

Sex Differences, but No Seasonal Variations in the Hippocampus of Food-Caching Squirrels: A Stereological Study

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ABSTRACT

Recent studies have described sex differences in the relative size of the hippocampus that are associated with sex differences in space use in birds and short-lived mammals. A correlation between spatial learning and increased hippocampal volume has also been demonstrated in food-caching animals. Such results suggest that sexually dimorphic spatial learning (sex differences in space use during the breeding season) and seasonal variations in food-caching behavior (spatial memory for cache locations) might correlate with morphological changes in the hippocampus of adult long-lived mammals. We used modern stereological techniques to examine the volume and neuron number of the structures forming the hippocampal complex (dentate gyrus, CA3, and CA1) of wild adult eastern gray squirrels (*Sciurus carolinensis*) throughout the year. We observed differences in brain size between samples collected at different times of the year (October, January, and June). Our analysis showed sex differences, but no seasonal variations, in the volume of CA1 stratum oriens and stratum radiatum. There were no sex differences or seasonal variations in the relative volume or the number of neurons of any other layer of the structures forming the hippocampal complex. These results confirm the existence of sex differences in the structure of the hippocampus; however, this sexual dimorphism does not vary seasonally in adulthood and is likely to result from developmental processes. These results do not support the hypothesis that seasonal variations in food-caching behavior might correlate with morphological changes, such as variations in volume or neuron number, in the hippocampal complex of adult long-lived mammals. *J. Comp. Neurol.* 425:152–166, 2000. © 2000 Wiley-Liss, Inc.

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A number of studies have shown that sex differences and seasonal variations in spatial behavior correlate with changes in hippocampal morphology in short-lived mammals and birds, but such correlations have never been examined in long-lived mammals. Recent reports describe sex differences in the relative size of the hippocampus that are associated with sex differences in space use. Voles, kangaroo rats, and cowbirds all exhibit a sex difference in relative size of the hippocampal complex (or hippocampal formation in birds) that is correlated with a sex difference in spatial behavior. Males of the polygynous meadow vole (*Microtus pennsylvanicus*) have a larger hippocampus than females, whereas there is no sex difference in hippocampal size in the monogamous pine vole (*M. pinoretum*; Jacobs et al., 1990). In kangaroo rats (*Dipodomys*

merriami and *D. spectabilis*), sex differences in mating strategies suggest that males of both species require more spatial ability than do females (Jacobs and Spencer, 1994). Accordingly, hippocampal size (relative to brain size) is larger in males than females in these two species. Similarly, differences in the size of home ranges of male and female Mongolian gerbils (*Meriones unguiculatus*) corre-

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late with differences in hippocampal size, with males being larger (Sherry et al., 1996). In brown-headed cowbirds (*Molothrus ater*), a brood-parasite, females search for host nests in which to lay their eggs during the breeding season. Males do not express such behavior and, as predicted, the relative size of the hippocampus is greater in females than males (Sherry et al., 1993). A similar study in shiny cowbirds (*M. bonariensis*) reported that these sex differences vary seasonally and that sexual dimorphism in relative hippocampal volume might be present only during the breeding season (Clayton et al., 1997). In meadow voles, sex differences in spatial learning vary seasonally and spatial performance is differentially affected by reproductive status (Galea et al., 1996). Additional research implicates gonadal hormones as a primary determinant of sexual dimorphism of the hippocampal complex (Foy et al., 1984; Galea et al., 1999; Gould et al., 1990a; Woolley and McEwen, 1992). These results suggest that hormonal changes might be responsible for the seasonal variations of sex differences in spatial learning by producing morphological changes in the hippocampal complex, a brain structure critically involved in spatial learning.

A correlation between spatial learning and increased hippocampal volume has also been demonstrated in food-caching animals (Sherry et al., 1992). In kangaroo rats, differences in foraging behavior indicate that the scatter-hoarding Merriam's kangaroo rats (*D. merriami*) have a larger hippocampus than the larder-hoarding bannertail kangaroo rats (*D. spectabilis*; Jacobs and Spencer, 1994). Similarly in birds, scatter-hoarders have a larger hippocampal formation relative to the telencephalon than species that do not store food (Krebs et al., 1989; Sherry et al., 1989). In the black-capped chickadee (*Parus atricapillus*), a scatter-hoarder that caches food and retrieves caches based on spatial memory of their locations, researchers reported an increase in volume of the hippocampal formation in the fall at the peak of caching behavior (Smulders et al., 1995; but see Krebs et al., 1995 and this discussion). In this species, recruitment of hippocampal neurons increases in the fall at the peak of caching behavior, but interestingly does not result in an increase in total number of neurons in the hippocampal formation (Barnea and Nottebohm, 1994, 1996).

Such results suggest that sexually dimorphic spatial behavior (sex differences in space use) and seasonal patterns of hippocampal-dependent learning (spatial memory for cache locations) might also correlate with morphological changes in the hippocampal complex of adult long-lived mammals. In eastern gray squirrels (*Sciurus carolinensis*), we recently demonstrated that seasonal variations in food caching behavior do not affect cell proliferation or total neuron number in the granule cell layer of the dentate gyrus (Lavenex et al., 2000). It remains to be determined, however, whether other morphological changes of the hippocampal complex might underlie sex differences and seasonal variations in spatial behavior. To test these hypotheses, we used modern stereological techniques to examine the volume and neuron number of the different layers of the structures forming the hippocampal complex (dentate gyrus, CA3, and CA1) of wild adult eastern gray squirrels throughout the year.

Gray squirrels represent an ideal model in which to study these questions. They live up to 6 years in the wild (Gurnell, 1987), they are seasonal breeders (Thompson, 1977), and they exhibit seasonal variations in food-

caching behavior (Thompson and Thompson, 1980). During the nonbreeding season, males maintain larger home ranges than females (Thompson, 1978), and sex differences in home range further increase during the breeding season, when males are actively searching for females (Thompson, 1977). Eastern gray squirrels usually have two breeding seasons per year, and most breeding occurs in December–February and May–July of each year (Merritt, 1987; Thompson, 1977). In thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*), males have been shown to rely on spatial memory when looking for receptive females (Schwagmeyer, 1995). In gray squirrels, the males' space use increases during the breeding season, but their reliance on spatial memory to locate receptive females has yet to be demonstrated. Caching activity and gray squirrels' dependence on food caches also varies seasonally (Gurnell, 1987; Koprowski, 1994; Thompson and Thompson, 1980; Vander Wall, 1990). Gray squirrels scatter-hoard their winter food supply of nuts in hundreds of locations and rely primarily on spatial memory to relocate these caches (Jacobs and Liman, 1991). We collected male and female adult eastern gray squirrels in Pennsylvania, at three times of the year: in October 1996, January 1997, and June 1997. October corresponded to the peak of the caching season, but did not coincide with either breeding season. In January, caching was completed but squirrels were still dependent on spatial memory to locate their caches; this period coincided with the first breeding season. In June, squirrels had depleted their caches and were not actively engaged in further caching activity; this period coincided with the second breeding season.

This study examines several hypotheses regarding sex differences and seasonality in adult long-lived mammals. The first hypothesis predicts that seasonal variations in food caching behavior in wild adult eastern gray squirrels might correlate with morphological changes, such as variations in volume of some layers of the structures forming the hippocampal complex. If this hypothesis is correct, one would expect to observe variations in the volume of some components of the hippocampal complex between October, January, and June, independently from the sex of the animals. The second hypothesis predicts that sex differences in space use might correlate with morphological differences, such as differences in volume and neuron number in the hippocampal complex, which are independent from the possible morphological changes linked to seasonal variations in food caching behavior. If such sex differences exist, two subsequent alternative hypotheses follow: (1) sexual dimorphism of the hippocampal complex varies seasonally in adult long-lived mammals, as previously reported in short-lived rodents, and correlates with seasonal variations in space use. In this case, the volume of the hippocampal complex should be larger in males than females during the two breeding seasons, whereas there should be no sex difference outside the breeding seasons; and (2) sexual dimorphism does not vary seasonally in adult long-lived mammals and does not correlate with seasonal variations in space use. This finding would suggest that seasonal variations of sex differences in the morphology of the hippocampal complex previously reported in short-lived rodents might not reflect true adult plasticity and might result from organizational or activational effects of gonadal hormones during development.

MATERIALS AND METHODS

Animals

Thirty wild adult eastern gray squirrels were trapped in Wilkes-Barre and Kingston, PA, at three different times of the year: (1) between October 3rd and 13th, 1996; (2) between January 24th and February 4th, 1997; and (3) between June 28th and July 4th, 1997. For each of the three sampling periods, 5 males and 5 females were live-trapped. The age of the animals was determined by using standard size measurements and obvious signs of sexual maturity (Gurnell, 1987). Females do not reproduce until 11 months and males become sexually mature at 15 months (Thompson, 1977). Subadults and juveniles were immediately released at the site of capture and were not used further in the study. Adult squirrels were housed in captivity for an average of 48 hours under artificial illumination matching the natural photoperiod and received two intraperitoneal injections of bromodeoxyuridine in order to enable the study of cell proliferation in the dentate gyrus (Lavenex et al., 2000). Squirrels were then anesthetized (1 ml Pentobarbital sodium [6 grains/ml]) and perfused transcardially with 100 ml of saline (0.9% NaCl), followed by 100 ml of a sodium sulfide solution (Na_2S 1.17% [w/v] + NaH_2PO_4 1.19% [w/v]) and 100 ml of 4% paraformaldehyde in phosphate-buffered saline (0.1 M PBS, pH 7.4). The animal was decapitated and the skin and muscles surrounding the skull removed. The brain within the skull was placed in 4% paraformaldehyde for at least 1 month until processing. The brain was then removed from the skull and cryoprotected in a 30% sucrose solution. Coronal sections were cut at 40 μm on a sliding, freezing microtome and collected in PBS. Sections to be stained for silver impregnation (1 in 4 series) and for Timm stain (1 in 4 adjacent series) were mounted directly from buffer onto gelatin-coated slides and processed. All experimental procedures were carried out following the NIH guidelines regarding the care and use of animals for experimental procedures and were approved by the University of California at Berkeley and Wilkes University Animal Care and Use Committees.

Silver impregnation processing

All sections of the series for silver impregnation were first mounted onto gelatin-coated slides and processing was performed following a technique modified from Sloviter et al. (1993). Sections were washed 3×5 minutes in dH_2O , pretreated 2×5 minutes in a 50:50 solution (v/v) of 9% sodium hydroxide (NaOH, w/v) and 1.2% ammonium nitrate (NH_4NO_3 , w/v), impregnated for 10 minutes in an impregnation solution made of 600 ml 9% NaOH + 400 ml 16% NH_4NO_3 + 6 ml 50% silver nitrate (AgNO_3 , w/v), rinsed 3×2 minutes in a washing solution made of 10 ml 1.2% NH_4NO_3 + 5 g of sodium carbonate anhydrous (Na_2CO_3) + 300 ml 95% EtOH + 700 ml dH_2O , treated for 1 minute in a developing solution made of 10 ml 1.2% NH_4NO_3 + 0.5 g citric acid anhydrous ($\text{C}_6\text{H}_8\text{O}_7$) + 15 ml 37% formalin + 500 ml 95% EtOH + dH_2O added to 1000 ml, washed 3×10 minutes in 0.5% acetic acid, dehydrated and coverslipped with Permount.

Timm stain

All sections of the series for Timm stain were first mounted onto gelatin-coated slides and processed according to techniques described in Danscher and Zimmer

(1978). Sections were incubated for 50 minutes in the dark at 36°C in a developing solution made of 20 ml of citrate solution (12.75 g citric acid monohydrate [$\text{C}_6\text{H}_5\text{O}_7 \cdot \text{H}_2\text{O}$] + 12.5 g tri-sodium citrate anhydrous [$\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$] in 50 ml dH_2O) + 60 ml hydroquinone solution (7.5 g hydroquinone in 150 ml dH_2O) and 120 ml arabic gum solution (200 g arabic gum in 400 ml dH_2O) + a few crystals of thymol + 1 ml of 17% silver nitrate (AgNO_3 , w/v); rinsed for 10 minutes in flowing tap water in the dark; rinsed twice in dH_2O , fixed for 1 minute in 1% sodium sulfate ($\text{Na}_2\text{S}_2\text{O}_3$); rinsed twice in dH_2O , dehydrated, and coverslipped with Permount.

Neuron counts and volume measurements

Volume and neuron count measurements were performed with StereoInvestigator (Microbrightfield Inc., Colchester, VT). The volume of each of the different components of the hippocampal complex (dentate gyrus, CA3, CA1, and their different layers) was measured according to the Cavalieri principle (Gundersen and Jensen, 1987; West and Gundersen, 1990) on the silver-impregnated sections or the Timm-stained sections. We followed the nomenclature of the hippocampus of the laboratory rat (*Rattus norvegicus*) described by Amaral and Witter (1995). Figure 1 presents silver-impregnated and Timm-stained coronal sections at four different rostrocaudal levels of the squirrel hippocampal complex, together with line drawings delineating the hippocampal fields and layers measured in this study. The CA2 field of the hippocampus proper was included in the CA1 field for all measurements, as the CA3/CA2 border was easily and reliably identifiable in both silver-impregnated and Timm-stained sections, whereas the CA2/CA1 border was more difficult to establish accurately and with confidence in the two stains.

We used the silver-impregnated sections to measure the volumes of the entire structure (all layers) of the dentate gyrus, CA3, and CA1. The volumes of the different layers of the dentate gyrus were also measured on the silver-impregnated sections. Unfortunately, our Timm reaction did not stain the different sublayers of the molecular layer in a reliable manner. Consequently, it was impossible to accurately distinguish between the different connectivity zones of the molecular layer, i.e., outer, middle, and inner portions of the molecular layer, that are innervated, respectively, by the lateral entorhinal cortex, the medial entorhinal cortex, and the commissural/associational zone (Amaral and Witter, 1995). We thus limited our analysis

Fig. 1. Low-power photomicrographs and line drawings at four different rostrocaudal levels of the squirrel hippocampal complex. Four by five negatives of photomicrographs taken on a Nikon multiphot were digitally scanned with a Polaroid PrintScan 45 scanner and levels were adjusted in Photoshop 5.0. **A–D**: Rostral to caudal. Coronal sections and line drawings delineating the structures measured with the silver-impregnated sections. DG, dentate gyrus; hilus, hilus; mol, molecular layer of the dentate gyrus; CA3, CA3 field; CA1, CA1 field. The layers in black represent the granule cell layer (within the dentate gyrus) and the pyramidal cell layer (within CA3 and CA1). **E–H**: Rostral to caudal. Coronal sections and line drawings delineating the structures measured with the Timm-stained sections. MF, mossy fibers; soCA3, stratum oriens CA3; srCA3, stratum radiatum CA3; soCA1, stratum oriens CA1; srCA1, stratum radiatum CA1; slmCA1, stratum lacunosum moleculare CA1. Scale bar = 1 mm.

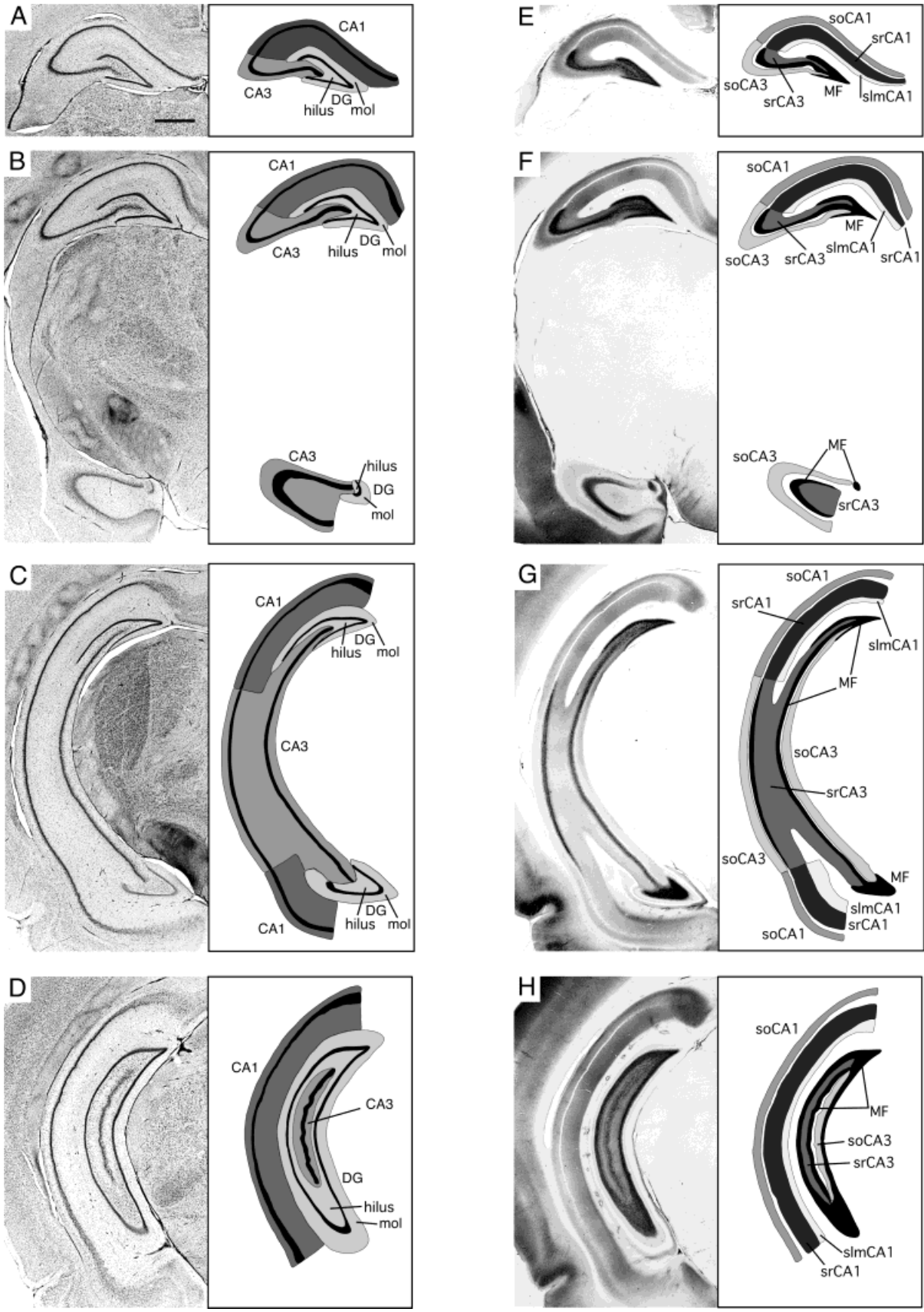


Figure 1

TABLE 1. Volume of the Brain, Hippocampal Complex, Dentate Gyrus, CA3, and CA1 Fields of the Hippocampus¹

	October	January	June	Average		By sex	CE	CE ² /CV ²
Brain	4,527 ± 50.0	4,318 ± 75.8	4,032 ± 65.4	4,292 ± 52.1	M	4,339 ± 76.9	0.030	0.20
Res ²	—	—	—	—	F	4,245 ± 70.9		
HC	210.71 ± 5.26	195.08 ± 4.47	184.23 ± 5.86	196.67 ± 3.54	M	201.62 ± 4.66	—	—
Res	0.009 ± 0.011	-0.005 ± 0.008	-0.003 ± 0.013		F	191.72 ± 5.18		
DG	56.42 ± 2.58	51.17 ± 2.44	49.53 ± 2.19	52.38 ± 1.45	M	53.81 ± 2.25	—	—
Res	0.004 ± 0.020	-0.014 ± 0.018	0.010 ± 0.017		F	50.94 ± 1.83		
CA3	68.91 ± 1.63	64.03 ± 1.17	58.50 ± 1.85	63.81 ± 1.18	M	64.65 ± 1.51	0.043	0.18
Res	0.013 ± 0.011	0.000 ± 0.009	-0.013 ± 0.013		F	62.98 ± 1.85		
CA1	85.83 ± 1.50	79.87 ± 1.56	76.21 ± 2.1	80.49 ± 1.20	M	83.16 ± 1.35	0.029	0.13
Res	0.006 ± 0.008	-0.005 ± 0.005	-0.001 ± 0.011		F	77.81 ± 1.76		

¹Values indicate group mean ± S.E.M. Absolute volume is expressed in cubic millimeters (mm³).

²Res, residuals from the regression analyses plotting the log of the volume of the structure of interest (e.g., log vol. [HC]) against the log of the volume of the brain minus the structure of interest (e.g., log vol. [Brain - HC]). The relative variance of individual estimates (CE) was not calculated for the hippocampal complex (HC) or the dentate gyrus (DG), because these volumes were calculated by summing the volumes of their components (HC = DG + CA3 + CA1; DG = gcl DG + mol DG + hilus; see Table 3). CE², observed relative variance of individual estimate; CV², observed relative variance of group.

to the volumes of the molecular layer, granule cell layer, and hilus as defined by the silver impregnation method. The volumes of the pyramidal cell layers of the CA3 and CA1 fields were also measured on the silver-impregnated sections. In contrast to what was observed in the dentate gyrus, the Timm reaction did provide clear boundaries of CA3 strata oriens and radiatum and of CA1 strata oriens, radiatum, and lacunosum moleculare. Therefore, Timm-stained sections were used to measure the volume of the different layers of CA3 and CA1. The CA3 stratum lucidum, although identifiable in silver-impregnated sections (in contrast to Nissl-stained sections), was not measured separately; instead the volume of the mossy fiber projections was measured with the Timm-stained sections. The mossy fibers are the projections of the granule cells of the dentate gyrus to the CA3 field of the hippocampus, which ultimately all reach (and help define) the stratum lucidum and stop at the CA3/CA2 border.

A total of 9 to 12 sections per animal (1 in 16 sections [640 µm apart]), with the first section selected randomly within the first four sections through the structure of interest, were used for these hippocampal volume measurements. Brain volume refers to the volume of the brain without the cerebellum, which was removed before sectioning. A total of 11 to 13 sections per animal (1 in 48 sections [1920 µm apart]), with the first section selected randomly within the first 12 sections through the olfactory bulb, were used for brain volume measurements. Sections were coded, so that the experimenter was blind to the period in which the animal was collected, as well as its sex, and the code was broken only after completion of the analyses.

The total number of neurons in the granule cell layer of the dentate gyrus or in the pyramidal cell layers of CA3 and CA1 was determined by using the optical fractionator method (West et al., 1991) on the silver-impregnated sections. This design-based method enables an estimation of the absolute number of neurons which is independent from the volume. A total of 9 to 12 sections per animal (1 in 16 sections [640 µm apart]), with the first section selected randomly within the first four sections through the structure of interest (granule cell layer of the dentate gyrus, pyramidal cell layer of CA3 or CA1), were used to count neurons. A 100× Neofluar oil objective (N.A. 1.30) was used on a Nikon Optiphot microscope linked to PC-based StereoInvestigator. Neuron number was measured

in the right granule cell layer or in the right pyramidal cell layers of CA3 and CA1 only, as volume measurements did not show any lateralization. The estimate of the total number of neurons bilaterally was calculated by doubling the total number of neurons counted on the right side. The data on neuron number in the granule cell layer have been previously published (Lavenex et al., 2000) and are presented here to provide a complete description of volumes and neuron numbers in the hippocampal complex of wild adult eastern gray squirrels.

Data analysis

Absolute volumes and total neuron numbers are presented as cubic millimeters (mm³) and number of neurons, respectively. Data on neuronal density are presented as number of neurons per mm³. We performed statistical analyses on the logarithm of the raw values to conform to the assumption of homogeneity of variance. For volume measures, we also performed regression analyses, plotting the logarithm of the volume of the structure of interest (e.g., log vol. hippocampal complex [HC]) against the logarithm of the brain volume minus the volume of the structure (e.g., log vol. [Brain - HC]). For neuron counts, we performed regression analyses, plotting the logarithm of the number of neurons in the structure of interest (log [Neuron number]) against the logarithm of the brain volume (log vol. [Brain]). We then performed analyses of variance (ANOVAs) on the residuals. These analyses take into account brain size differences, and compare relative values of volume or neuron number rather than absolute values, in order to demonstrate variations affecting specifically the structure of interest (e.g., Healy et al., 1996). For all comparisons, we performed factorial ANOVAs with a Fisher PLSD post-hoc test. The significance level was set at $P < 0.05$. We present the statistical analyses conducted on the logarithm of the raw values (absolute values), and those conducted on the residuals that take into account variations in brain size (relative values).

RESULTS

The hippocampal complex and its different fields

Table 1 presents the measures of the absolute and relative values for the volume of the brain (without the

TABLE 2. Statistical Analyses for the Brain, Hippocampal Complex, Dentate Gyrus, CA3, and CA1 Fields of the Hippocampus¹

	Season				Sex				Interaction		
	df	F	P		df	F	P		df	F	P
Brain Res	2/24	14.559	0.0001	Oct > Jan > June	1/24	1.483	0.2351	—	2/24	0.370	0.0694
HC Res		6.432 0.485	0.0058 0.6216	Oct > June —		2.804 1.313	0.1070 0.2632	— —		0.318 0.900	0.7305 0.4197
DG Res		2.064 0.453	0.1489 0.6410	— —		0.911 0.298	0.3493 0.5903	— —		0.572 1.314	0.5720 0.2874
CA3 Res		10.195 1.221	0.0006 0.3127	Oct = Jan > June —		0.926 0.087	0.3455 0.7700	— —		0.536 0.882	0.5917 0.4268
CA1 Res		8.126 0.495	0.0020 0.6154	Oct > Jan = June —		8.411 5.590	0.0079 0.0265	M>F M>F		0.071 0.212	0.9320 0.8109

¹For abbreviations, see Table 1.

cerebellum), the hippocampal complex and its different fields (dentate gyrus, CA3, and CA1) for the three different samples collected throughout the year, and by sex averaged over the year. Table 2 presents the results of the statistical analyses conducted on these measures. We observed differences in brain volume between samples collected at different times of the year, but no sex difference (October > January > June). We observed differences in the volume of the hippocampal complex between the October and June samples, whereas the January sample was not significantly different from the October or June samples; there was no sex difference. We observed no season or sex effect on the volume of the dentate gyrus. We observed a season, but no sex effect on the volume of CA3 (October = January > June). We observed season and sex effects on the volume of CA1 (October > January = June; males > females).

Because brain size varied between samples collected at different times of the year, we performed regression analyses on the absolute volume measures, plotting the logarithm of the volume of the structure of interest against the logarithm of brain volume (minus the volume of the structure of interest) (Fig. 2). The ANOVA performed on the residuals (Table 2) revealed no season or sex effect on the relative volume of the hippocampal complex, the relative volume of the dentate gyrus, or the relative volume of CA3. We observed a sex effect, but no season effect on the relative volume of CA1 (males > females).

Dentate gyrus

Table 3 presents the measures of the absolute and relative values for the volume of the different layers of the dentate gyrus analyzed separately (granule cell layer, molecular layer, and hilus), the number of neurons and neuron density in the granule cell layer, and the volume of the mossy fibers for the three different samples collected throughout the year, and by sex averaged over the year. Table 4 presents the results of the statistical analyses conducted on these measures. We observed no season or sex effect on the volume of the granule cell layer, the number of neurons in the granule cell layer, the density of neurons in the granule cell layer, the volume of the molecular layer, or the volume of the hilus. We observed differences in the volume of the mossy fibers between the October and June samples, whereas the January sample was not significantly different from the October or June samples; there was no sex difference.

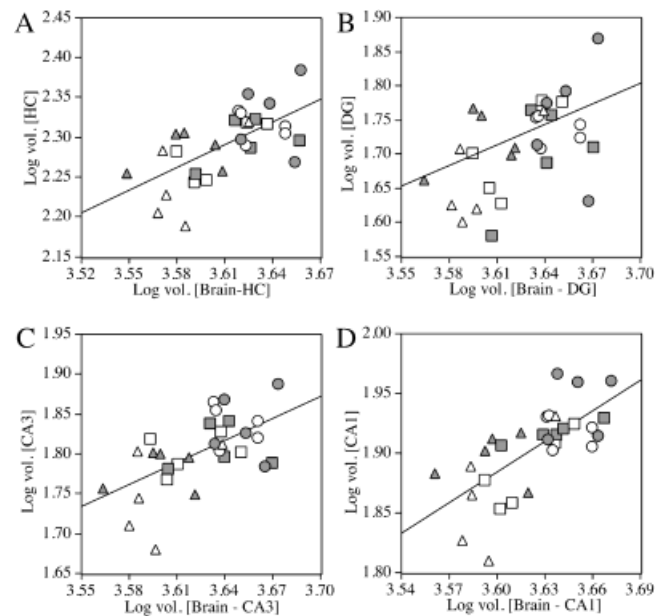


Fig. 2. Regression plots of the volume of the hippocampal complex and its different fields (logarithm of the absolute value) against the volume of the brain minus the volume of the structure of interest (logarithm of the absolute value). **A:** Hippocampal complex volume: (log vol. [HC]) versus (log vol. [Brain - HC]). **B:** Dentate gyrus volume: (log vol. [DG]) versus (log vol. [Brain - DG]). **C:** CA3 volume: (log vol. [CA3]) versus (log vol. [Brain - CA3]). **D:** CA1 volume: (log vol. [CA1]) versus (log vol. [Brain - CA1]). Circles, October sample; squares, January sample; triangles, June sample. Males in gray; females in white.

Figure 3 presents the individual measurements of the different parameters measured within the dentate gyrus in relation to the volume of the brain. The ANOVA performed on the residuals (Table 4) revealed no season or sex effect on the relative volume of the granule cell layer, the relative number of neurons in the granule cell layer, the relative volume of the molecular layer, the relative volume of the hilus, or the relative volume of the mossy fibers.

CA3 field

Table 5 presents the measures of the absolute and relative volumes of the different layers of CA3 analyzed

TABLE 3. Volume and Neuron Number of the Different Layers of the Dentate Gyrus¹

	October	January	June	Average		By sex	CE	CE ² /CV ²
gcl DG	8.70 ± 0.32	8.02 ± 0.52	7.62 ± 0.41	8.12 ± 0.25	M	8.41 ± 0.34	0.060	0.13
Res ²	0.005 ± 0.017	-0.010 ± 0.025	0.005 ± 0.022		F	7.82 ± 0.36		
Neuron number	5.88 ± 0.20	5.76 ± 0.41	5.61 ± 0.38	5.75 ± 0.19	M	5.76 ± 0.26	0.092	0.26
Res	-0.004 ± 0.013	-0.004 ± 0.031	0.008 ± 0.028		F	5.73 ± 0.29		
Neuron density	6.82 ± 0.28	7.25 ± 0.40	7.38 ± 0.37	7.15 ± 0.20	M	6.92 ± 0.30	—	—
Res	—	—	—		F	7.38 ± 0.28		
mol DG	33.81 ± 1.62	30.87 ± 1.56	29.94 ± 1.24	31.54 ± 0.88	M	32.34 ± 1.41	0.051	0.11
Res	0.003 ± 0.021	-0.013 ± 0.019	0.010 ± 0.016		F	30.74 ± 1.07		
Hilus	13.91 ± 0.77	12.28 ± 0.53	11.96 ± 0.62	12.72 ± 0.39	M	13.06 ± 0.59	0.076	0.20
Res	0.003 ± 0.021	-0.018 ± 0.015	0.015 ± 0.018		F	12.38 ± 0.52		
MF	23.73 ± 1.09	20.78 ± 0.77	20.40 ± 1.03	21.63 ± 0.61	M	22.53 ± 0.93	0.052	0.12
Res	0.012 ± 0.018	-0.019 ± 0.012	0.007 ± 0.023		F	20.74 ± 0.75		

¹Values indicate group mean ± S.E.M. Absolute volume is expressed in cubic millimeters (mm³); total neuron number is expressed in millions (10⁶), and the density of neurons (neuron number/volume) in the granule cell layer of the dentate gyrus (gcl DG) in hundreds of thousands (10⁵) per mm³.

²Res, residuals from the regression analyses plotting the log of the volume of the structure of interest (e.g., log vol. [gcl DG]) against the log of the volume of the brain minus the structure of interest (e.g., log vol. [Brain - gcl DG]). The relative variance of individual estimates (CE) was not calculated for the density of neurons in the granule cell layer, because neuron density was calculated by dividing total neuron number by the volume of gcl DG. For abbreviations, see Figure 1 and Table 1.

TABLE 4. Statistical Analyses for the Volume and Neuron Number of the Different Layers of the Dentate Gyrus¹

	Season				Sex				Interaction		
	df	F	P		df	F	P		df	F	P
gcl DG	2/24	1.965	0.1621	—	1/24	1.686	0.2065	—	2/24	2.129	0.1408
Res		0.198	0.8220	—		0.887	0.3557	—		3.423	0.0492
Neuron number		0.266	0.7689	—		0.015	0.9042	—		0.543	0.5882
Res		0.069	0.9331	—		0.013	0.9086	—		0.727	0.4939
Neuron density		0.536	0.5918	—		0.557	0.4628	—		0.244	0.7853
Res		—	—	—		—	—	—		—	—
mol DG		1.642	0.2146	—		0.662	0.4238	—		0.634	0.5393
Res		0.387	0.6832	—		0.180	0.6755	—		1.308	0.2888
Hilus		2.401	0.1120	—		0.811	0.3768	—		0.077	0.9258
Res		0.762	0.4777	—		0.175	0.6791	—		0.221	0.8034
MF		3.409	0.0498	Oct > June		2.435	0.1318	—		0.939	0.4048
Res		0.878	0.4284	—		1.349	0.2569	—		1.799	0.1871

¹For abbreviations, see Table 3.

separately (stratum oriens, pyramidal cell layer, stratum radiatum), the number of neurons and neuron density in the pyramidal cell layer for the three different samples collected throughout the year, and by sex averaged over the year. Table 6 presents the results of the statistical analyses conducted on these measures. We observed a season, but no sex effect on the volume of the CA3 pyramidal cell layer (October = January > June). We observed no season or sex effect on the number of neurons in the CA3 pyramidal cell layer or the density of neurons in the CA3 pyramidal cell layer. We observed season and sex effects on the volume of the CA3 stratum oriens (males > females). There were volume differences in the CA3 stratum oriens between the October and June samples, whereas the January sample was not significantly different from the October or June samples. We observed a season, but no sex effect on the volume of the CA3 stratum radiatum. There were volume differences in the CA3 stratum radiatum between the October and June samples, whereas the January sample was not significantly different from the October or June samples.

Figure 4 presents the individual measurements of the different parameters measured within the CA3 field in relation to the volume of the brain. The ANOVA performed on the residuals (Table 6) revealed no season or

sex effect on the relative volume of the CA3 pyramidal cell layer, the relative number of neurons in the CA3 pyramidal cell layer, the relative volume of the CA3 stratum oriens, or the relative volume of the CA3 stratum radiatum.

CA1 field

Table 7 presents the measures of the absolute and relative values for the volume of the different layers of CA1 analyzed separately (stratum oriens, pyramidal cell layer, stratum radiatum, stratum lacunosum moleculare), the number of neurons and neuron density in the pyramidal cell layer for the three different samples collected throughout the year, and by sex averaged over the year. Table 8 presents the results of the statistical analyses conducted on these measures. We observed a season, but no sex effect on the volume of the CA1 pyramidal cell layer (October = January > June). We observed no season or sex effect on the number of neurons in the CA1 pyramidal cell layer, or the density of neurons in the CA1 pyramidal cell layer. We observed season and sex effects on the volume of the CA1 stratum oriens (October = January > June; males > females) and the volume of the CA1 stratum radiatum (October = January > June; males > females). We observed a season, but no sex effect on the

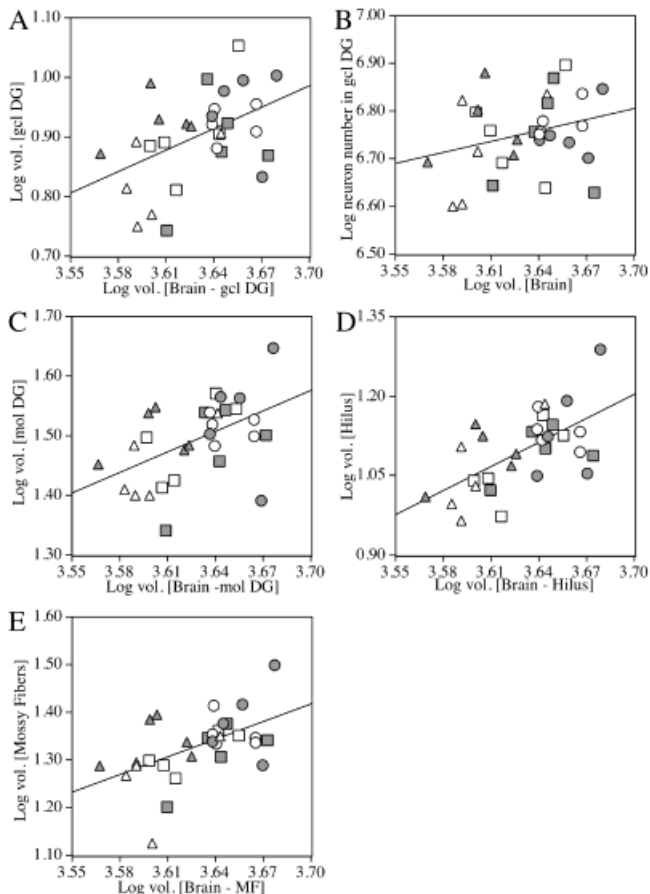


Fig. 3. Regression plots of the volume and neuron number of the different layers of the dentate gyrus (logarithm of the absolute value) against the volume of the brain, minus the volume of the structure of interest for the volume measurements (logarithm of the absolute value). **A:** Granule cell layer volume: (log vol. [gcl DG]) versus (log vol. [Brain - gcl DG]). **B:** Number of neurons in the granule cell layer: (log neuron number in gcl DG) versus (log vol. [Brain]). **C:** Molecular layer volume: (log vol. [mol DG]) versus (log vol. [Brain - mol DG]). **D:** Hilus volume: (log vol. [hilus]) versus (log vol. [Brain - hilus]). **E:** Mossy fibers volume: (log vol. [MF]) versus (log vol. [Brain - MF]). Circles, October sample; squares, January sample; triangles, June sample. Males in gray; females in white. For abbreviations, see Figure 1 legend.

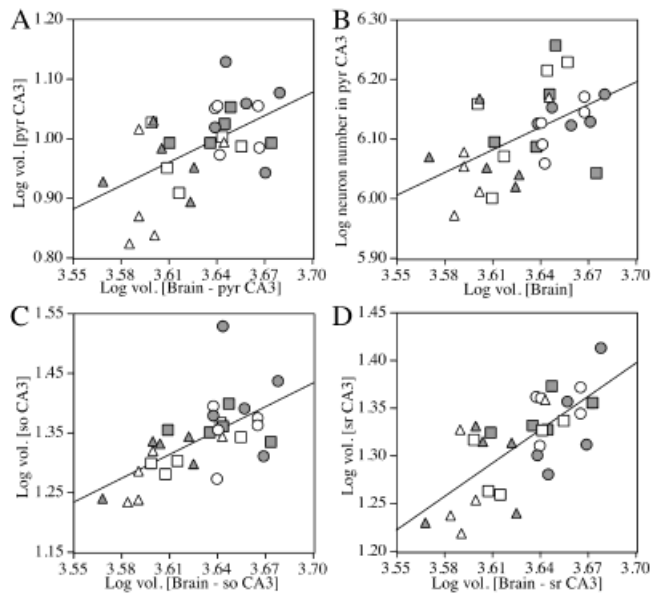


Fig. 4. Regression plots of the volume and neuron number of the different layers of CA3 (logarithm of the absolute value) against the volume of the brain, minus the volume of the structure of interest for the volume measurements (logarithm of the absolute value). **A:** CA3 pyramidal cell layer volume: (log vol. [pyr CA3]) versus (log vol. [Brain - pyr CA3]). **B:** Number of neurons in the CA3 pyramidal cell layer: (log neuron number in pyr CA3) versus (log vol. [Brain]). **C:** CA3 stratum oriens volume: (log vol. [so CA3]) versus (log vol. [Brain - so CA3]). **D:** CA3 stratum radiatum volume: (log vol. [sr CA3]) versus (log vol. [Brain - sr CA3]). Circles, October sample; squares, January sample; triangles, June sample. Males in gray; females in white.

volume of the CA1 stratum lacunosum moleculare. There were volume differences in the CA1 stratum lacunosum moleculare between the October and June samples, whereas the January sample was not significantly different from the October or June samples.

Figure 5 presents the individual measurements of the different parameters measured within the CA1 field in relation to the volume of the brain. The ANOVA performed on the residuals (Table 8) revealed no season or sex effect on the relative volume of the CA1 pyramidal cell layer or the relative number of neurons in the CA1 pyra-

TABLE 5. Volume and Neuron Number of the Different Layers of the CA3 Field¹

	October	January	June	Average		By sex	CE	CE ² /CV ²
pyr CA3	10.94 ± 0.44	9.91 ± 0.28	8.68 ± 0.46	9.85 ± 0.28	M	10.23 ± 0.38	0.069	0.19
Res ²	0.017 ± 0.019	0.003 ± 0.014	-0.020 ± 0.023		F	9.47 ± 0.40		
Neuron number	1.36 ± 0.03	1.39 ± 0.09	1.17 ± 0.06	1.30 ± 0.04	M	1.32 ± 0.05	0.115	0.49
Res	-0.009 ± 0.009	0.021 ± 0.026	-0.012 ± 0.019		F	1.29 ± 0.06		
Neuron density	1.25 ± 0.05	1.39 ± 0.07	1.36 ± 0.04	1.33 ± 0.03	M	1.29 ± 0.04	—	—
Res	—	—	—		F	1.37 ± 0.05		
so CA3	24.38 ± 1.29	21.96 ± 0.57	19.91 ± 0.64	22.09 ± 0.60	M	23.19 ± 0.97	0.057	0.15
Res	0.010 ± 0.023	-0.003 ± 0.010	-0.007 ± 0.010		F	20.98 ± 0.62		
sr CA3	22.06 ± 0.64	21.06 ± 0.53	19.30 ± 0.73	20.80 ± 0.41	M	21.05 ± 0.58	0.071	0.43
Res	-0.002 ± 0.011	0.003 ± 0.008	-0.002 ± 0.014		F	20.56 ± 0.60		

¹Values indicate group mean ± S.E.M. Absolute volume is expressed in cubic millimeters (mm³); total neuron number is expressed in millions (10⁶), and the density of neurons (neuron number/volume) in the CA3 pyramidal cell layer (pyr CA3) in hundred of thousands (10⁵) per mm³.

²Res, residuals from the regression analyses plotting the log of the volume of the structure of interest (e.g., log vol. [pyr CA3]) against the log of the volume of the brain minus the structure of interest (e.g., log vol. [Brain - pyr CA3]). The relative variance of individual estimates (CE) was not calculated for the density of neurons in the pyramidal cell layer, because neuron density was calculated by dividing the total number of neurons by the volume of pyr CA3. For abbreviations, see Figure 1 and Table 1.

TABLE 6. Statistical Analyses for the Volume and Neuron Number of the Different Layers of the CA3 Field¹

	Season				Sex				Interaction		
	df	F	P		df	F	P		df	F	P
pyr CA3	2/24	7.948	0.0022	Oct = Jan > June	1/24	2.777	0.1086	—	2/24	0.138	0.8719
Res		0.938	0.4052	—		1.075	0.3101	—		0.256	0.7764
Neuron number		3.394	0.0503	—		0.180	0.6748	—		0.098	0.9073
Res		0.809	0.4572	—		0.030	0.9560	—		0.304	0.7408
Neuron density		1.796	0.1876	—		1.494	0.2334	—		0.430	0.6553
Res		—	—	—		—	—	—		—	—
so CA3		7.366	0.0032	Oct > June		5.261	0.0309	M > F		0.246	0.7842
Res		0.370	0.6945	—		2.737	0.1111	—		0.264	0.7705
sr CA3		4.899	0.0164	Oct > June		0.448	0.5099	—		1.150	0.3333
Res		0.059	0.9425	—		0.001	0.9723	—		1.068	0.3596

¹For abbreviations, see Table 5.TABLE 7. Volume and Neuron Number of the Different Layers of the CA1 Field¹

	October	January	June	Average		By sex	CE	CE ² /CV ²
pyr CA1	6.42 ± 0.47	6.07 ± 0.23	5.24 ± 0.24	5.91 ± 0.21	M	6.22 ± 0.35	0.054	0.08
Res ²	0.007 ± 0.033	0.013 ± 0.015	-0.020 ± 0.019		F	5.59 ± 0.20		
Neuron number	1.70 ± 0.08	1.64 ± 0.11	1.51 ± 0.12	1.62 ± 0.06	M	1.61 ± 0.08	0.101	0.24
Res	-0.015 ± 0.016	0.001 ± 0.025	0.013 ± 0.032		F	1.62 ± 0.09		
Neuron density	2.82 ± 0.28	2.72 ± 0.18	2.89 ± 0.18	2.81 ± 0.12	M	2.70 ± 0.17	—	—
Res	—	—	—		F	2.92 ± 0.17		
so CA1	20.83 ± 0.96	20.22 ± 0.61	18.59 ± 0.75	19.88 ± 0.47	M	21.56 ± 0.60	0.037	0.08
Res	-0.001 ± 0.019	0.007 ± 0.013	-0.006 ± 0.016		F	18.21 ± 0.41		
sr CA1	36.51 ± 1.34	34.50 ± 0.52	31.78 ± 0.98	34.26 ± 0.67	M	35.75 ± 1.00	0.034	0.10
Res	0.005 ± 0.015	0.002 ± 0.005	-0.007 ± 0.013		F	32.77 ± 0.73		
slm CA1	12.21 ± 0.44	11.38 ± 0.30	10.77 ± 0.48	11.45 ± 0.25	M	11.81 ± 0.36	0.050	0.17
Res	0.009 ± 0.016	-0.003 ± 0.011	-0.006 ± 0.018		F	11.09 ± 0.35		

¹Values indicate group mean ± S.E.M. Absolute volume is expressed in cubic millimeters (mm³); total neuron number is expressed in millions (10⁶), and the density of neurons (neuron number/volume) in the CA1 pyramidal cell layer (pyr CA1) in hundreds of thousands (10⁵) per mm³.²Res, residuals from the regression analysis plotting the log of the volume of the structure of interest (e.g., log vol. [pyr CA1]) against the log of the volume of the brain minus the structure of interest (e.g., log vol. [Brain - pyr CA1]). The relative variance of individual estimates (CE) was not calculated for the density of neurons in the pyramidal cell layer, because neuron density was calculated by dividing the total number of neurons by the volume of pyr CA1. For abbreviations, see Figure 1 and Table 1.TABLE 8. Statistical Analyses for the Volume and Neuron Number of the Different Layers of the CA1 Field¹

	Season				Sex				Interaction		
	df	F	P		df	F	P		df	F	P
pyr CA1	2/24	3.880	0.0347	Oct = Jan > June	1/24	2.591	0.1206	—	2/24	1.946	0.1647
Res		0.601	0.5565	—		1.374	0.2526	—		1.823	0.1833
Neuron number		0.938	0.4054	—		0.006	0.9364	—		0.261	0.7727
Res		0.271	0.7653	—		0.181	0.6741	—		0.192	0.8268
Neuron density		0.157	0.556	—		0.820	0.3741	—		1.356	0.2767
Res		—	—	—		—	—	—		—	—
so CA1		4.296	0.0254	Oct = Jan > June		26.160	0.0001	M > F		0.610	0.5515
Res		0.260	0.7730	—		19.838	0.0002	M > F		0.727	0.4936
sr CA1		7.642	0.0027	Oct = Jan > June		8.751	0.0069	M > F		1.335	0.2820
Res		0.375	0.6911	—		5.361	0.0295	M > F		1.806	0.1859
slm CA1		3.452	0.0481	Oct > June		2.567	0.1222	—		1.271	0.2987
Res		0.295	0.7475	—		1.353	0.2562	—		1.319	0.2862

¹For abbreviations, see Table 7.

midal cell layer. We observed a sex, but no season effect on the relative volume of the CA1 stratum oriens (males > females) and the relative volume of the CA1 stratum radiatum (males > females). We observed no season or sex effect on the relative volume of the CA1 stratum lacunosum moleculare.

Precision of measures

To estimate the precision of the sampling method, coefficients of error were calculated for both volume and

neuron-count measurements, as described in Gundersen and Jensen (1987) and West et al. (1996). The relative variance of individual estimates (CE) was calculated following the quadratic approximation (Gundersen and Jensen, 1987), and by taking into account the Nugget effect (West et al., 1996). The relative variance of the group (CV) for the different parameters was calculated for each group as CV = standard deviation (S.D.)/mean.

Average CEs and CE²/CV² ratios are presented together with the volume and neuron number data in Tables 1, 3, 5,

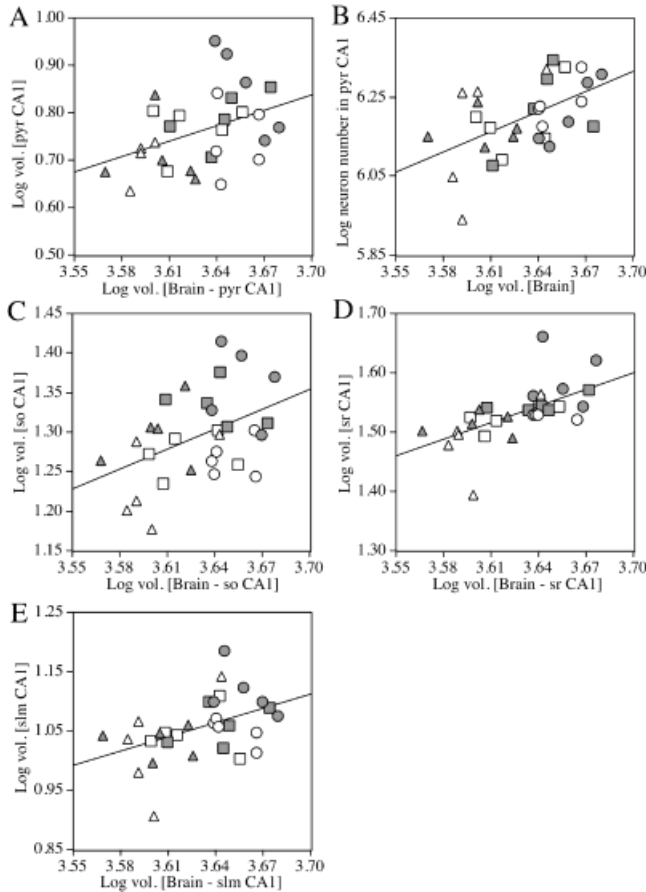


Fig. 5. Regression plots of the volume and neuron number of the different layers of CA1 (logarithm of the absolute value) against the volume of the brain, minus the volume of the structure of interest for the volume measurements (logarithm of the absolute value). **A:** CA1 pyramidal cell layer volume: (log vol. [pyr CA1]) versus (log vol. [Brain - pyr CA1]). **B:** Number of neurons in the CA1 pyramidal cell layer: (log neuron number in pyr CA1) versus (log vol. [Brain]). **C:** CA1 stratum oriens volume: (log vol. [so CA1]) versus (log vol. [Brain - so CA1]). **D:** CA1 stratum radiatum volume: (log vol. [sr CA1]) versus (log vol. [Brain - sr CA1]). **E:** CA1 stratum lacunosum moleculare volume: (log vol. [slm CA1]) versus (log vol. [Brain - slm CA1]). Circles, October sample; squares, January sample; triangles, June sample. Males in gray; females in white.

and 7. For most of the measures, the observed relative variance of the group ($CV^2 = ICV^2 + CE^2$) was essentially due to the inherent relative variance within a group (biological variance, ICV^2) rather than to the variance of the estimates. In a few cases, the variance of the estimate accounted for almost as much of the observed relative variance of the group as the biological variance. In such cases, however, the CV^2 was also very low, indicating very low biological variance. In all cases, the variance of the estimates remained very low, and, together with the number of subjects per group (10 per season, 15 per sex), substantiates the reliability of our methods.

Figure 6 provides a summary of the sex differences observed in absolute volume in the CA1 field and the corresponding analyses on the relative values, taking into account differences in brain size. It is important to present not only the absolute values of the different measures, but

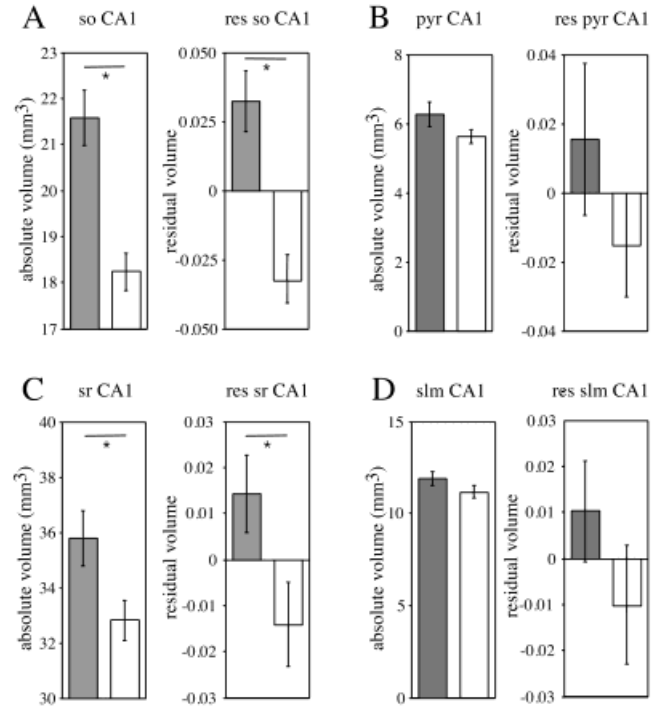


Fig. 6. Absolute and relative (residuals from a regression analysis accounting for differences in brain size) volumes of the different layers of the CA1 field of the hippocampal complex segregated by sex: **A:** Volume of the CA1 stratum oriens. **B:** Volume of the CA1 pyramidal cell layer. **C:** Volume of the CA1 stratum radiatum. **D:** Volume of the CA1 stratum lacunosum moleculare. Males in gray, females in white. The asterisks indicate significant sex differences. res, residuals; so, stratum oriens; pyr, pyramidal cell layer; sr, stratum radiatum; slm, stratum lacunosum-moleculare.

also the statistical analyses conducted on both absolute and relative values in order to provide a complete description of the differences observed between groups and conditions. These comparisons are necessary to identify the morphological changes that are specific to a particular brain structure and that might correlate with differences in behavior between groups. Such analyses indicate that the only differences that can not be explained simply by differences in brain size between conditions are the sex differences observed in CA1, and are limited to CA1 stratum oriens and stratum radiatum.

DISCUSSION

The present study is the first to provide a detailed analysis of the volume and neuron number of the different layers of the structures forming the hippocampal complex in adult food-storing mammals throughout the year. We observed brain volume differences in wild adult eastern gray squirrels caught at different times of the year. Our results show, however, that seasonal changes in behavior associated with increased spatial memory processing do not correlate with morphological changes, such as variations in volume or neuron number, in the different structures forming the hippocampal complex in adult long-lived mammals. Our results show sex differences in the volume of the CA1 stratum oriens and stratum radiatum that did

not vary seasonally. There were no sex differences, or any seasonal variations in relative volume or neuron number in any other layer of the structures forming the hippocampal complex.

Differences in brain volume

Our results suggest seasonal variations in brain size in adults of a long-lived species. Yaskin (1988, 1989) reported seasonal changes in brain and skull sizes in wild-caught narrow-skulled voles (*Microtus gregalis*). Similarly, Dark et al. (1987) showed that photoperiod influenced both DNA content and brain weight in meadow voles (*Microtus pennsylvanicus*). However, because voles rarely live more than one and a half years (Merritt, 1987), these variations were likely to reflect developmental changes regulated by environmental factors rather than adult plasticity. Indeed, Dark et al. (1987) showed that day length affected brain mass during early postnatal development, but not when treatment was initiated in adulthood.

In contrast, our results suggest that life span might not be a limiting factor to such changes (see also Yaskin, 1994). There are, however, two points to consider regarding the differences in brain size observed in squirrels. First, although all the brains from all the samples had been equally well perfused and postfixed for at least 1 month, there were differences in the time the brains from the different samples remained in fixative prior to processing. Indeed, the brains from the October sample remained in fixative for a total of 4 months, whereas the brains of the January and June samples remained in fixative for 2 and 1 month(s), respectively. Although, it is well known that different types of perfusions can produce an initial differential shrinkage, there is no report indicating that equally well-perfused (using the same type of perfusion) and sufficiently postfixed brains later display differential shrinkage. However, whether or not these differences in postfixation time affected the absolute values of volume, they certainly did not affect cell count measurements or the relative values that take into account brain size differences. Second, although unlikely, it is possible that for some reason the trapping of the animals might have been biased. All animals were collected in the same general areas; the October sample was collected first and the June sample last. We evaluated the age of the animals and excluded juveniles and subadults (i.e., animals not sexually mature and those that had not experienced at least one caching season), so that age and experience were not influencing factors. In this regard, it is important to note that there were no differences in body weight or any size measurements between the three samples of animals collected (not shown). It is possible, however, that some other unknown factors might have influenced trapping rates and that different animals were more likely to be trapped at different times of the year.

Changes in brain size are particularly important when evaluating the effects of seasonality on the morphological characteristics of different brain structures. We provide absolute values of volume and cell number, and statistical analyses conducted both on absolute and relative values in order to provide a detailed description of volume and cell number of the hippocampal complex in wild adult eastern gray squirrels. However, only changes specifically and selectively affecting the hippocampal complex, and thus

only the analyses conducted on the relative values, are informative when considering the relation between seasonal changes in spatial behavior and morphological changes in the hippocampal complex. Future experiments aimed at studying the relations between seasonal changes in behavior and variations in particular brain structures will have to account for possible variations in brain size, and are needed to understand the mechanisms responsible for the changes underlying such brain plasticity in adult mammals.

Seasonal variations in food-caching behavior and hippocampal structure

Our results show that in eastern gray squirrels, increased demand in spatial memory processing (i.e., caching in the fall) does not correlate with changes in relative volume or total neuron number in any layer of the structures forming the hippocampal complex (i.e., dentate gyrus, CA3, CA1). Previous studies in wild-caught chickadees reported that the volume (Smulders et al., 1995; but see Krebs et al., 1995), but not the total number of neurons (Barnea and Nottebohm, 1994, 1996) of the hippocampal formation might fluctuate and correlate with seasonal variations in caching behavior. The absence of seasonal variations in total neuron number in the CA3 and CA1 pyramidal cell layers in squirrels was as predicted, as pyramidal cells are generated mainly prenatally (Bayer, 1980), and adult neurogenesis in the hippocampal formation of mammals is limited to the dentate gyrus (Altman and Das, 1965). We have previously described the absence of seasonal variations in cell proliferation and total neuron number in the dentate gyrus of gray squirrels (Lavenex et al., 2000). This lack of change in total neuron number is consistent with the study of seasonal plasticity in wild-caught scatter-hoarding black-capped chickadees (Barnea and Nottebohm, 1994, 1996), and with the study of experience-dependent changes in the dentate gyrus of 6- and 18-month-old mice (Kempermann et al., 1998). Thus, even if neurogenesis varies seasonally in mature rodents, discernible changes in the total number of neurons in the granule cell layer in adulthood are absent. Such findings, however, do not exclude the possibility that in squirrels, a higher rate of turnover of neurons (i.e., birth and death of neurons) in the fall, at the peak of the caching season, could underlie differential neurogenesis in the granule cell layer, resulting in the constant neuron number observed in this study. This possibility remains to be investigated.

The absence of variations in the relative volume of any layer of the structures forming the hippocampal complex in squirrels contrasts with the only report suggesting seasonal variations in the hippocampal formation of an adult vertebrate with respect to food-caching behavior (Smulders et al., 1995). A closer look at the different experiments, however, can explain the discrepancies and provide a coherent explanation. Smulders et al. (1995) reported seasonal variations in the volume of the hippocampal formation of black-capped chickadees. Their conclusions, however, were based on results from multidimensional analyses that included data from juveniles. Moreover, "telencephalon" and "times of the year" were not truly independent factors in their model, as telencephalon volume varied throughout the year. The authors also indicated that the seasonal variations in the volume of the hippocampal formation followed the seasonal variations in

the telencephalon. Analyses limited to the data from adult animals, and that considered the relative size of the hippocampus (i.e., taking into account variations in the size of the telencephalon) at different times of the year, were unfortunately not presented. Our reanalysis of the data presented for the adult birds in Smulders et al.'s (1995) study, however, indicates that the variations of the volume of the hippocampal formation mirrored the variations observed in the telencephalon so closely that these variations do not appear to be specific to the hippocampal formation.

Our results in gray squirrels, however, agree with those of another study in black-capped chickadees, in which modulation of the photoperiod induced changes in food-caching behavior that were not associated with volumetric changes of the hippocampal formation (Krebs et al., 1995). Because changes in food-caching activity were induced by changes in photoperiod, this indicates that only the morphology of the brain, specifically the volume of the hippocampal formation, remained unaffected by their experimental manipulations. Together with data from previous studies in black-capped chickadees (Barnea and Nottebohm, 1994, 1996; Krebs et al., 1995; Smulders et al., 1995), our results in wild adult eastern gray squirrels demonstrate that seasonal variations in food-caching activity do not correlate with variations in the relative volume or the total number of neurons of the hippocampal formation in adult vertebrates.

Sex differences in space use and hippocampal structure

Our results in gray squirrels show sex differences in the volume of the CA1 field of the hippocampus, and thus provide further evidence for the hypothesis that sexual dimorphism of the hippocampal complex is related to sex differences in space use in mammals and birds. The sex differences that we observed in the hippocampal complex of gray squirrels, however, did not vary seasonally and thus did not correlate with variations in space use during the breeding and nonbreeding seasons in adult long-lived mammals.

Our results support previous findings showing a positive correlation between sex differences in space use and sexual dimorphism of the hippocampal structure in wild rodents. In two species of kangaroo rats, sex differences in mating strategies suggest that males of both species require more spatial ability than do females, and hippocampal size (relative to brain size) is larger in males than females (Jacobs and Spencer, 1994). In meadow voles, a polygynous species, breeding males have range sizes 4–5 times larger than those of females; this difference in range size is absent among immature voles and among adults outside of the breeding season. In this species, a sex difference in hippocampal volume, favoring males, is observed in animals collected during the breeding season (Jacobs et al., 1990). In contrast, pine voles, a monogamous species, lack such sex differences in ranging behavior, regardless of age or reproductive status, and show no sex difference in the volume of the hippocampus (Jacobs et al., 1990). In eastern gray squirrels too, males have larger home ranges than females, mostly due to an increase in their range during the breeding season (Thompson, 1977, 1978). Our results, however, revealed that in squirrels only the CA1 field of the hippocampal complex exhibited a

sexual dimorphism, and that these sex differences were not dependent on the reproductive status (i.e., there were no variations related to seasonal breeding).

Our results in gray squirrels seem, at first, to contrast with the studies in shiny cowbirds and voles suggesting that these sex differences might vary seasonally and that sexual dimorphism in relative hippocampal volume might be present only during the breeding season (Clayton et al., 1997; Jacobs et al., 1990). A closer look at these other experiments, however, can explain the discrepancies and reconcile the different interpretations. In cowbirds, there is a female advantage in hippocampal size that relates to a higher demand in spatial abilities for reproduction (Sherry et al., 1993). Clayton et al. (1997) reported that these sex differences varied seasonally and were only visible during the breeding season. According to the authors, however, their results regarding the seasonality of the phenomenon were inconclusive due to a possible confound in the age distribution of their samples. Similarly, in meadow voles, previous reports presented a correlation between sex differences in space use and size of the hippocampus during the breeding season (Jacobs et al., 1990). The absence of sex differences in space use in immature and adult animals outside the breeding season (Gaulin and FitzGerald, 1986) was suggestive of seasonal variations of the hippocampal structure. Anatomical data from adult animals outside the breeding season were, however, not available to support this hypothesis. Thus, to date, there are no conclusive neuroanatomical data showing seasonal variations of sex differences in hippocampal morphology in adult vertebrates.

Developmental versus adult plasticity

Our results suggest that previously observed effects of seasonality on hippocampal structure represent developmental changes rather than true adult plasticity. Previous studies in deer mice and voles suggested that sexually dimorphic spatial learning is correlated with reproductive status (Galea et al., 1996), and that hippocampal volume, dentate gyrus width, and cell proliferation rate in the granule cell layer correlate with levels of sexual hormones in voles (Galea and McEwen, 1999; Galea et al., 1999). Collectively, these findings prompted researchers to conclude that these parameters varied seasonally, together with space use, in adult animals in the wild. Although gonadal hormone-induced changes in hippocampal morphology have been demonstrated in pubescent and adult rats (Foy et al., 1984; Gould et al., 1990a; Woolley and McEwen, 1992), differences in adult hippocampal volume have been shown to result from differences in perinatal exposure to hormones (Isgor and Sengelaub, 1998). Consistent with these findings, perinatal testosterone treatment modifies sex differences in spatial abilities and induces development of a male hippocampus in females, indicating that gonadal hormones during brain development play a role in the development of sex differences in spatial memory (Roof and Havens, 1992; Roof, 1993). Furthermore, the short lifespan of voles (one and a half years, Merritt, 1987) potentially confounds any correlation between seasonality and adult plasticity. Voles and several other small rodents born in the spring grow rapidly, reach sexual maturity, and reproduce within several months of birth. By contrast, animals born in late summer or early fall do not achieve adult size, nor do they reproduce, in the

season of their birth (Iverson and Turner, 1974). Instead, they overwinter in a reproductively quiescent state, showing a growth spurt and reproductive development the following spring. It is possible, then, that a sample of animals trapped outside the breeding season might be biased towards immature animals, confounding any relation between seasonality and volumetric changes in adult animals (see also Clayton et al., 1997).

Thus, the data collected in birds and short-lived mammals, together with the present results in squirrels, indicate that the morphological characteristics of the hippocampal complex (hippocampal formation in birds), such as volume and neuron number, do not vary seasonally in adult vertebrates, and suggest that sex differences in the hippocampal formation appear through developmental differentiation.

Sex differences in CA1 strata oriens and radiatum

Our results showed sex differences limited to the volume of the stratum oriens and stratum radiatum of the CA1 field of the hippocampus, and thus provide further evidence for sexual dimorphism of the hippocampal complex in mammals. Numerous studies have shown the influence of gonadal steroids and sex differences in the morphology of the hippocampal complex in laboratory rats, such as the number of neurons in the granule cell layer of the dentate gyrus (Madeira et al., 1988; Wimer and Wimer, 1985, 1989), the width of the granule cell layer (Roof and Havens, 1992), the dendritic trees of granule cells (Juraska et al., 1985, 1989), the size of the mossy fiber projections (Madeira et al., 1991), the dendritic morphology of pyramidal neurons in CA3 (Juraska et al., 1989; Woolley and McEwen, 1993), the number of neurons in the CA1 region (Gould et al., 1990b), and the growth of dendritic spines in the CA1 area of the hippocampus (Meyer et al., 1978). Moreover, estradiol mediates fluctuation in synapse density in the CA1 field of the hippocampus during the estrous cycle (Woolley and McEwen, 1992). However, one can not assume a direct relationship between synaptic density and volume, and there are no reports of changes in volume of CA1 throughout the cycle. Given this wide range of findings, it is beyond the scope of this paper to offer a hypothesis explaining all of the findings regarding the implications of sex differences in morphology observed at different levels of the hippocampal complex. Rather, we limit our discussion to the sex differences observed in squirrels in relation to the connectivity and physiological properties of the CA1 field of the hippocampus proper.

The CA1 field of the hippocampus receives both direct and indirect projections through the perforant path, which are largely segregated between the different strata. The Schaffer collaterals (projections from CA3 pyramidal cells to CA1 pyramidal cells) are associated with the apical dendrites of CA1 cells in the stratum radiatum and the basal dendrites in the stratum oriens (Amaral and Witter, 1995). Pyramidal cells in CA1 also receive direct neocortical projections from the entorhinal cortex that terminate in the stratum lacunosum moleculare. In contrast to the other fields of the hippocampal complex, there are very few associational connections within CA1 (Amaral et al., 1991; Lavenex and Amaral, 2000). The main difference between male and female squirrels, then, might arise from differences in the strength of the connectivity between

CA3 and CA1 pyramidal cells. In terms of inputs, this might indicate a difference in strength of the tri-synaptic inputs to the CA1 pyramidal cells, whereas the monosynaptic input from the entorhinal cortex might not differ (i.e., we observed no differences in CA1 stratum lacunosum moleculare). Functionally, it is difficult to predict what would be the consequences of such morphological differences. However, it may be interesting to hypothesize that the sexual dimorphism in volume of CA1 strata oriens and radiatum is also accompanied by sex differences in the physiological properties of CA1 pyramidal cells. Thus, for example, the spatial firing properties of CA1 pyramidal cells (O'Keefe and Dostrovsky, 1971) might exhibit sex differences reflecting these potential differences in the relative strengths of monosynaptic or trisynaptic inputs between males and females.

Conclusion

Our results in wild adult eastern gray squirrels further demonstrate that seasonal variations in food-caching activity do not correlate with variations in the relative volume or the total number of neurons of the hippocampal formation in adult vertebrates. Our findings confirm the existence of sex differences in the morphology of the hippocampal complex that do not fluctuate in adult animals. They support the hypothesis that sexual selection can affect the morphology of the hippocampal complex in a very specific and limited manner. Our results, together with those of other studies, indicate that the morphological characteristics of the hippocampal complex (hippocampal formation in birds), such as volume and neuron number, do not vary seasonally in adult vertebrates. Altogether, these results suggest that the seasonal changes previously reported in short-lived species do not represent true adult plasticity, and that modulation of sex hormone levels might lead to seasonal sexual dimorphism in the hippocampal complex through organizational or activational effects during development. Seasonal variations in space use during the breeding season are unlikely to have activational effects that produce detectable changes in volume or cell number in adult vertebrates. More subtle changes, such as those observed throughout the estrous cycle in rats are, however, possible. The sexual dimorphism observed in this study in CA1 stratum oriens and stratum radiatum raises questions about the functional implications regarding the physiology of CA1 pyramidal cells in relation to space use and spatial mapping in particular (Nadel, 1991). Future studies combining well-defined behavioral paradigms and thorough neuroanatomical and functional studies are clearly needed to elucidate the relationship between sex differences and seasonal variations in spatial behavior and their neurobiological correlates in the hippocampal complex of mammals.

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