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The seasonal pattern of cell proliferation and neuron number in the dentate gyrus of wild adult eastern grey squirrels

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Abstract

The dentate gyrus is one of two areas in the mammalian brain that produces neurons in adulthood. Neurogenesis (proliferation, survival, and differentiation of new neurons) is regulated by experience, and increased neurogenesis appears to be correlated with improved spatial learning in mammals and birds. We tested the hypothesis that in long-lived mammals that scatter-hoard food, seasonal variations in spatial memory processing (i.e. increased processing during caching season in the autumn) might correlate with changes in neurogenesis and neuron number in the granule cell layer of the dentate gyrus (gcl DG). We investigated the rate of cell proliferation and the total number of neurons in the granule cell layer of wild adult eastern grey squirrels (*Sciurus carolinensis*) at three different times of the year (October, January and June). We found no seasonal differences in cell proliferation rate or in total neuron number in the granule cell layer. Our findings are in agreement with those of previous studies in laboratory mice and rats, and in free-ranging, food-caching, black-capped chickadees, as well as with current hypotheses regarding the relationship between neurogenesis and learning. Our results, however, are also in agreement with the hypothesis that neurogenesis in the dentate gyrus represents a maintenance system that may be regulated by environmental factors, and that changes in total neuron number previously reported in rodents represent developmental changes rather than adult plasticity. The patterns observed in mature wild rodents, such as free-ranging squirrels, may represent more accurately the extent of hippocampal plasticity in adult mammals.

Introduction

New neurons are produced continuously in the dentate gyrus of adult mammals (Altman & Das, 1965; Bayer, 1982; Eriksson *et al*., 1998) and in the ventricular zone below the hippocampal formation of birds (Barnea & Nottebohm, 1994). Neurogenesis (cell proliferation, survival, and differentiation of new neurons; Gage *et al*., 1998) is regulated by numerous factors. Cell proliferation is influenced by enriched conditions in 129/SvJ mice (Kempermann *et al*., 1998a, but not in C57BL/6 mice, Kempermann *et al*., 1997), voluntary activity (van Praag *et al*., 1999) and stressful experience (Gould *et al*., 1998), and is possibly under hormonal control (Galea & McEwen, 1999). In marsh tits, spatial learning can induce cell proliferation in the developing hippocampus (Patel *et al*., 1997). Survival of new granule cells is enhanced by participation in hippocampus-dependent learning tasks in rats (Gould *et al*., 1999a). Similarly, 2-month-old C57BL/6 mice maintained in enriched conditions exhibit increased neurogenesis, as compared to littermates housed in deprived conditions, and this increased neurogenesis correlates with shorter escape paths in the Morris navigation task (Kempermann *et al*., 1997). These enriched conditions induce increases in total neuron number and volume of the granule cell layer, which persist into adulthood.

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A correlation between spatial learning and increased neurogenesis has been observed in food-caching animals. In the black-capped chickadee (*Parus atricapillus*), a scatter-hoarder that caches food and retrieves caches based on spatial memory of their locations, recruitment of hippocampal neurons increases in the autumn at the peak of caching behaviour (Barnea & Nottebohm, 1994, 1996). Such results suggest that seasonal patterns of hippocampus-dependent learning (spatial memory for cache locations) might also correlate with differential patterns of neurogenesis, impacting the number of neurons in the dentate gyrus of adult long-lived mammals. We examined cell proliferation and total neuron number in the dentate gyrus of wild adult eastern grey squirrels (*Sciurus carolinensis*) throughout the year. Caching activity and the squirrels' dependence on food caches varies seasonally (Thompson & Thompson, 1980; Gurnell, 1987; Vander Wall, 1990; Koprowski, 1994). Grey squirrels scatter-hoard their winter food supply of nuts in hundreds of locations and rely primarily on spatial memory to relocate these caches (Jacobs & Liman, 1991). We collected adult squirrels at three times of the year: in October, at the peak of the caching season; in January, when caching is complete but squirrels are still dependent on spatial memory to locate their caches; and in June, after squirrels have depleted their caches and are not actively engaged in further caching activity. One hypothesis predicts that seasonal changes in behaviour affect neurogenesis (potentially through variations in cell proliferation), leading to variations in total number of neurons in the granule cell layer of the dentate gyrus (gcl DG). An alternative hypothesis

predicts that whether or not neurogenesis varies throughout the year, it will not lead to variations in neuron number, and that changes in total neuron number previously reported in rodents represent developmental changes rather than adult plasticity.

Materials and methods

Animals

Thirty wild adult eastern grey squirrels were trapped in Wilkes-Barre and Kingston, PA, at three different times of the year: (i) October 1996; (ii) January 1997; and (iii) June 1997. For each sampling period, five males and five females were live-trapped and maintained in captivity for an average of 48 h (range 36–120 h). The age of the animal was determined using standard size measurements and obvious signs of sexual maturity (Gurnell, 1987). Subadults and juveniles were released at the capture site and were not used in the study. Adult squirrels were kept indoors with food and water *ad libitum* in the live-traps filled with straw, and under artificial illumination matching the natural photoperiod. All experimental procedures were carried out following the NIH guidelines regarding the care and use of experimental animals and were approved by the UC Berkeley and Wilkes University Animal Care and Use Committees.

Treatment protocol

The captive squirrels received two intraperitoneal injections of 50 mg/kg bromodeoxyuridine (BrdU; Boehringer Mannheim, Indianapolis, IN), the second injection 12 h after the first. The BrdU was dissolved in 0.9% NaCl with 1–4 drops of sodium hydroxide (NaOH) per mL added. Squirrels were sacrificed 24 h after the second injection, similar to previous experiments investigating cell proliferation in laboratory rodents (e.g. Kempermann *et al*., 1998a; Gould *et al*., 1999a; van Praag *et al*., 1999). Squirrels were anaesthetized with 1 mL sodium pentobarbitol (at a concentration of 6 grains/mL) and perfused transcardially with 100 mL of saline (0.9% NaCl), followed by 100 mL of a sodium sulphide solution (Na_2S, 1.17% w/v /NaH_2PO_4, 1.19% [w/v]) and 100 mL of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). Animals were decapitated and the brain within the skull was placed in 4% paraformaldehyde until processing. Brains were then extracted from the skulls 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) were mounted directly from buffer onto slides and processed. Sections for BrdU immunohistochemistry (1 in 4 adjacent series) were stored at –20 °C in a cryoprotectant solution (300 g sucrose + 10 g polyvinylpyrrolidone [PVP-40] + 300 mL ethylene glycol + dH_2O added to 1000 mL) until processing.

Silver impregnation processing

All sections of the silver impregnation series were mounted on gelatin-coated slides and processed following a technique modified from Sloviter *et al*. (1993). Sections were washed (3 × 5 min) in dH_2O and pretreated (2 × 5 min) in a 50 : 50 solution (v/v) of 9% NaOH (w/v) and 1.2% ammonium nitrate (NH_4NO_3, w/v). The sections were impregnated for 10 min in a solution comprised of 600 mL of 9% NaOH, 400 mL of 16% NH_4NO_3 and 6 mL of 50% silver nitrate (w/v) and rinsed (3 × 2 min) in a solution comprised of 10 mL of 1.2% NH_4NO_3, 5 g of anhydrous sodium carbonate, 300 mL of 95% EtOH and 700 mL of dH_2O. Sections were developed for 1 min in a solution of 10 mL of 1.2% NH_4NO_3, 0.5 g of anhydrous citric acid, 15 mL of 37% formalin, 500 mL of 95% EtOH and dH_2O added to 1000 mL. Slides were washed in 0.5% acetic acid (3 × 10 min), dehydrated and mounted with Permount.

BrdU labelling

BrdU immunohistochemistry was performed simultaneously for all samples, using the same stock solutions. The procedure was performed with free-floating sections at room temperature and following the reagent suppliers' recommendations. Sections were first rinsed in 0.1 M PBS (4 × 10 min), treated for 1 h in 2 N HCl, rinsed for 15 min in 0.1 M borate buffer pH 8.5, washed in PBS (2 × 10 min) and incubated for 30 min in 1 µg/mL proteinase K (Sigma Chemicals, St Louis, MO) in PBS. The sections were then washed in PBS (3 × 5 min), treated for 30 min in 0.5% hydrogen peroxide (H_2O_2) in PBS, further washed in PBS (3 × 5 min) and incubated for 1 h in a blocking solution of 0.1 M PBS containing 1 mg/mL bovine serum albumin (BSA), IgG from the animal producing the secondary antibody (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) and 0.3% Triton X-100. The sections were then incubated overnight in primary anti-BrdU antibody (Becton Dickinson, San Jose, CA; 1 : 100 in PBS/BSA/Triton), washed in PBS (3 × 5 min) and incubated for 1 h in anti-mouse biotinylated secondary antibody in 0.1 M PBS/BSA/Triton (Vectastain ABC kit). Following this, the sections were washed in PBS (3 × 5 min), incubated for 1 h in ABC reagent (Vectastain ABC kit, in PBS / BSA / Triton), washed in PBS (3 × 5 min) and reacted for 4 min in a solution of 0.5 mg/mL 3,3′-diaminobenzidine (Sigma Chemicals, St Louis, MO) in 0.1 M KPBS, pH 7.4 + 0.01% H_2O_2. The sections were finally rinsed in PBS (3 × 5 min), mounted on gelatin-coated slides, dehydrated and cover slipped with Permount.

Neuron counts and volume measurements

Neuron counts and volume measurements were performed with StereoInvestigator 3.15a (Microbrightfield Inc., Colchester, VT). Volume measurements were performed according to the Cavalieri principle (Gundersen & Jensen, 1987; West & Gundersen, 1990) on the silver impregnated sections. Nine 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 dentate gyrus, were used for the measurement of the volume of the granule cell layer and the hilus. Brain volume refers to the volume of the brain without the cerebellum, which was removed before sectioning. Eleven 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.

The total number of neurons in the granule cell layer was determined using the optical fractionator method (West *et al*., 1991). This design-based method enables an estimation of the absolute number of neurons that is independent from the volume. Nine 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 dentate gyrus, were used for the neuron counts. A 100X Neofluar oil objective (N.A. 1.30) was used on a Nikon Optiphot microscope linked to a PC-based StereoInvestigator 3.15a. Neuron number was measured in the right granule cell layer only, as volume measurements did not show any difference between the left and the right. The estimate of the total number of neurons bilaterally was calculated by doubling the number of neurons counted on the right side.

BrdU-labelled cell counts

All BrdU-labelled cells within the subgranular zone (SGZ; the proliferative zone at the border of the granule cell layer and the hilus) and the hilus of the dentate gyrus were counted in every section

TABLE 1. Density of BrdU-labelled cells

	SGZ (cells/mm^3)		Hilus (cells/mm^3)		SGZ + Hilus (cells/mm^3)	
	All subjects ($n = 10$)	Subgroup ($n = 7$)*	All subjects ($n = 10$)	Subgroup ($n = 7$)*	All subjects ($n = 10$)	Subgroup ($n = 7$)*
October	136.1 ± 24.0	169.6 ± 24.3	27.4 ± 5.2	35.0 ± 5.1	68.6 ± 11.3	85.7 ± 10.4
January	129.2 ± 19.2	159.9 ± 15.3	33.4 ± 3.8	39.0 ± 3.7	73.4 ± 8.1	85.4 ± 7.8
June	159.9 ± 12.4	176.9 ± 11.3	34.9 ± 5.6	40.3 ± 6.9	83.8 ± 6.2	93.7 ± 4.1
Average	141.7 ± 10.9	168.8 ± 9.9	31.9 ± 2.8	38.1 ± 3.0	75.3 ± 5.0	88.3 ± 4.4

Values indicate group means ± SEM of density of BrdU-labelled cells. The first values ($n = 10$ columns) represent group means that include all subjects. *The second values ($n = 7$ columns) represent group means after removal of the three lowest scores for each group. The volume of reference to calculate the density of BrdU-labelled cells in the SGZ is that of the granule cell layer. The volume of reference for the density of BrdU-labelled cells in SGZ + hilus is the volume of the granule cell layer + hilus.

processed for BrdU immunohistochemistry. Nine 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 dentate gyrus, were used to estimate the number of BrdU-labelled cells. Because of the low number of labelled cells, we did not use a stereological probe to count the number of BrdU-labelled cells. Direct counting was performed with a Leitz Dialux 20 microscope under dark-field illumination at ×200 magnification. Therefore, all labelled cells were counted through the entire thickness of the section. This method, which does not take into account the problems of oversampling and lost caps, does not affect the conclusions drawn from our study, as all samples were treated identically. The number of BrdU-labelled cells counted in the sampled sections was multiplied by the inverse of the section sampling fraction (ssf = 1/16) to obtain the total number of BrdU-labelled cells.

Data analysis

Data on volumes and neuron numbers are presented as cubic millimeters (mm^3) and number of neurons, respectively. Data on cell proliferation rates are presented as number of labelled cells per mm^3. For the cell proliferation rate in the SGZ, we used the volume of the granule cell layer as the reference volume. We performed statistical analyses on the logarithm of the raw values to conform to the assumption of homogeneity of variance. For volume measures and neuron counts, we performed regression analyses plotting the logarithm of the volume of the granule cell layer or the number of neurons against the logarithm of the brain volume. We then performed analyses of variance (ANOVAs) on the residuals. For all comparisons, factorial ANOVAs were performed, followed by a Fisher PLSD *post hoc* test. The significance level was set at $P < 0.05$.

Results

We found that cell proliferation occurs in the dentate gyrus of wild eastern grey squirrels throughout the year. There were no sex differences in any of the parameters analysed; therefore data from both sexes were pooled for analysis. We found differences in brain size between the three samples of animals collected at different times of the year ($F_{2,27} = 14.99$, $P < 0.0001$; October, 4527 ± 50 mm^3 > January, 4318 ± 76 mm^3 > June, 4031 ± 65 mm^3, Fisher PLSD all significant with $P < 0.05$; average, 4292 ± 52 mm^3).

Cell proliferation

We found no differences in the number of BrdU-labelled cells in the SGZ or in the hilus at different times of the year (Table 1 [$n = 10$ per group]; SGZ, $F_{2,27} = 0.709$, $P = 0.50$; hilus, $F_{2,27} = 0.647$, $P = 0.53$; SGZ and hilus combined, $F_{2,27} = 0.786$, $P = 0.46$). Three squirrels from the October sample (two males, one female) showed very low cell proliferation rates in both the SGZ and the hilus (less than half the average rate for the group). Because all squirrels received two BrdU injections, it is possible that one injection was not administered correctly, resulting in the delivery of a lower dose of BrdU and thus reducing artificially the number of BrdU-labelled cells. We therefore recalculated the number of BrdU-labelled cells, omitting the three lowest scores in each group. We found no seasonal changes in the number of BrdU-labelled cells either in the SGZ or the hilus following this correction (Table 1 [$n = 7$ per group]; SGZ, $F_{2,18} = 0.229$, $P = 0.79$; hilus, $F_{2,18} = 0.352$, $P = 0.70$; SGZ and hilus combined, $F_{2,18} = 0.273$, $P = 0.76$).

Neuron number

Although cell proliferation did not appear to vary, differential survival and recruitment of newly generated neurons might have varied across seasons and produced, as a consequence, variations in the total number of neurons in the granule cell layer. Because brain size differed between samples, we performed ANOVAs on the residuals from regression analyses plotting the logarithm of neuron number (Fig. 1a) or the logarithm of the granule cell layer volume (Fig. 1b) against the logarithm of brain volume. The total number of neurons in the granule cell layer did not differ between squirrels caught at three different times of the year (Table 2; $F_{2,27} = 0.074$, $P = 0.92$). Similarly, there were no seasonal variations in the volume of the granule cell layer ($F_{2,27} = 0.168$, $P = 0.84$), nor did the density of neurons within the granule cell layer (total neuron number/volume gcl DG) differ between animals collected in October, January and June ($F_{2,27} = 0.626$, $P = 0.54$).

Precision of measurements

To estimate the precision of the sampling method, coefficients of error were calculated for volume and neuron-count measurements. The relative variance of individual estimates (CE) was calculated following the quadratic approximation (Gundersen & 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 = SD/mean. For the measurements of neuron count, the average CE was 0.092 (range 0.068–0.121), whereas the CV for each group was 0.106 in October, 0.223 in January and 0.214 in June. This resulted in CE^2/CV^2 ratios of 0.72, 0.17 and 0.19, respectively. For the volume measurements, the average CE was 0.060 (range 0.039–0.072, using a shape factor of 16 determined empirically on outlines of the granule cell layer), whereas the CV for each group was 0.116 in October, 0.204 in January and 0.169 in June. This resulted in CE^2/CV^2 ratios of 0.29, 0.08 and 0.12, respectively. From these results, it follows that the observed relative variance of the group ($CV^2 = ICV^2 + CE^2$) for all the measures was essentially due to the inherent relative variance within a group

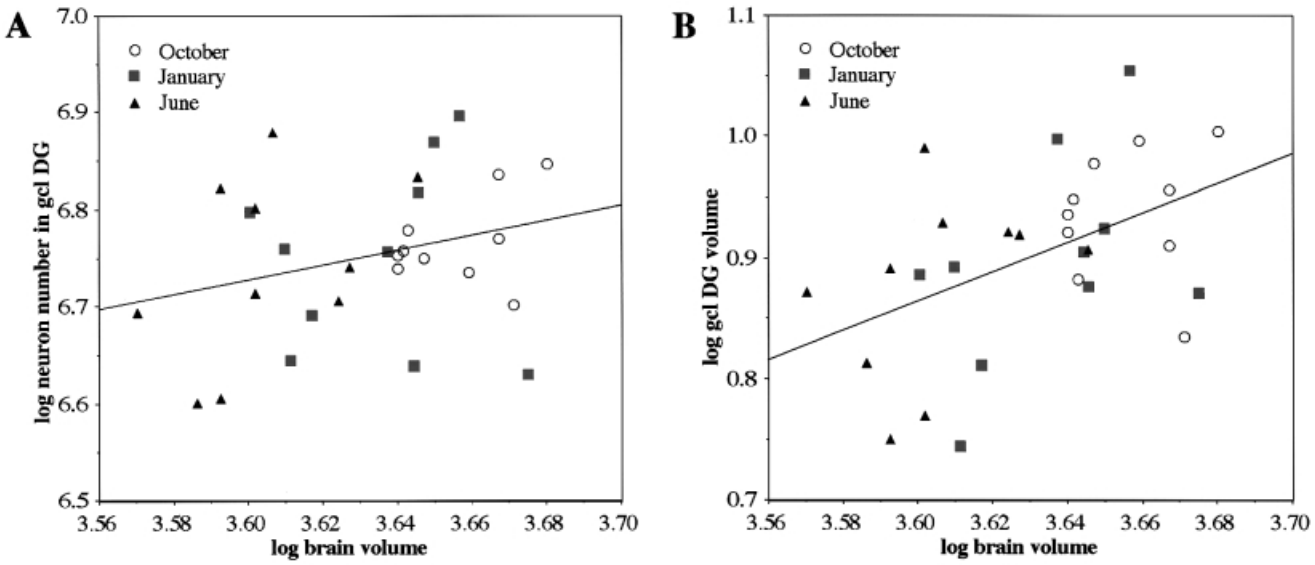

FIG. 1. Regression plots of the volume and the number of neurons in the granule cell layer against the volume of the brain. (A) Logarithm of the number of neurons in the granule cell layer (log neuron number in gcl DG) vs. logarithm of brain volume. (B) Logarithm of the granule cell layer volume (log gcl DG volume) vs. logarithm of brain volume.

(biological variance, ICV^2) rather than to the variance of the estimates (CE^2). We can therefore be confident that the lack of differences between groups (10 subjects per group), reflects a true absence of seasonal variations in cell proliferation, neuron number and volume of the granule cell layer of the dentate gyrus (gcl DG) of the grey squirrel, and is not due to a lack of sensitivity of our methods.

TABLE 2. Total number and density of neurons in the granule cell layer

	Neuron number ($\times 10^6$)	Volume of gcl DG (mm^3)	Neuron density ($\times 10^5 / mm^3$)
October	5.88 ± 0.20	8.70 ± 0.32	6.82 ± 0.28
January	5.76 ± 0.41	8.02 ± 0.52	7.25 ± 0.40
June	5.61 ± 0.38	7.62 ± 0.41	7.38 ± 0.37
Average	5.75 ± 0.19	8.12 ± 0.25	7.15 ± 0.20

Values indicate group mean ± SEM. '×' indicates that actual values are 10^6 and 10^5 times values shown in first and third columns respectively. Neuron density = neuron number/volume gcl DG.

Discussion

Our results show that in a mammalian species whose natural life span is greater than one year (up to six years in the wild, Gurnell, 1987), seasonal changes in behaviour associated with increased spatial memory processing (food-caching in the autumn) do not correlate with changes in cell proliferation rate, or with variations in total neuron number in the granule cell layer of the dentate gyrus in adulthood. Our results agree with those from previous experiments with food-caching birds (Barnea & Nottebohm, 1994, 1996) and with current hypotheses regarding the relationship between neurogenesis and learning. Our results, however, do not support the hypothesis that increased demand in learning and memory processing are correlated with variations in total neuron number in adult mammals, particularly in species that are highly reliant on hippocampus-dependent memory for survival.

Cell proliferation

In squirrels, cell proliferation is not affected by increased demand in spatial memory processing (i.e. seasonal variations in caching behaviour). This is consistent with results in C57BL/6 mice showing that cell survival, but not cell proliferation, is increased in enriched conditions (Kempermann *et al*., 1997, 1998b). However, other reports showing that both voluntary running in C57BL/6 mice (van Praag *et al*., 1999) and enriched conditions in 129/SvJ mice (Kempermann *et al*., 1998a) can increase cell proliferation indicate that differential behaviour can modulate neurogenesis via changes in cell proliferation. In our study, squirrels were not released into their natural environment after BrdU injections. Because we did not see any differences, this raises the possibility that cell proliferation may be regulated by differential caching behaviour on a short time scale (i.e. while performing the behaviour and possibly for a short time thereafter). If this hypothesis is correct, no differences in cell proliferation would be expected between the squirrels caught at different times of the year, as they were no longer able to express differential behaviours (across seasons) after BrdU injections (they were kept in captivity for 24 h until perfusion, and for an average of 48 h from trapping to perfusion). A lack of differential cell proliferation, however, is supported by the results of Gould *et al*. (1999a), which did not show proliferation differences in Sprague–Dawley rats required to perform different learning tasks that were either hippocampus-dependent or not.

Alternatively, maintaining the squirrels in captivity may have caused a stress-induced decrease in cell proliferation (Gould *et al*., 1998), as compared to animals not stressed in this manner. As the three groups of animals in our study were treated identically, however, the effects of stress on cell proliferation would not be expected to have a differential impact. Although cell proliferation rates may be decreased, differences between groups would be expected to be maintained, unless, however, there was a basal rate of cell proliferation (from which further down-regulation can not occur), that was reached by all three groups. Such a basal rate has never been suggested or described. Indeed, a recent study in voles (Galea & McEwen, 1999) showed that maintaining voles in captivity for 24 h after BrdU injection (48 h from trapping to perfusion) did not

eliminate differences in the rate of cell proliferation in the dentate gyrus. Thus, we are confident that our results represent accurately the relative rate of cell proliferation in wild-caught squirrels throughout the year.

Neuron number

Increased demand in spatial memory processing did not correlate with changes in total neuron number in the granule cell layer in adult squirrels. Our results contrast with those of Kempermann *et al*. (1997) in C57BL/6 mice, who showed an increase in neuron number in 2-month-old mice living in enriched conditions that correlated with shorter escape paths in the Morris navigation task. However, the lack of change in total neuron number is consistent with the only other studies of neurogenesis in wild-caught animals (scatter-hoarding black-capped chickadees; Barnea & Nottebohm, 1994, 1996) and with those studies using 6- and 18-month-old mice (Kempermann *et al*., 1998b). The absence of seasonal variations in cell proliferation in squirrels does not rule out the possibility of differential survival and recruitment of newly generated neurons in the granule cell layer at different times of the year. A recent study (Gould *et al*., 1999a) has shown that survival of newly generated cells is enhanced by hippocampus-dependent learning in rats. This is consistent with the results showing that environmental enrichment has a survival-promoting effect on proliferating neuronal precursors in young and old C57BL/6 mice (Kempermann *et al*., 1997; 1998b). In adult squirrels, as in 6- and 18-month-old mice, however, there were no discernible differences in total neuron number between groups. Thus, whether or not survival and recruitment of proliferating neuronal precursors vary seasonally in mature rodents, such changes fail to produce any discernible changes in total neuron number in the dentate gyrus in adulthood. Changes in total neuron number are thus observed only in juveniles and are likely to represent developmental changes rather than adult plasticity. In squirrels, a higher turnover of neurons in the autumn, at the peak of the caching season, could underlie differential neurogenesis in the granule cell layer, resulting in a constant total neuron number. This scenario, consistent with that observed in chickadees (Barnea & Nottebohm, 1994, 1996), remains to be investigated.

New neurons, total neuron number and learning

Our results in wild, mature long-lived rodents agree with those from previous experiments in food-caching birds and with current hypotheses regarding the positive relationship between neurogenesis and learning (for a review see Gould *et al*., 1999b). Our results, as well as those described above, however, are also in agreement with an alternative hypothesis regarding the relationship between spatial information processing and changes in neurogenesis and neuron number in adult mammals. Neurogenesis in the dentate gyrus of the hippocampal formation, a structure critically involved in learning and memory processes, might simply represent a maintenance system. Cell proliferation, survival, and differentiation of new neurons can be regulated by experience and environmental conditions, but they do not lead to variations in total neuron number in adult, fully mature animals, regardless of the environmental context (e.g. Barnea & Nottebohm, 1994; Kempermann *et al*., 1998b; this study). A higher turnover of neurons, correlated with increased memory processing within the dentate gyrus, is therefore expected.

Current interpretations suggest that these new neurons are involved in hippocampal learning (Gould *et al*., 1999b). As noted by Gould and colleagues, however, changes in the number of new cells can not have immediate functional consequences. If more new neurons are produced while performing a particular (hippocampus-dependent) behaviour, there will be a delay until these neurons become functional and capable of contributing to information processing in the hippocampal formation. Enriched conditions (Kempermann *et al*., 1997) or hippocampus-dependent learning (Patel *et al*., 1997) might correlate with increased neurogenesis in developing animals and produce an increase in neuron number in the dentate gyrus of these animals in adulthood. In adults, however, newly generated neurons do not increase the number of existing neurons, nor do they alter the hippocampal circuitry for the specific learning event that triggered the increase in neurogenesis. Indeed learning, and the formation of new memories, can be subserved by more subtle neuronal changes, such as synaptic reorganization (Weiler *et al*., 1995; Nordeen & Nordeen, 1997) or changes in synaptic strength via mechanisms, such as long term potentiation (Bliss & Collingridge, 1993) and long term depression (Abraham *et al*., 1994) that do not necessitate the addition of new neurons to the neural circuitry. Instead, the new neurons may replace older neurons that no longer exhibit the plasticity necessary for optimal learning. The survival of these newly generated neurons could thus be upregulated to compensate for increased cell death that may underlie increased information processing in the dentate gyrus during hippocampus-dependent learning. Future studies combining well-defined behavioural paradigms and variable survival times are clearly needed to elucidate the relationship between cell proliferation, survival of newly generated neurons and cell death in the adult, fully mature dentate gyrus and hippocampus-dependent learning. Seasonal studies on food-caching animals in semi-naturalistic settings represent a model that may provide invaluable insights to this issue.

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Abbreviations

Ammonium nitrate, NH_4NO_3; BrdU, bromodeoxyuridine; gcl DG, granule cell layer of dentate gyrus; PBS, phosphate buffered saline; NaOH, sodium hydroxide; SGZ, subgranular zone.

References

Abraham, W.C., Christie, B.R., Logan, B., Lawlor, P. & Dragunow, M. (1994) Immediate early gene expression associated with the persistence of heterosynaptic long-term depression in the hippocampus. *Proc. Natl Acad. Sci. USA*, **91**, 10049–10053.

Altman, J. & Das, G.D. (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J. Comp. Neurol.*, **124**, 319–336.

Barnea, A. & Nottebohm, F. (1994) Seasonal recruitment of hippocampal neurons in adult free-ranging black-capped chickadees. *Proc. Natl Acad. Sci. USA*, **85**, 11217–11221.

Barnea, A. & Nottebohm, F. (1996) Recruitment and replacement of hippocampal neurons in young and adult chickadees: an addition to the theory of hippocampal learning. *Proc. Natl Acad. Sci. USA*, **93**, 714–718.

Bayer, S.A. (1982) Changes in the total number of dentate gyrus cells in juvenile and adult rats: a correlated volumetric and 3H-thymidine autoradiography study. *Exp. Brain Res.*, **46**, 315–323.

Bliss, T.V.P. & Collingridge, G.L. (1993). A synaptic model of memory: Long-term potentiation in the hippocampus. *Nature*, **361**, 31–39.

Eriksson, P.S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A.M., Nordborg, C., Peterson, D.A. & Gage, F.H. (1998) Neurogenesis in the adult human hippocampus. *Nature Med.*, **4**, 1313–1317.

Gage, F.H., Kempermann, G., Palmer, T.D., Peterson, D.A. & Ray, J. (1998) Multipotent progenitor cells in the adult dentate gyrus. *J. Neurobiol.*, **36**, 249–266.

Galea, L.A.M. & McEwen, B.S. (1999) Sex and seasonal differences in the rate of cell proliferation in the dentate gyrus of adult wild meadow voles. *Neuroscience*, **89**, 955–964.

Gould, E., Tanapat, P., McEwen, B.S., Flügge, G. & Fuchs, E. (1998) Proliferation of granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress. *Proc. Natl Acad. Sci. USA*, **95**, 3168–3171.

Gould, E., Beylin, A., Tanapat, P., Reeves, A.J. & Shors, T.J. (1999a) Learning enhances neurogenesis in the hippocampal formation. *Nature Neurosci.*, **2**, 260–265.

Gould, E., Tanapat, P., Hastings, N.B. & Shors, T.J. (1999b) Neurogenesis in adulthood: a possible role in learning. *Trends Cognit. Sci.*, **3**, 186–192.

Gundersen, H.J.G. & Jensen, E.B. (1987) The efficiency of systematic sampling in stereology and its prediction. *J. Microscop.*, **147**, 229–263.

Gurnell, J.C. (1987) The natural history of squirrels. New York, N.Y. Facts on File.

Jacobs, L.F. & Liman, E.R. (1991) Grey squirrels remember the locations of buried nuts. *Anim. Behav.*, **41**, 103–110.

Kempermann, G., Kuhn, G. & Gage, F.H. (1997) More hippocampal neurons in adult mice living in an enriched environment. *Nature*, **386**, 493–495.

Kempermann, G., Brandon, E.P. & Gage, F.H. (1998a) Environmental stimulation of 129/SvJ mice causes increased cell proliferation and neurogenesis in the adult dentate gyrus. *Curr. Biol.*, **8**, 939–942.

Kempermann, G., Kuhn, H.G. & Gage, F.G. (1998b) Experienced-induced neurogenesis in the senescent dentate gyrus. *J. Neurosci.*, **18**, 3206–3212.

Koprowski, J.L. (1994) Sciurus carolinensis. *Mamm. Spec.*, **480**, 1–9.

Nordeen, K.W. & Nordeen, E.J. (1997) Anatomical and synaptic substrates for avian song learning. *J. Neurobiol.*, **33**, 532–548.

Patel, S.N., Clayton, N.S. & Krebs, J.R. (1997) Spatial learning induces neurogenesis in the avian brain. *Behav. Brain Res.*, **89**, 115–128.

Sloviter, R.S., Sollas, A.L., Dean, E. & Neubort, S. (1993) Adrenalectomy-induced granule cell degeneration in the rat hippocampal dentate gyrus: Characterization of an in vivo model of controlled neuronal death. *J. Comp. Neurol.*, **330**, 324–336.

Thompson, D.C. & Thompson, P.S. (1980) Food habits and caching behavior of urban grey squirrels. *Can. J. Zool.*, **58**, 701–710.

Vander Wall, S.B. (1990) *Food Hoarding in Animals*. University of Chicago Press, Chicago, IL.

van Praag, H., Kempermann, G. & Gage, F.H. (1999) Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nature Neurosci.*, **2**, 266–270.

Weiler, I.J., Hawrylak, N. & Greenough, W.T. (1995) Morphogenesis in memory formation: Synaptic and cellular mechanisms. *Behav. Brain Res.*, **66**, 1–6.

West, M.J. & Gundersen, H.J.G. (1990) Unbiased stereological estimation of the number of neurons in the human hippocampus. *J. Comp. Neurol.*, **296**, 1–22.

West, M.J., Slomianka, L. & Gundersen, H.J.G. (1991) Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. *Anat. Rec.*, **231**, 482–497.

West, M.J., Ostergaard, K., Andreassen, O.A. & Finsen, B. (1996) Estimation of the number of somatostatin neurons in the striatum: an in situ hybridization study using the optical fractionator method. *J. Comp. Neurol.*, **370**, 11–22.