the neurons counted by the reciprocal of the fraction of the structure examined. As discussed elsewhere (West, 1994), a noteworthy feature of

this approach is that it yields an unambiguous measure for examining potential neuron loss in the aged brain. Cell density measures used widely in earlier research, by comparison, can be influenced substantially by volumetric change and other variables in the absence of actual neuron death.

The optical fractionator approach was implemented using a computer-aided morphometry system (StereoInvestigator; MicroBright-Field Inc., Colchester, VT) and all analyses were conducted blind with respect to the age and cognitive status of the experimental subjects. Starting at a randomly selected level within the first sampling interval, the cytoarchitectonic borders of the entorhinal, perirhinal and postrhinal cortices were digitized under low-power magnification (4–10× objectives on a Leitz DMRB microscope; Leica, Heidelberg, Germany), in an evenly spaced series of histological sections (200 or 400 μm intersection interval) that spanned the rostro-caudal extent of the parahippocampal region. This design yielded a minimum of 10 sections from each brain for quantification, although the number containing the relatively short anterior–posterior extent of the postrhinal cortex was substantially lower (mean = 4 sections per brain). Using a 100× oil-immersion objective, cells were counted while viewing live color video images of the histology on a high-resolution computer monitor. The motorized stage of the micro-scope was moved in evenly spaced x–y intervals under computer control, surveying the regions of interest in each section according to a systematic random sampling scheme. The x–y step size was 280 × 280 μm for the lateral entorhinal cortex, 250 × 250 μm for the medial entorhinal cortex, 200 × 200 μm for the perirhinal cortex and 180 × 180 μm for the post-rhinal cortex. At each sampling site, an unbiased counting frame was displayed over the live video image of the histology and counts were confined to an optical disector 25 μm in height, positioned within a middle portion of the z-axis of the section. The x–y dimensions of the counting frame were 30 × 30 μm for all regions examined. Total section thickness was measured at several sampling sites in every histological section and region of interest by focusing on the top and bottom of the preparations and recording the z-axis movement of the microscope stage. The counting procedure utilized a dynamic rule such that the top-most nucleolus associated with a neuronal nucleus was counted only when it first came into focus within each optical disector, provided it did not encroach on the exclusion lines of the counting frame (Sterio, 1984). Glia were excluded from quantification on the basis of size and cytological criteria.

Using the present sampling parameters, the total number of optical disectors examined per brain (mean = 936) and the number of neurons counted across the parahippocampal region (mean = 880/brain) greatly exceeded the recommended minimum for achieving highly precise estimates of total neuron number. The numbers of dissectors and neurons surveyed in each individual region of interest were also relatively high in comparison with standard procedures (e.g. an average of >200 cells per brain was counted in each of the four cortical areas examined). This intensive design was adopted to increase the precision of the neuron number estimates for individual subjects and to provide a basis for relating the cell count results to individual variability in spatial learning ability. The total neuron number estimates used in all analyses were calculated as the sum of the neurons counted per region of interest, multiplied by the reciprocal of the fraction of the structure sampled (i.e. the fraction of histological sections selected for examination, the fraction of the x–y step size covered by the counting frame and the fraction of the total section thickness occupied by the optical disector height). Statistical comparisons failed to reveal lateralized differences in total neuron number in any region examined (all P values >0.2) and this factor was not considered further in the analysis.

Approximately a third of the 24 brains examined in the present study (three each per young, aged-unimpaired and aged-impaired groups) were analyzed by two independent operators, using the sampling parameters described above. This partial replication revealed that one examiner had applied an overly conservative criterion to identify neurons for counting. Because the magnitude of the difference was highly consistent across the two estimates (e.g. for the nine brains examined twice, the standard error of the mean percentage difference across the two estimates was <3%), the counts from this examiner were multiplied by a fixed factor, reflecting the percentage of cells mistakenly excluded. Note that because this treatment comprised a simple linear transform, the pattern and relative
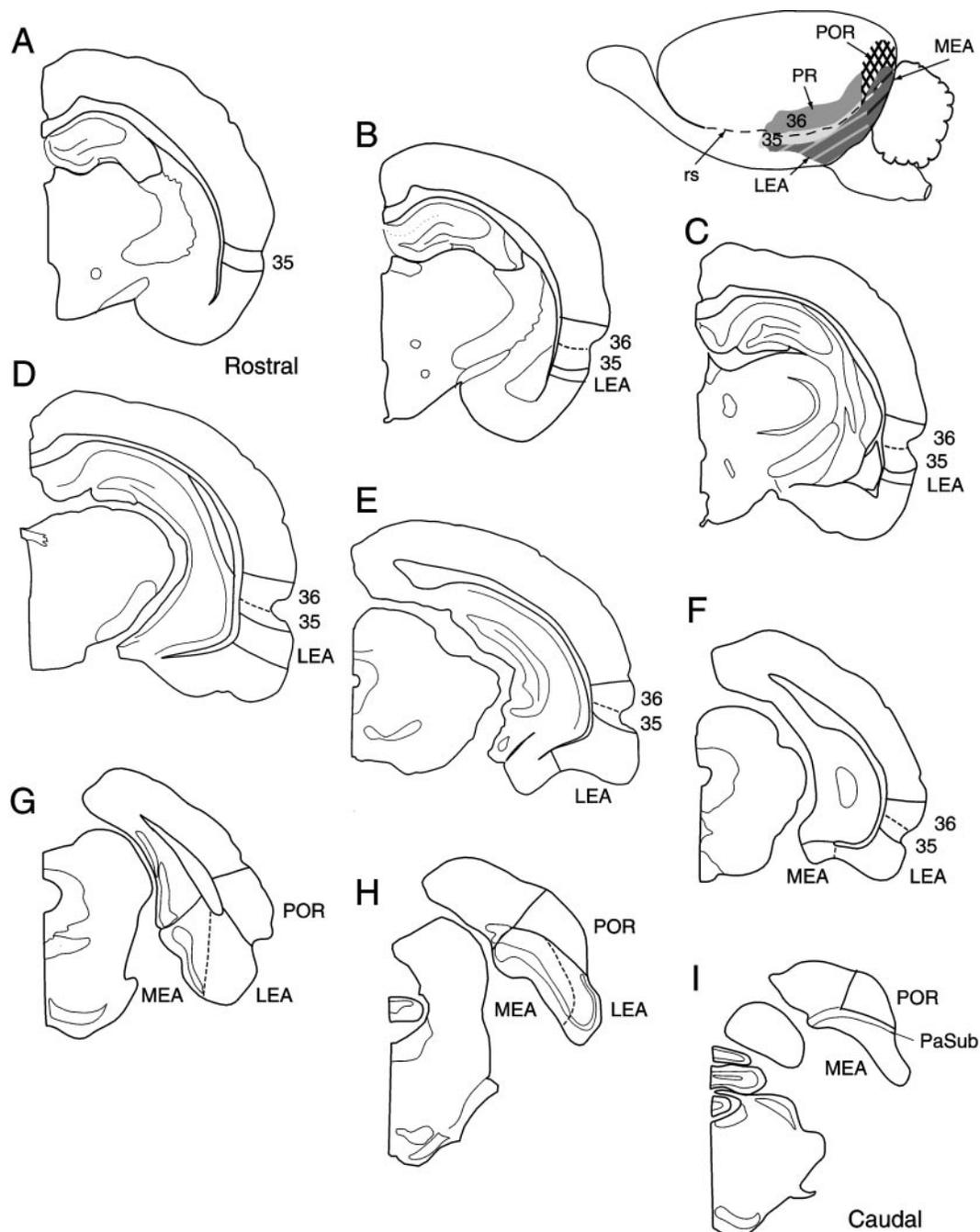
Figure 1. Schematic representation of the topographic organization of the perirhinal (PR; areas 35 and 36) and postrhinal (POR) cortices, and the lateral (LEA) and medial (MEA) entorhinal areas in the rat brain. Upper right inset presents a whole brain lateral view (rs, rhinal sulcus). Outlines of coronal sections are arranged rostral to caudal (*A–I*) through the parahippocampal region. PaSub, parasubiculum. [Illustration adapted from Burwell (Burwell, 2001).]

magnitude of differences between individuals and groups was completely unaffected.

Cytoarchitectonic Delineation of the Entorhinal, Perirhinal and Postrhinal Cortices

The following sections describe the main criteria that were used to delineate the borders of the cortical regions examined in the present study. Figure 1 schematically represents the organization of areas comprising the parahippocampal region of the rat brain, including a lateral surface view of the entorhinal, perirhinal and postrhinal cortices (Fig. 1, upper right). Figure 2 presents photomicrographic documentation of the neuroanatomical borders as they were applied in histological material analyzed in the present study.

The borders of the entorhinal cortex were defined according to previously published cytoarchitectonic criteria (Insausti *et al.*, 1997). For the present study, the entorhinal cortex was subdivided into lateral and medial areas (LEA and MEA, respectively) on the basis of classical and more recent cytoarchitectonic descriptions (Krieg, 1946; Blackstad, 1956; Dolorfo and Amaral, 1998). The LEA is bordered rostrally by the piriform and periamygdaloid cortices, and dorsally by the perirhinal and postrhinal cortices (Fig. 1). At caudal levels, the LEA is bordered medially and posteriorly by the MEA. There are subregional variations, but, in general, the LEA can be distinguished from the MEA by its layer II, which is thinner than in the MEA (Fig. 2*F–I*). The rostro-caudal strip of the LEA nearest the rhinal sulcus has a narrow layer I with cells that appear displaced from layer II (Insausti *et al.*, 1997). These displaced, or ectopic,
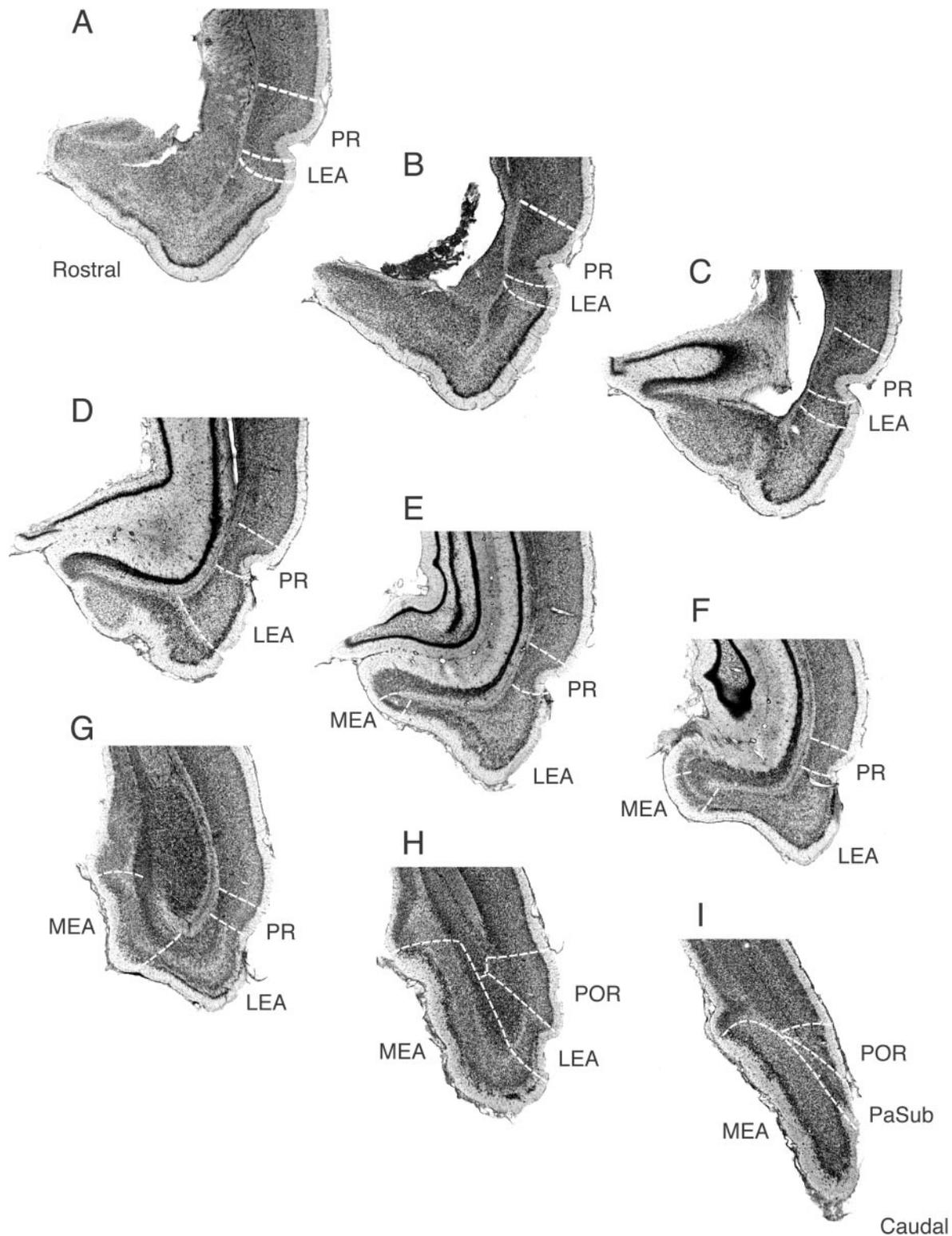
Figure 2. Scanning digital photomicrographs of Nissl-stained sections through the parahippocampal region in the brain of a young rat included in the present analysis. Sections are arranged rostral to caudal (*A–I*). Dashed white lines delineate the borders of the regions quantified. PR includes both areas 35 and 36 of the perirhinal cortex (other abbreviations as in Fig. 1).

cells, visible in Figure 2*B*, were used to demarcate the rostral limit of the LEA. The highly distinctive amygdalo-entorhinal transitional area was included as a part of the LEA (Insausti *et al.*, 1997). This area, shown in Figure 2*E*, is characterized by a scalloped border between layers I and II

on the ventromedial surface of the brain lateral to the MEA. The MEA is interposed between the parasubiculum (medially) and caudal LEA (laterally) (Dolorfo and Amaral, 1998). This entorhinal subregion was distinguished from the LEA in the present study by differences in layer II

and by its generally more prominent lamina dissecans, or cell-sparse layer IV. Laterally, the strip of MEA located near the border with LEA exhibits a clumpy layer II, as compared to the more homogeneous layer II of the adjacent LEA (Fig. 2*H*). The MEA border with the parasubiculum medially is marked by a layer II that thickens into a characteristic club-shaped formation (Figs 1*H–I* and 2*G–I*).

The borders of the perirhinal and postrhinal cortices were defined according to previously published criteria (Burwell, 2001), modified to accommodate subtle strain differences noted in the Long–Evans rat. The perirhinal region, comprising areas 35 and 36, occupies a strip of cortex associated with the most deeply invaginated portion of the rhinal sulcus (Figs 1 and 2*A–F*). The region is bordered rostrally by the insular cortex, which overlies the claustrum (Burwell, 2001). Layer V of the posterior insular region has cell-sparse gaps on either side (not shown), distinguishing it from the perirhinal cortex, which has a more homogeneous layer V (Fig. 2*A*). In general, the perirhinal cortex is dysgranular (area 36) or agranular (area 35) cortex characterized by large heart-shaped pyramidal cells in deep layer V. A prominent characteristic of the region that proved useful in the present study is its patchy layer II, visible in Figure 2*A*. This feature defined the dorsal perirhinal border. The prominent ectopic layer II cells distributed into layer I of the LEA, along with other laminar features (Burwell, 2001), were used to define the perirhinal–entorhinal border.

The postrhinal cortex (corresponding to the parahippocampal cortex in primates) is located caudal to the perirhinal cortex, dorsal to the rhinal sulcus. It is bordered ventrally by the LEA and medially by agranular retrosplenial cortex, as shown in Figure 1 (Burwell, 2001). The rostral border with the perirhinal cortex is marked by the presence of ectopic layer II cells in ventral postrhinal cortex. In coronal sections cut perpendicular to the flat skull stereotaxic position, this feature generally appears at the level of the caudal limit of the angular bundle (Fig. 2*H*). The postrhinal cortex is further characterized by the lack of prominent laminar features as compared to the neighboring cortices. In the dorsally adjacent cortex, layers II and III are more easily distinguished from each other and layer IV is relatively more prominent. At rostral levels, the postrhinal cortex is distinguished from the medially adjacent entorhinal cortex by the absence of the lamina dissecans (Fig. 2*H*). Caudally, postrhinal cortex is distinguished from the ventrally adjacent parasubiculum by its larger cells and patchy layer II (Fig. 2*I*).

Data Analysis
A multi-stage analytic strategy was adopted for evaluating parahippocampal neuron number in relation to chronological age and cognitive status. First, the quantitative morphometric data were compared across the young and aged groups, independent of spatial learning ability. Comparisons were included for neuron number summed across the cortical areas belonging to the parahippocampal region and for the various regions of interest considered individually. The former treatment minimizes variability attributable to imprecision in distinguishing the transitional cytoarchitectonic borders between adjacent cortical fields, increasing the sensitivity for detecting differences in cell number as a function of age. Parallel analyses then compared parahippocampal neuron number in the young group relative to aged rats classified as impaired or unimpaired on the basis of their performance in the spatial version of the Morris water maze (Gallagher *et al.*, 1993; Rapp and Gallagher, 1996). Parametric statistics [factorial analysis of variance (ANOVA)] were used throughout, with the significance level set at $P = 0.05$. Finally, Pearson r correlation coefficients were computed to test the possibility that individual differences in parahippocampal neuron number predict variability in the effects of aging on cognitive capacities mediated by the hippocampal system. All statistical analyses were conducted using the StatView 5.0 software package (SAS Institute Inc., Cary, NC).

Results

Spatial Learning in the Aged Rat
Subjects in the present experiment were the same as in a previous report and the outcome of behavioral testing is detailed elsewhere (Rapp and Gallagher, 1996). Relative to young rats,
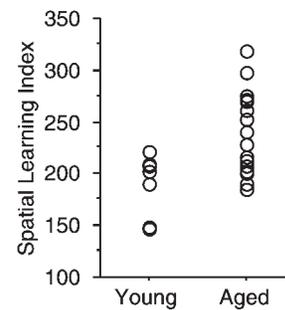

Figure 3. Learning index scores for individual young ($n = 8$) and aged ($n = 16$) rats derived during probe testing in the hippocampus-dependent, spatial version of the Morris water maze. This performance measure reflects an animal's distance from the escape location during searching and lower scores therefore represent better learning — see Materials and Methods and previous work (Gallagher *et al.*, 1993). Note that half the aged rats scored outside the range of the young group (index scores > 230), exhibiting substantial spatial learning impairment.

the aged group was significantly impaired in learning the hidden escape platform location in the place version of the Morris water maze. Substantial variability was observed between the aged individuals and half performed within the range of values for the young group, as assessed by the learning index measure derived from probe testing (Fig. 3). The remaining aged rats displayed substantial impairment, scoring outside this normative range. In contrast, the young and aged groups performed similarly on the non-spatial, cued version of the water maze (data not shown). These findings are consistent with a large body of published data from this study population, demonstrating that a substantial proportion of aged rats exhibit a pattern of spatial learning impairment qualitatively similar to the effects of direct hippocampal damage (Gallagher *et al.*, 1993; Rapp and Gallagher, 1996; Gallagher and Rapp, 1997; Rapp *et al.*, 1999; Smith *et al.*, 2000).

Neuron Number in the Parahippocampal Region
Results from the stereological analysis of neuron number in the parahippocampal region are illustrated in Figure 4. Independent of age and cognitive status, total neuron number summed across the entorhinal, perirhinal and postrhinal cortices averaged 1 033 941 (SE = 16 654) in the Long–Evans rat. Mean values for the young and aged groups differed by <3.5% and were statistically indistinguishable [young mean (SE) = 1 057 143 (31 002); aged mean (SE) = 1 022 340 (19 666), ANOVA; $F < 1$, $P > 0.3$; Fig. 4, top panel). Negative results were also obtained when the entorhinal and peri-/postrhinal cortices were considered separately (all F-values <1, P-values >0.4) and when all four regions of interest were analyzed individually (all F-values <1, P-values >0.3). These results indicate that chronological aging in the rat can occur in the absence of substantial neuron death in cortical components of the hippocampal system.

Notably, variability in the neuron number estimates was generally low. For the parahippocampal region as a whole, the interanimal coefficient of variation (i.e. SD/mean total neuron number) was <9% in both the young and aged groups. We also calculated the coefficient of error for the neuron number estimates according to the quadratic approximation formula (Gundersen and Jensen, 1987), providing a standardized measure for gauging the precision of the morphometric results. This parameter was well under 5% for all regions examined, substantially exceeding recommended stereological guidelines. Finally, the sensitivity of the experimental approach was explored further by performing a series of statistical power
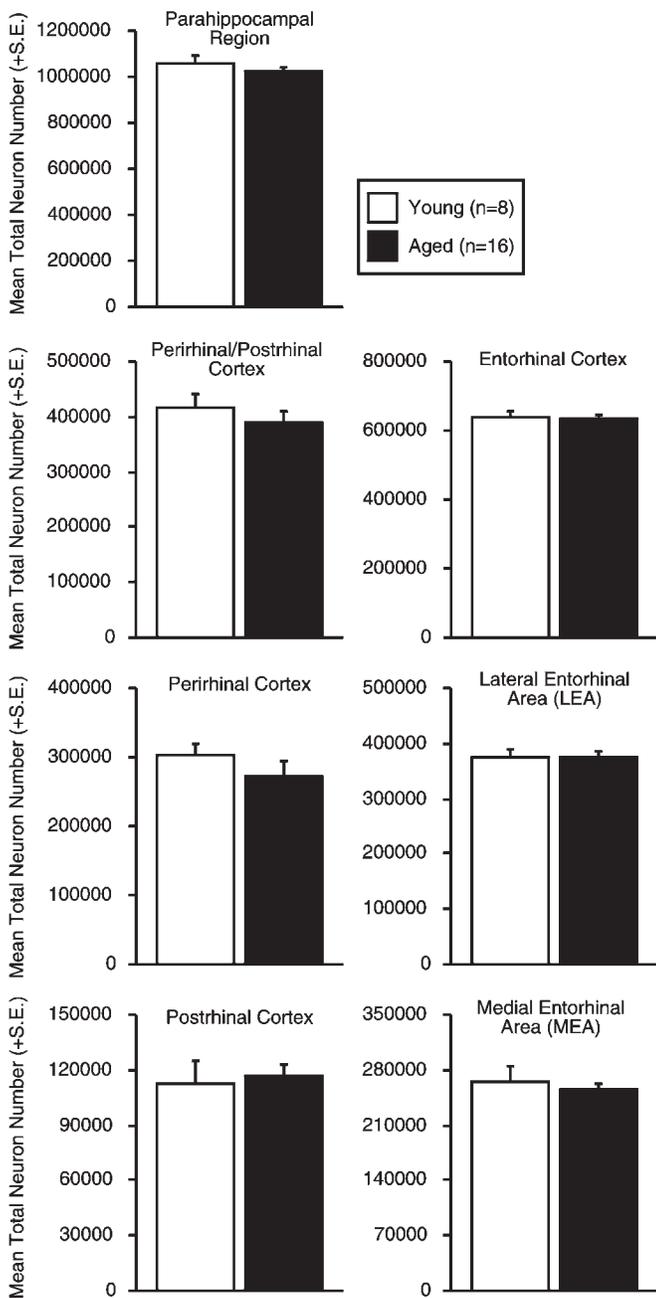
Figure 4. Mean estimated total neuron number (+SE) in the parahippocampal region of behaviorally characterized young adult and aged rats. Results are presented for neuron number summed across the component areas of the parahippocampal region (upper left), for the peri-/postrhinal and entorhinal cortices considered separately, and for the perirhinal, postrhinal, lateral entorhinal and medial entorhinal areas individually. Differences in neuron number between age groups were not statistically significant in any comparison.

Figure 5. Mean estimated total neuron number (+SE) in the parahippocampal region for young adult rats, and aged animals with ('Aged-Imp'), or without ('Aged-Un') spatial learning impairment. Panels are organized as in Figure 4. Statistical analyses failed to reveal reliable differences in neuron number as a function of age or cognitive status in any region examined.

calculations. Taking into account the number of subjects examined and the standard deviations of the quantitative estimates, this analysis indicated that a difference in parahippocampal neuron number between the young and aged groups as small as 8.0% would have been reliably detected, with 70% power. The corresponding detection limits for the entorhinal and peri-/postrhinal cortices considered individually were 7.5 and 13.7%, respectively. Together, these measures confirm that the stereo-
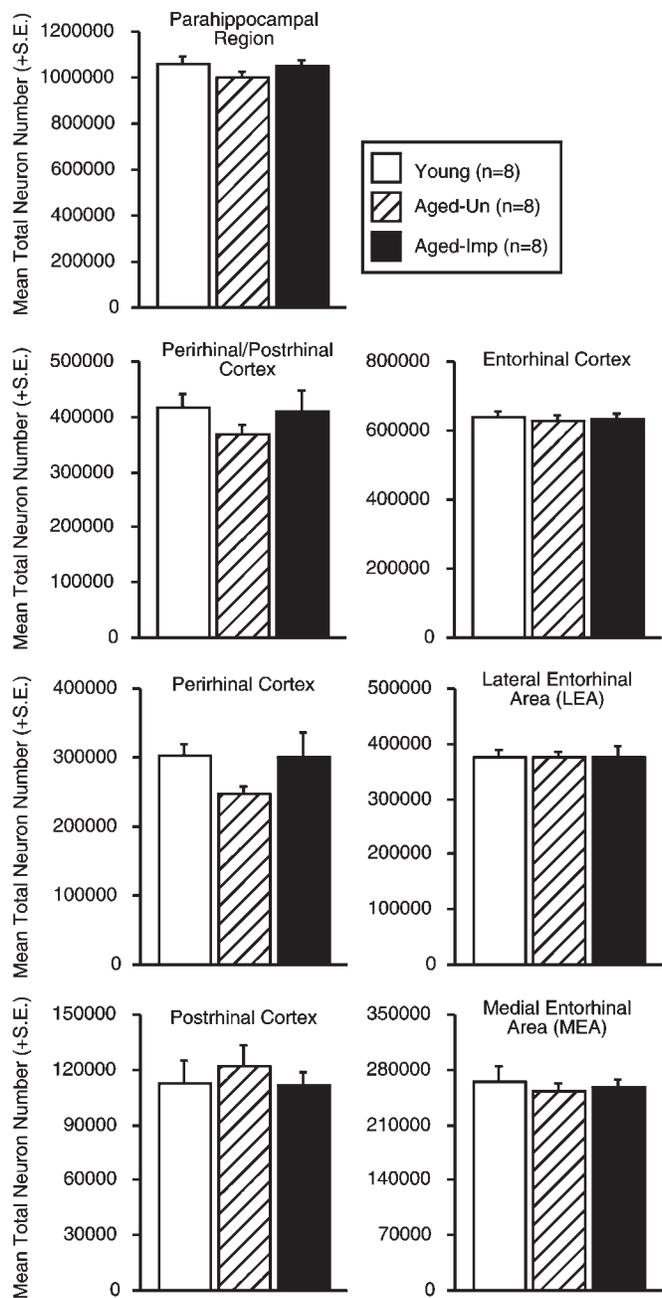
logical design was successful in yielding precise estimates of neuron number with low variability and that the experimental strategy we adopted was sufficiently sensitive to detect even a modest degree of age-related neuron loss.

Parallel analyses tested the possibility that parahippocampal neuron loss is selectively present among aged rats with hippocampal learning impairment. Mean total neuron number failed to differ in any comparison between the young, aged-unimpaired and aged-impaired groups, regardless of whether the morphometric data were summed across the cortical areas examined, or

considered separately for the individual regions (all *P*-values >0.2; Fig. 5). The only substantial numerical difference between groups was observed in the perirhinal cortex, where average neuron number was ~18% greater for young adults and aged-impaired rats relative to aged animals with preserved spatial learning. Interestingly, estimated neuron number for the postrhinal cortex was somewhat greater in the latter group ('Aged-Un' in Fig. 5) compared with values for young and aged-impaired rats. The boundary between the adjacent peri- and postrhinal cortices appears as a subtle transition rather than a sharp demarcation and the non-significant group differences seen across these areas may partly reflect inconsistencies in defining this border. With respect to the central hypothesis under consideration in the present investigation, however, the more illuminating observation is that total neuron number in the perirhinal cortex differed by <2% between young rats and aged animals with robust spatial learning impairment ('Aged-Imp' in Fig. 5). Accordingly, the overall pattern of results counts against the interpretation that perirhinal neuron death is sufficient to account for the effects of aging on hippocampal learning.

Finally, a linear correlation approach was used to examine whether variability in parahippocampal neuron number predicts individual differences in spatial learning capacity. In order to gain maximal statistical power, all 24 young and aged rats were considered together in the analysis. None of the correlation coefficients evaluating the morphometric results in relation to the learning index scores were statistically reliable, regardless of whether neuron number was summed across regions, or considered separately for each area (all *r* values between −0.24 and 0.15, all *P*-values >0.6). Together with the group comparisons reported above, these findings provide strong support for the conclusion that age-related spatial learning impairment in the rat can occur in the absence of substantial neuron loss in cortical components of the hippocampal system.

Discussion

A long-standing view of cognitive aging is that the hippocampus is particularly vulnerable to neuron death and that pyramidal cell loss importantly contributes to age-related decline in the function of this system. Support for this proposal includes early evidence that decreases in pyramidal cell density in the aged hippocampus are coupled with the severity of spatial learning impairment in aged rats and that manipulations conferring protection against neuronal loss improve the cognitive outcome of aging (Meaney *et al.*, 1988; Issa *et al.*, 1990). More recent investigations using stereological methods, however, have made it clear that substantial age-related learning impairment can occur in the absence of detectable neuron death in the hippocampus. Using a design similar to the present study, a number of laboratories have documented that total neuron number in the principal cell fields of the hippocampus (i.e. the granule cell layer and the CA3–CA1 pyramidal cell fields) is statistically indistinguishable in aged rats that exhibit robust spatial learning deficits relative to both young animals and age-matched rats with intact learning (Rapp and Gallagher, 1996; Rasmussen *et al.*, 1996). Similar preservation has been noted in behaviorally characterized aged monkeys and in humans (Morrison and Hof, 1997; Peters *et al.*, 1998).

An alternative account, based on converging anatomical and behavioral evidence, is that neuron death associated with age-related learning impairment might instead be localized to the parahippocampal region, disrupting the principal circuitry by which the hippocampus interacts with the neocortex. The findings reported here discount this proposal and demonstrate

that total neuron number across the entorhinal, perirhinal and parahippocampal cortices differs by <3.5% in young and aged rats. We also failed to detect statistically significant or numerically substantial age-related neuron loss in any of the individual cortical areas examined. Most importantly, no evidence was obtained for selective or disproportionate neuron death among aged rats with documented deficits in learning mediated by the hippocampal system, regardless of whether the individual regions of interest were considered together or separately. Thus, taken together with earlier analyses focusing on the hippocampus (Rapp and Gallagher, 1996; Rasmussen *et al.*, 1996), the present investigation establishes that age-related cognitive decline can occur in the absence of detectable neuron loss in any major, cytoarchitectonically defined component of the hippocampal system.

The present study was not designed to test for potential neuron loss among small subpopulations of specific cell types within the regions examined. A potential concern, for example, is that age-related neuron death might be restricted to superficial layers of the entorhinal cortex that project to the hippocampus, comprising only a minor fraction of this cortical field as a whole. Although laminar-specific cell loss of this sort could, in principle, escape detection by the approach used here, the pattern of results actually obtained mitigates this concern. Total neuron number in the entorhinal cortex differed by <1% across the young and aged-impaired groups, representing an absolute difference of <6000 cells. To place this finding in context, an earlier stereological analysis reported that layer II of the rat entorhinal cortex contains ~96 000 neurons (Merrill *et al.*, 2001). Accordingly, even in the event that the group difference we observed in the entorhinal cortex is reliable and entirely attributable to a selective decline in layer II, the total loss would represent only 6% of the neuronal population that resides in this layer. There is limited empirical basis for supposing that neuron death of this magnitude, restricted to neurochemically or regionally specific subpopulations of cells within the hippocampal system, could account for the effects of aging on cognitive capacities supported by this system.

Rigorous quantitative data on neuron number provide an important framework for interpreting the effects of aging on a wide range of other neurobiological parameters. Electrophysiological results from both *in vivo* preparations and awake, behaving rats converge on the view that the function of the hippocampus is significantly altered in aged subjects with spatial learning impairment (Barnes *et al.*, 1997; Shen *et al.*, 1997; Tanila *et al.*, 1997a,b; Barnes, 2001). Related morphometric investigations in the brains of behaviorally characterized rats indicate that the strictly ordered organization of hippocampal circuitry is sensitive to aging and that across the principal relays of hippocampal connectivity, inputs arising in the entorhinal cortex may be particularly susceptible (Geinisman *et al.*, 1995; Rapp *et al.*, 1999; Smith *et al.*, 2000). A reasonable account of these findings is that the physiological and anatomical effects of aging observed in the hippocampus may be secondary to neuron loss in afferent systems, either in the entorhinal cortex itself, or in the peri-/postrhinal cortices. The data reported here, in contrast, suggest that age-related learning impairment arises as a consequence of functional compromise among the surviving neurons of the hippocampal system. Candidate substrates include deficits in the capacity for synaptic morphological modification and defects in signaling pathways known to be critical for learning-related cellular plasticity. By comparison with Alzheimer's disease and other age-related pathologies in which neuron death is prominent, impairments in cell biological function associated

with normal cognitive aging may prove more tractable targets for intervention.

Beyond our primary focus on the neurobiological consequences of aging, results of the present investigation extend previous normative data on the number of neurons in the rat hippocampal memory system. Studies using the optical fractionator stereological approach have reported that the entorhinal cortex comprises ~690 000 neurons in young female Wistar rats (Mulders *et al.*, 1997) and 663 000 neurons in young Fischer 344 females (Merrill *et al.*, 2001). The estimate obtained here for male Long–Evans rats is similar, totaling ~635 000 neurons across the lateral and medial subdivisions of the entorhinal cortex. This is the first investigation that we are aware of to provide a parallel analysis of other components of the parahippocampal region, i.e. the peri- and postrhinal cortices. The findings demonstrate that these cortical areas collectively contain ~400 000 neurons, substantially fewer than the entorhinal cortex. Previous results from the same brains indicate that there are ~1.8 million neurons across the principal cell fields of the hippocampus, i.e. the granule cell layer of the dentate gyrus and the CA3/2 and CA1 pyramidal cell fields (Rapp and Gallagher, 1996). Together with published estimates for the hilus and subiculum (Rasmussen *et al.*, 1996), the accumulated evidence from investigations using stererological methods therefore indicates that ~3.2 million neurons comprise the rat hippocampal memory system as a whole. Normative data of this sort are an essential foundation for developing biologically constrained, realistic simulations of hippocampal information processing. Ultimately, computational modeling could provide a valuable strategy for testing the functional significance of perturbations in hippocampal integrity associated with aging and other conditions.

Notes

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