Where's my dinner? Adult neurogenesis in free-living food-storing rodents

J. M. Barker[†], J. M. Wojtowicz[‡] and R. Boonstra[*,§]

[†]*Centre for the Neurobiology of Stress,* [‡]*Department of Physiology, and* [§]*Centre for the Neurobiology of Stress, University of Toronto, Scarborough, Ontario, Canada*
Corresponding author: R. Boonstra, Centre for the Neurobiology of Stress, University of Toronto, 1265 Military Trail, Scarborough, Ontario, Canada M1C 1A4. E-mail: boonstra@utsc.utoronto.ca

Postnatal hippocampal neurogenesis in wild mammals may play an essential role in spatial memory. We compared two species that differ in their reliance on memory to locate stored food. Yellow-pine chipmunks use a single cache to store winter food; eastern gray squirrels use multiple storage sites. Gray squirrels had three times the density of proliferating cells in the dentate gyrus (determined by Ki-67 immunostaining) than that found in chipmunks, but similar density of young neurons (determined by doublecortin immunostaining). Three explanations may account for these results. First, the larger population of young cells in squirrels may increase the flexibility of the spatial memory system by providing a larger pool of cells from which new neurons can be recruited. Second, squirrels may have a more rapid cell turnover rate. Third, many young cells in the squirrels may mature into glia rather than neurons. The densities of young neurons were higher in juveniles than in adults of both species. The relationship between adult age and cell density was more complex than that has been found in captive populations. In adult squirrels, the density of proliferating cells decreased exponentially with age, whereas in adult chipmunks the density of young neurons decreased exponentially with age.

The functional significance of neurogenesis in adult, free-living mammals remains unknown. It occurs both in the rostral migratory stream, which feeds new neurons to the olfactory bulb, and in the subgranular zone (SGZ) of the dentate gyrus (DG), which generates granule neurons in the hippocampus (recent reviews Alvarez-Buylla & Garcia-Verdugo 2002; Gould & Gross 2002; Gross 2000). In the laboratory, cell proliferation in the adult brain declines with age (Bizon & Gallagher 2003; Kuhn *et al*. 1996), declines under the conditions of high glucocorticoid concentrations (Cameron & Gould 1994; Tanapat *et al*. 2001) and increases under conditions of higher ovarian steroid concentrations (Ormerod & Galea 2001; Tanapat *et al*. 1999). The survival of these newly generated cells can be enhanced by certain types of learning (Ambrogini *et al*. 2000; Gould *et al*. 1999b) and by environmental enrichment (Kempermann *et al*. 1997; Kempermann *et al*. 1998a; Nilsson *et al*. 1999). How these laboratory findings may be used to determine the utility of this universal trait in free-living animals is unclear (Boonstra *et al*. 2001a). For example, the decline of hippocampal cell proliferation rates in older rats in the laboratory (Bizon & Gallagher 2003; Cameron & McKay 1999; Kempermann *et al*. 2002; Kuhn *et al*. 1996) may have very real consequences for animals living in a natural environment. Whereas animals in the laboratory are maintained in protected environments with access to abundant food and water, and without predators, free-living animals have no such security. If adult neurogenesis is beneficial to survival, then an age-related decline in proliferation rates may not be detectable in wild populations, because those animals whose proliferation rates declined below a critical minimum would not survive.

Adult neurogenesis in the SGZ appears to be related to spatial memory. The hippocampus may be involved in certain spatial memory tasks related to memory of contextual cues (O'Keefe & Nadel 1978; Rosenbaum *et al*. 2001). Increased or decreased levels of neurogenesis are correlated with an improved or impaired spatial memory, respectively (recent reviews Gould *et al*. 1999b; Lee *et al*. 1998; but see Shors *et al*. 2002), though the survival of proliferating cells may be more strongly related to learning and memory than is the proliferation rate (Ambrogini *et al*. 2000; Gould *et al*. 1999b; Kempermann *et al*. 1998b; Nilsson *et al*. 1999; but see Drapeau *et al*. 2003). Virtually, all these studies have used laboratory rats and mice. Hamsters (Huang *et al*. 1998), eastern gray squirrels (Lavenex *et al*. 2000a), voles (Galea & McEwen 1999), tree shrews (Gould *et al*. 1997) and primate species (Gould *et al*. 1998; Gould *et al*. 1999a; Gould *et al*. 2001) including humans (Eriksson *et al*. 1998) have also been used, but most studies share the same limitations of those on rats and mice. The individuals in animal studies are held in

highly controlled conditions not reflective of the natural environment where they are normally found. The human tissue, though acquired from a non-captive population, was acquired from cancer patients and so the results cannot be considered representative of a normal, healthy population. Animals in a laboratory setting may be exposed to limited or inappropriate environmental stimuli, including a surplus of food and water, unusually close proximity to conspecifics, and an absence of predators, competitors, mutualistic interactions with other species and seasonal cues (Boonstra et al. 2001a). Additionally, caution must be exercised when extrapolating from the laboratory to the natural world, as the laboratory counterparts of wild equivalents are often less aggressive, less aware of their environment and explore less but are more social and respond more strongly to stressors (Künzl et al. 2003). Some studies have sought to partially rectify these problems by bringing wild individuals into the laboratory, injecting them with a mitotic marker such as bromodeoxyuridine and then assessing differences [e.g. sex and seasonal differences in voles (Galea & McEwen 1999) and gray squirrels (Lavenex et al. 2000b)]. However, live capture of wild animals is known to be stressful (Boonstra et al. 2001b; Boonstra & Singleton 1993), and because stress is known to inhibit the proliferation of neurons (Fuchs et al. 1997; Gould & Tanapat 1999; Tanapat et al. 1998), this may compromise the subsequent determination of neurogenesis. Thus, techniques are needed that minimize observer-induced artifacts.

To assess the impact of differences in spatial memory requirements on adult hippocampal neurogenesis, we examined two free-living rodent species with profoundly different requirements for memory of food storage locations. We relied on methods that could not be confounded by capture-induced stress. Ki-67 and doublecortin (DCX) are both endogenous proteins that are highly conserved among species and serve as indicators of neurogenesis. Ki-67 is present in all cells that are actively cycling and is involved in the disintegration and reformation of nucleoli (Schmidt et al. 2003; Scholzen & Gerdes 2000), whereas DCX is a microtubule-associated protein that is expressed in young migrating neurons (Brown et al. 2003; Francis et al. 1999; Gleeson et al. 1999; Rao & Shetty 2004). We assumed that Ki-67 and DCX present in cells at the time of capture would remain in cells until the time of killing, even if further development of the cells became arrested due to trapping stress.

We selected two rodent species from the same family (Sciuridae) that both cache food prior to winter, but use storage strategies at the extremes of a continuum. These strategies are not learned behaviors, but rather are innate features. Yellow-pine chipmunks (Tamias amoenus) cache food at a single underground storage site (larder hoard). They remain in this burrow from September to April/May, hibernating for approximately 6 days at a time (Geiser et al. 1997; Sutton 1992). During their short waking periods, they simply move to their cache to eat. Eastern gray squirrels (Sciurus carolinensis) cache food at multiple above-ground storage sites (scatter hoard) and remain active all winter (Koprowski 1994; Pereira et al. 2002). Throughout the winter, they rely primarily on their spatial memory to find hundreds of food caches (Jacobs & Liman 1991; Macdonald 1997; McQuade et al. 1986). Any process that would improve the capacity of gray squirrels to remember spatial information should therefore be enhanced through natural selection, relative to the chipmunks, because the squirrels rely so heavily on spatial memory for survival through the winter. Thus, if adult hippocampal neurogenesis does serve to improve spatial memory capacity, duration or retrieval, we expect that eastern gray squirrels should have much higher rates of adult neurogenesis than yellow-pine chipmunks during the critical autumn season. We therefore predict that the squirrels should have higher densities of both proliferating cells and young neurons in the hippocampus. We also expect that the maintenance of neurogenesis is under extremely strong selection pressure and that a minimum rate of production of new neurons is crucial for survival. We therefore predict that the densities of these cell types will not decline with age in either species, as occurs in laboratory rodents.

Materials and methods

Animal collection and processing

We captured 25 yellow-pine chipmunks in the mountain valleys of the Rocky Mountains of Kananaskis Country, Alberta, Canada, early in September 2001 using Longworth live traps (Penlon-Britannia Medical Supplies, Mississauga, Ontario, Canada). These are small (40–60 g) rodents; although longevity data are currently unavailable for this species, a related species (Tamias ruficaudus) can live up to 8 years old in the wild, though it is rare for individuals to survive longer than 5 years (Best 1999). The times of capture correspond to times when individuals were actively collecting and storing food for the winter (Sutton 1992).

We captured 13 eastern gray squirrels outside of Toronto, Ontario, Canada, in October 2001 using Tomahawk live traps (14 × 14 × 40 cm or 16.5 × 16.5 × 48 cm; Tomahawk Live Trap Company, Tomahawk, WI). These are medium-sized (400–900 g) rodents that can live up to 12 years old in the wild, though it is rare for individuals to survive longer than 6 years (Barkalow & Soots 1975). The times of capture correspond to times when individuals were actively collecting and storing food for the winter (Koprowski 1994; Thompson 1977). All other processing was done in the same manner for both species.

Within 2 h of capture, we removed the animal from the trap and anesthetized it with halothane (Halocarbon Laboratories, River Edge, NJ). Squirrels were removed from the trap into a mesh bag, anesthetized and weighed to the nearest 5 g using a 1-kg spring scale. Chipmunks were restrained by hand while an overdose of the anesthetic was administered and weighed to the nearest 0.5 g using a 100-g spring scale.

The sex and sexual condition of the animal (scrotal or abdominal testes for males; non-breeding, pregnant or lactating for females) were determined, and the animal was killed by terminal bleeding via cardiac puncture.

We removed the brain immediately and fixed one hemisphere by submersion in a solution of 4% paraformaldehyde in 0.2 M phosphate buffer (pH 7.4). After 24 h, the brain was removed from the fixative and stored in a 0.1% sodium azide in phosphate-buffered saline (PBS) solution (pH 7.2). The tissue was cut into 40-μm sections, stored in 0.1% sodium azide in PBS and immunostained.

After removal of the brains, the bodies were frozen at −20 °C until dissection to confirm sex and sexual condition. We determined the ages of individual chipmunks by counting annuli in femoral sections (Barker et al. 2003). We distinguished adult squirrels from juveniles by testes weight in the males (juveniles < 500 mg, adult > 500 mg) and by the presence of uterine scars in females (which indicated past pregnancy and therefore that the animal was an adult) and determined the ages of the adults by counting annuli in femoral sections (Barker et al. 2003).

Immunohistochemistry

For all immunostaining, we used coronal brain sections that included the dorsal length of the hippocampus, because damage to this region is more disruptive to spatial learning than damage to other hippocampal regions (Ferbinteanu & McDonald 2001; Ferbinteanu et al. 2003; Moser et al. 1995). To observe cells containing Ki-67, we randomly selected one section from the first four anterior sections in which the two blades of the dentate met. We then selected every fourth section from then on, until the hippocampus was no longer oriented horizontally. The selected sections were rinsed three times in PBS, then incubated for 20 min in 3% normal goat serum in 0.3% Triton-X-100 in PBS to reduce background staining. We then removed the goat serum solution and incubated the sections with rabbit anti-human-Ki-67 immunoglobulin G (IgG) primary antibody (Vector Laboratories, Burlington, Ontario, Canada) in 0.3% Triton-X-100 in PBS (1 : 500) at 3 °C for 48 h. We then incubated the sections with secondary antibody (fluorescein-conjugated goat anti-rabbit IgG, Molecular Probes, Burlington, Ontario, Canada) in 0.3% Triton-X-100 in PBS (1 : 200) at room temperature for 2 h.

To observe cells containing DCX, we selected sections in the same manner as for Ki-67 staining, except that sections stained for Ki-67 were not used for DCX staining. The sections selected for DCX staining were rinsed three times in PBS, then incubated with goat anti-human-DCX IgG primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in 0.3% Triton-X-100 in PBS (1 : 200) at 3 °C for 48 h. We then incubated the sections with secondary antibody (fluorescein-conjugated donkey anti-goat IgG, Molecular Probes) in 0.3% Triton-X-100 in PBS (1 : 200) at room temperature for 2 h.

To correct for the size difference between the two species, we counted total numbers of NeuN^{+} cells (mature neurons) in the granule cell layer (GCL). To observe cells containing NeuN, we selected sections adjacent to those used for Ki-67 and DCX staining. The selected sections were rinsed three times in PBS, then incubated with mouse anti-NeuN monoclonal IgG primary antibody (Chemicon, Temecula, CA) in 0.3% Triton-X-100 in PBS (1 : 200) at 3 °C for 24 h. We then incubated the sections with secondary antibody (fluorescein-conjugated goat anti-mouse IgG, Molecular Probes) in 0.3% Triton-X-100 in PBS (1 : 200) at room temperature for 2 h.

Cell counting

We counted cells in the DG stained with Ki-67 or DCX under a fluorescent Optiphot-2 microscope (Nikon Canada Inc., Mississauga, ON). Because biases may result from counting cells non-stereologically (Keuker et al. 2001; West 1993, 1999), we also counted positively stained cells (Ki-67^{+} or DCX[+]) in a random subsample of sections stereologically using an optical dissector method (West 1993) on a confocal microscope (Zeiss, Thornwood, NY). Because the counts from both microscopes agreed to within 10%, the fluorescent microscope was used throughout.

We counted Ki-67^{+} cells within the SGZ of the DG that we defined as an area two-cell diameter wide along the inner edge of the GCL. We counted cells containing DCX throughout the GCL and SGZ, provided their entire nucleus was visible.

To account for size differences between the two species, we counted cells in the GCL stained with NeuN using the optical dissector method (West 1993) on a confocal microscope in sections adjacent to those in which Ki-67 or DCX were counted. We then expressed cell counts for both Ki-67 and DCX as number of cells counted per 1000 NeuN^{+} cells.

We first applied the Brown–Forsythe test to confirm homogeneity of variance using the program JMP (SAS Institute, Inc., Cary, NC). All further statistical analyses were carried out using the program STATVIEW (Caldarola et al. 1998). We initially analyzed cell data with three-way ANOVA (species, sex and age). Exponential regressions (of the form $Y = b0 \times e^{b1 \times X}$) were run on data for adults to examine age effects in adulthood. An exponential relationship between age and cell densities was assumed based on evidence from laboratory rats showing exponential decay of neurogenesis with age (Seki & Arai 1995), confirmed in our laboratory (J. M. Wojtowicz, unpublished data). We considered differences or regressions statistically significant if $P < 0.05$, but we also note that we used fairly small sample sizes and thus differences or regressions with $P < 0.10$ may be biologically significant (Yoccoz 1991).

Results

Body condition

In the chipmunks, females were approximately 10% heavier than males, but there was no difference in size between

juveniles and adults (Table 1). All females were in non-breeding condition (not in estrous, pregnant or lactating), and all males had abdominal testes. Of the adult chipmunks, eight were 1 year old, three were 2 years old, three were 3 years old, two were 4 years old and one was 5 years old.

In the squirrels, there was no difference in body weight between males and females, though adults were approximately 8% heavier than juveniles (Table 1). All the female squirrels were in non-breeding condition; all adult males and one juvenile male had scrotal testes. Of the adult squirrels, one was 1 year old, two were 2 years old, one was 3 years old, one was 5 years old, two were 6 years old and one was 8 years old.

**Table 1:** Age structure and body mass of animals captured

	Females		Males	
	Juveniles	Adults	Juveniles	Adults
Yellow-pine chipmunks				
Number	5	8	3	9
Mass (g)	56.4 ± 1.7	56.3 ± 1.0	50.2 ± 1.1	51.6 ± 1.5
Eastern gray squirrels				
Number	3	6	2	2
Mass (g)	543 ± 3	587 ± 16	568 ± 28	620 ± 70

Animals were separated into age classes of adult (over 1 year old) and juvenile (under 1 year old). Data are presented as means ± 1 standard error.
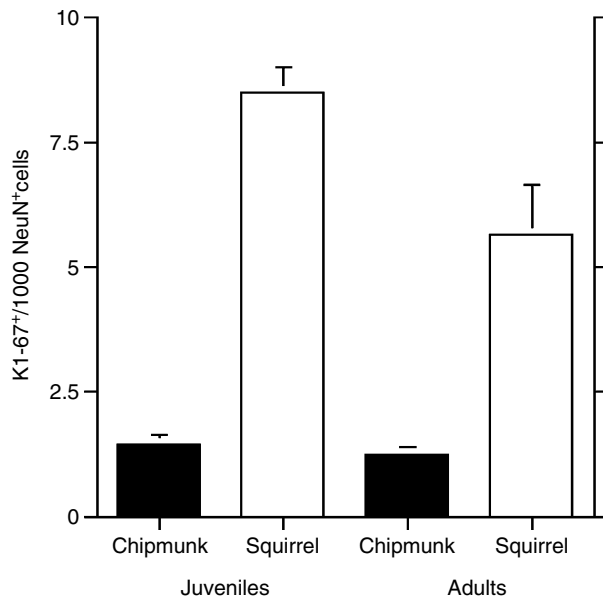
Ki-67 cell counts

Cells containing Ki-67 were present in the SGZ of both chipmunks and squirrels (Fig. 1). We examined data for the main effects of species, age and sex and their interactions. There was no significant effect of sex on the proportion of Ki-67$^+$ cells ($F = 0.01$, df = 1, 30, $P = 0.94$), nor were any of the interactions including sex significant (sex × species: $F = 0.71$, df = 1, 30, $P = 0.41$; sex × age: $F = 0.08$, df = 1, 30, $P = 0.77$; sex × species × age: $F = 0.001$, df = 1, 30, $P = 0.97$). Therefore, the data from the two sexes were pooled and the analysis run as a two-way ANOVA (age × species). The proportion of cells expressing Ki-67 was approximately three times greater in eastern gray squirrels than in yellow-pine chipmunks ($F = 129$, df = 1, 34, $P < 0.0001$) (Fig. 2). There was a significant age effect on proportions of Ki-67$^+$ cells, with juveniles having higher proportions of Ki-67 cells ($F = 9.40$, df = 1, 34, $P = 0.004$). There was also a species–age interaction effect ($F = 6.69$, df = 1, 34, $P = 0.014$). To examine the nature of this interaction, we analyzed each species separately using one-way ANOVA (age). In chipmunks, there was no age effect on the proportion of Ki-67$^+$ cells ($F = 0.83$, df = 1, 23, $P = 0.37$). In squirrels, the proportion of Ki-67$^+$ cells was 50% higher in juveniles than in adults ($F = 4.65$, df = 1, 11, $P = 0.05$).

In chipmunks, there was no significant relationship between adult age and proportion of cells expressing Ki-67 in the DG ($b0 = 1.65$, $b1 = -0.25$, $r^2 = 0.16$, $P = 0.18$) (Fig. 3a). In squirrels, there was an exponential decline in the proportion of cells expressing Ki-67 in the DG with increasing adult age ($b0 = 10.91$, $b1 = -0.19$, $r^2 = 0.69$, $P = 0.011$) (Fig. 3b).
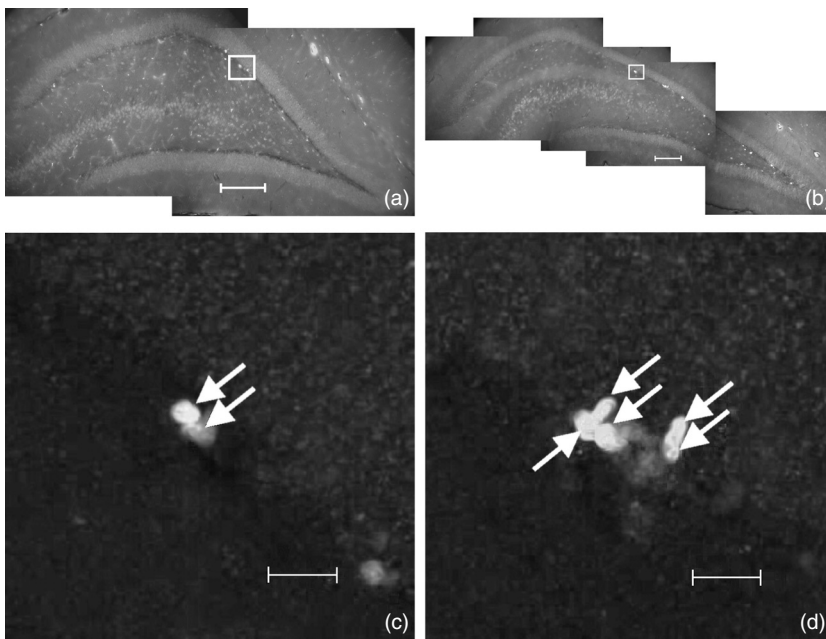
Figure 1: Photomicrographs of hippocampal sections immunostained against Ki-67 protein in yellow-pine chipmunk and eastern gray squirrel. Cells positive for Ki-67 that were located within two-cell widths of the subgranular zone were considered potential neurons. The entire dentate gyrus of an adult chipmunk is shown in (a) and of an adult squirrel in (b) (scale bars: 200 µm); the white boxes in (a) and (b) surround the areas magnified in (c) and (d), respectively (scale bars: 20 µm). The arrows in (c) and (d) indicate individual positively stained nuclei of proliferating cells.
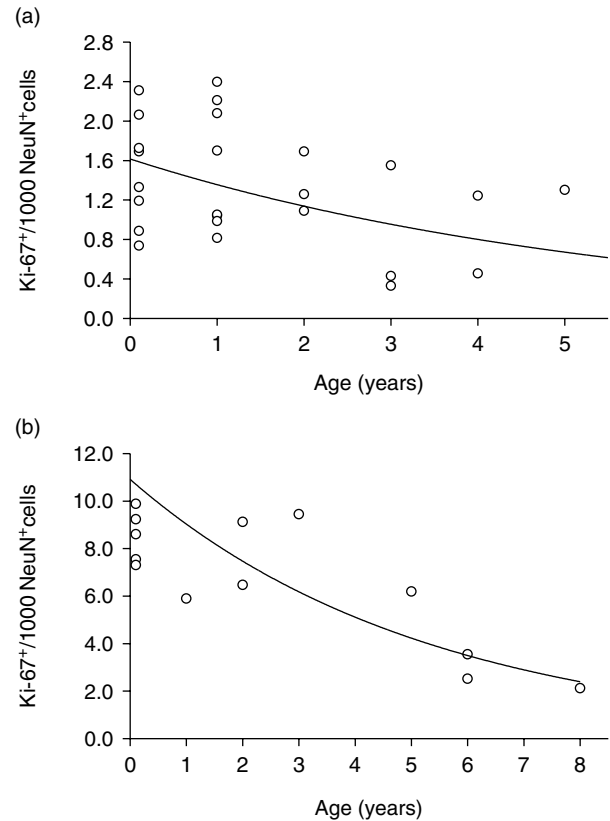
Figure 2: Proportions of cells expressing Ki-67 in the subgranular zone of yellow-pine chipmunks and eastern gray squirrels. Animals were separated into age classes of adult (over 1 year old) and juvenile (under 1 year old). The vertical axis represents the number of cells that expressed Ki-67 per thousand mature neurons (cells that expressed NeuN) in the dentate gyrus. Columns show untransformed means ± 1 standard error. Squirrels had significantly higher densities of cells expressing Ki-67 than chipmunks.

DCX cell counts

Cells containing DCX were present in the DG of both chipmunks and squirrels (Fig. 4). We examined data for the main effects of species, age and sex and their interactions. There was no significant effect of sex on proportions of DCX^{+} cells ($F = 1.06$, df $= 1$, 30, $P = 0.31$), nor were any of the interactions including sex significant (sex × species: $F = 0.12$, df $= 1$, 30, $P = 0.74$; sex × age: $F = 0.16$, df $= 1$, 30, $P = 0.70$; sex × species × age: $F = 0.34$, df $= 1$, 30, $P = 0.47$). Therefore, the data from the two sexes were pooled and the analysis run as a two-way ANOVA (age × species). There was no significant species effect ($F = 3.39$, df $= 1$, 34, $P = 0.074$) and no interaction effect ($F = 0.11$, df $= 1$, 34, $P = 0.74$), though the difference between the species (approximately 35% higher in squirrels than in chipmunks) may be biologically significant. Proportions of DCX^{+} cells were approximately 75% higher in juveniles than in adults (Fig. 5; $F = 11.4$, df $= 1$, 34, $P = 0.0018$). Thus age, but not species, affected the proportion of cells expressing DCX (Fig. 5).

In chipmunks, there was an exponential decline in the proportion of cells expressing DCX in the DG with increasing adult age ($b0 = 32.8$, $b1 = -0.31$, $r^2 = 0.48$, $P = 0.002$) (Fig. 6a). In squirrels, there was no significant relationship between adult age and proportion of cells expressing DCX


Figure 3: Exponential regression of proportion of cells expressing Ki-67 in the subgranular zone of adult yellow-pine chipmunks (a) and eastern gray squirrels (b). Data from juveniles are presented but were not included in the analysis when determining the regression lines presented. The vertical axis represents the number of cells that expressed Ki-67 per thousand mature neurons (cells that expressed NeuN) in the dentate gyrus. The relationship is statistically significant in squirrels, but not in chipmunks.

in the DG ($b0 = 42.4$, $b1 = -0.16$, $r^2 = 0.29$, $P = 0.17$) (Fig. 6b).

Discussion

This is the first study to examine adult neurogenesis in free-living animals in which handling-stress effects have not confounded the interpretation of the assessment of neurogenesis. Two counterintuitive results were obtained. First, though the prediction of greater neurogenesis in the species with the greater need was confirmed at one level (greater proliferation of cells in the SGZ in the scatter-hoarding squirrels than in the larder-hoarding chipmunks, Fig. 2), it was not confirmed at another, as these incipient neurons did not translate into more young neurons (Fig. 5). Thus, a simple linear relationship between neurogenesis rates or neuronal survival and spatial memory requirements may not be present in free-living animals. Second, though sharp declines
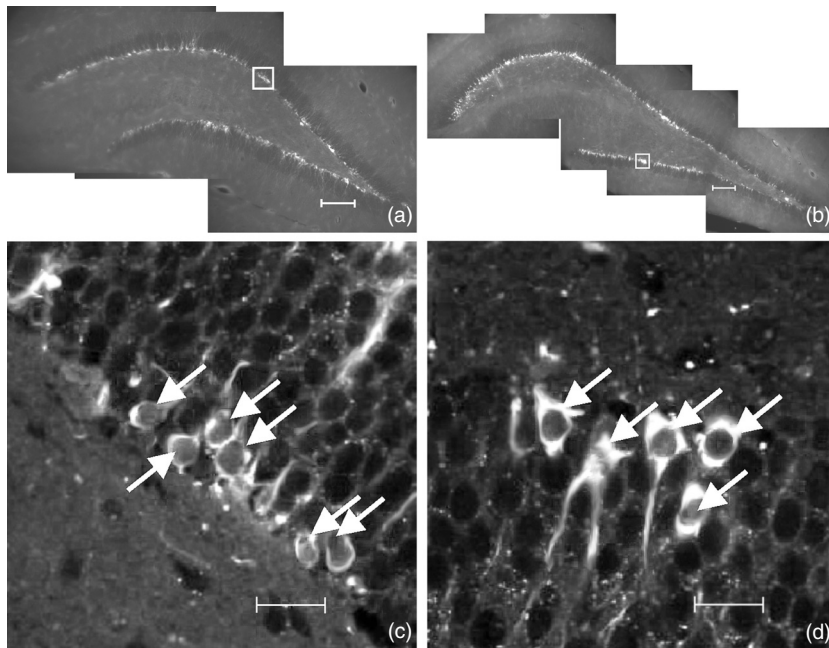
Figure 4: Photomicrographs of hippocampal sections immunostained against doublecortin protein in yellow-pine chipmunk and eastern gray squirrel. Cells positive for doublecortin that were located within the granule cell layer and subgranular zone were considered young neurons. The entire dentate gyrus of an adult chipmunk is shown in (a) and of an adult squirrel in (b) (scale bars: 200 μm); the white boxes in (a) and (b) surround the areas magnified in (c) and (d), respectively (scale bars: 20 μm). The arrows in (c) and (d) indicate individual positively stained cells.

in neurogenesis with increasing age are seen in laboratory animal models (Seki & Arai 1995), the situation in wild rodents is more complex. Squirrels showed an exponential age-related decline in the proportion of proliferating cells in the DG, but not in the proportion of young neurons. Conversely, chipmunks showed an exponential age-related decline in the proportion of young neurons in the DG, but not in the proportion of proliferating cells. Neither of these changes was as marked as those seen in the laboratory rodents.

Commensurate with their greater reliance on spatial memory for locating food stores, gray squirrels showed higher rates of hippocampal cell proliferation in the DG than did yellow-pine chipmunks, a species with less need for this memory (Fig. 2). Squirrels had three times the density of proliferating cells (containing Ki-67) than did chipmunks. Similar results were obtained when Ki-67$^+$ cell counts were expressed per unit GCL volume (juveniles: squirrels – 4448 ± 287 cells/mm^3, chipmunks – 1077 ± 155 cells/mm^3; adults: squirrels – 2641 ± 406 cells/mm^3, chipmunks – 1070 ± 110 cells/mm^3). These results are consistent with our prediction that the former should have much greater proliferation of incipient neurons given their great need for memory of food storage locations. However, these differences did not translate into differences in the densities of young neurons (containing DCX) that were similar between the species (Fig. 5). Our conclusions about the lack of differences in DCX$^+$ cells between species must be tempered by the relatively small sample sizes in our study. The data in Fig. 5 suggest there may be a biologically significant species difference, with squirrels having higher densities of DCX$^+$ cells than chipmunks. However, when DCX$^+$ cell counts are expressed per unit GCL volume, chipmunks have slightly

higher DCX$^+$ cell densities than squirrels (juveniles: squirrels – $26\,164 \pm 3874$ cells/mm^3, chipmunks – $26\,329 \pm 3763$ cells/
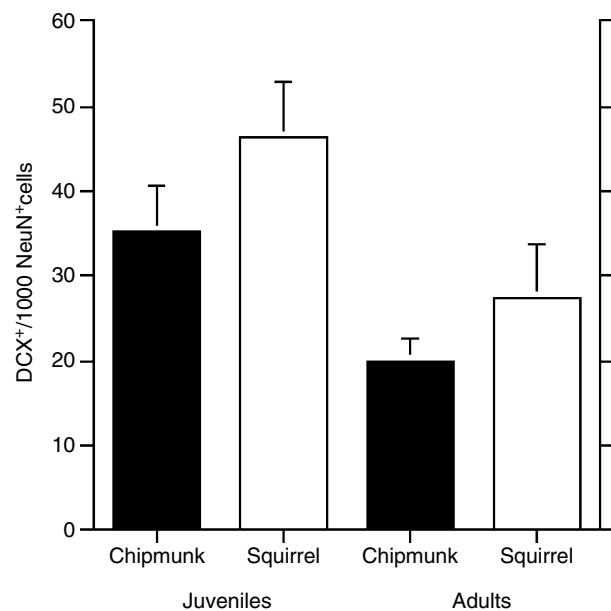
Figure 5: Proportions of cells expressing doublecortin (DCX) in the dentate gyrus (DG) of yellow-pine chipmunks and eastern gray squirrels. Animals were separated into age classes of adult (over 1 year old) and juvenile (under 1 year old). The vertical axis represents number of cells that expressed DCX per thousand mature neurons (cells that expressed NeuN) in the DG. Columns show untransformed means ± 1 standard error. Juveniles had significantly higher densities of cells expressing DCX than adults.

(a)

(b)
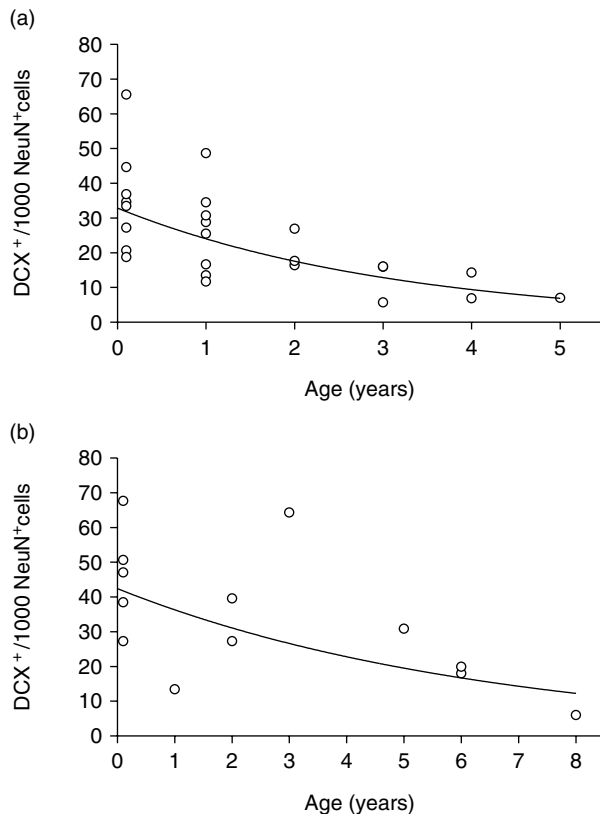
Figure 6: Exponential regression of proportion of cells expressing doublecortin (DCX) in the subgranular zone of adult yellow-pine chipmunks (a) and eastern gray squirrels (b). Data from juveniles are presented, but were not included in the analysis when determining the regression lines presented. The vertical axis represents the number of cells that expressed DCX per thousand mature neurons (cells that expressed NeuN) in the dentate gyrus. The relationship is statistically significant in chipmunks, but not in squirrels.

mm^3; adults: squirrels – $11\,810 \pm 2144$ cells/mm^3, chipmunks – $16\,647 \pm 1792$ cells/mm^3). The two-way ANOVA of these data indicate no species effect ($F = 0.039$, df $= 1$, 34; $P = 0.84$), but proportions of DCX$^+$ cells were higher in juveniles than in adults (by 58% in chipmunks and by 121% in squirrels). Assuming the lack of difference is real therefore, there are three possible explanations for the differences in the two measures of neurogenesis.

First, the greater number of cells containing Ki-67 in the squirrels may provide them with a larger population from which to recruit new neurons. This would allow for greater flexibility within the DG of squirrels, because the potential would exist to recruit additional cells as required and the location of the new cells within the DG could be precisely selected, while suboptimal cells are allowed to die or become quiescent, to optimize efficiency of the system. Assuming adult neurogenesis in the SGZ is involved in spatial memory, this improved efficiency should translate to either

increased capacity for memory of spatial information or increased speed of information retrieval or both. This could also explain why, in both species, juveniles had higher densities of young neurons than adults. For the squirrels, although it may be the case that the juveniles' brains were still developing, these 'juvenile' squirrels were nearing full adult size and sexual maturity (Table 1, Koprowski 1994). Indeed, for the chipmunks, the 'juveniles' had already reached full adult size at the time of capture. In chipmunks, although the densities of proliferating cells did not differ between age classes (Fig. 2), juveniles did have higher densities of young neurons than adults (Fig. 5), suggesting increased rates of cell survival. This would be consistent with data obtained in the laboratory indicating that learning increases cell survival rates (Ambrogini *et al.* 2000; Cecchi 2001; Gould *et al.* 1999b). We therefore suggest that the increased proliferation rates, including increased densities of young neurons in juveniles, reflect the need for young animals to learn more new spatial information about their environment than do adults. To survive, juveniles must learn not only food storage sites but all spatial aspects of their home range that adults already know: where cover is, where conspecifics reside and what sites to avoid to minimize predation risk. Juvenile mammals normally suffer much higher mortality rates than adults (e.g. Byrom & Krebs 1999; Paradis *et al.* 1993).

Second, there may be species-specific differences in the cell turnover rate in the hippocampus. Proliferating cells in the SGZ of squirrels may be maturing at a faster rate than those in chipmunks. In that case, DCX may be present in the young neurons for a shorter time in the squirrels than in the chipmunks. This could also cause the differences between adults and juveniles in densities of cells expressing DCX. If DCX is present for a longer period of time in cells of younger brains than in cells of older brains, then juveniles would have more cells expressing DCX than would adults. Though this explanation is plausible, there is no data available at present to assess it.

Third, the higher density of actively cycling cells in the SGZ of the squirrels may be due to an increased production of glia, rather than neurons. The gray squirrel is an arboreal species, whereas the chipmunk is primarily terrestrial. Thus, the squirrels may be more prone to minor physical damage, for example from falls, than the chipmunks and therefore need additional glia to help in removing damaged cell fragments from the brain. Such increased gliogenesis in response to inflammation has been demonstrated in mice (Vallières *et al.* 2002) and rats (Ekdahl *et al.* 2003; Monje *et al.* 2003). This seems unlikely, because the large majority of cells generated in the SGZ go on to become neurons rather than glia (Cameron *et al.* 1993; Palmer *et al.* 2000). Additionally, there is evidence that astrocytes can generate neuronal precursors (Alvarez-Buylla *et al.* 2001; Seri *et al.* 2001), and so the distinction between neuronal precursors and glial precursors generated in the SGZ may not be meaningful. Finally,

the proliferation of precursor cells, but not glia, is increased after mechanical injury to the brain (Dash *et al.* 2001; Gould & Tanapat 1997; Kernie *et al.* 2001; Rice *et al.* 2003).

Because continued adult hippocampal neurogenesis is related to spatial memory abilities in the laboratory, we predicted that adults in free-living populations should not show an age-related decline in densities of proliferating cells or young neurons in the hippocampus. Our findings only partially bear out this prediction. We found in chipmunks a significant age-related decline in the proportion of young neurons in the DG, and we found in squirrels a significant age-related decline in the proportion of proliferating cells in the DG. There are two explanations to account for the lack of consistency in age-related declines. First, there may be mechanisms in free-living animals that maintain neurogenesis rates throughout adulthood. Such mechanisms could include the complexity of the environment, because age-related declines in numbers of young neurons in laboratory rats can be countered by enriching their normal, 'impoverished' living conditions or allowing them the opportunity to engage in physical activity (Kempermann *et al.* 1998b; Kempermann *et al.* 2002; vanPraag *et al.* 1999). Free-living animals normally live in such highly 'enriched' environments, and so would be expected to maintain densities of young neurons throughout their adult lives. Environmental enrichment in the laboratory primarily affects survival, rather than proliferation rate, of proliferating hippocampal cells (Kempermann *et al.* 1998b). In our study, therefore, environmental enrichment could account for the lack of age-related decline in young neurons in squirrels. However, living in an 'enriched' environment may not have the same effect on all free-living species, because the chipmunks in our study showed an age-related decline in densities of young neurons.

Unlike laboratory animals, free-living species have been continually subjected to natural selection and thus may also possess internal mechanisms that maintain an optimal proliferation rate or pool of proliferating cells. This may be the case in the chipmunks in this study, who showed no evidence of an age-related decline in proliferating cell densities. However, the squirrels in our study showed a negative relationship between proliferating cell density and age. This would run counter to our prediction that free-living animals should not show such a decline if individuals rely on the new cells for spatial learning and, ultimately, survival. However, such results are consistent with evidence that it is the survival of new cells rather than the proliferation rate that relates to spatial learning (Ambrogini *et al.* 2000; Gould *et al.* 1999b).

Second, any animal in the wild that did indeed experience an age-related decline in its neurogenesis rate may not survive. Thus, taking a cross-section of survivors, as we did, would not detect the decline observed in captive populations. If neurogenesis is involved with an ability that increases survival, e.g. locating vital food stores, then a decline in this ability would decrease survival, removing animals from the population as their neurogenesis rates

decreased. The cost of a declining rate of neurogenesis should be more serious for scatter-hoarding squirrels, because accessing multiple storage sites is vital to their survival (Thompson & Thompson 1980), than for the larder-hoarding chipmunks which always use the same storage site. Our results show that chipmunks experience an age-related decline in survival rates of proliferating hippocampal cells. Adult squirrels do not show such an age-related decline in young neurons, despite the age-related decline in proliferating cells discussed above. A similar reaction to decreased numbers of precursor cells in the DG has been shown in young rats (Ciaroni *et al.* 2002), and our results suggest that aging squirrels maintain this ability to compensate for the loss of neuronal precursors. Because squirrels appear to be maintaining their pool of young neurons as they age, which chipmunks are not, these young neurons may be required for learning, storing or recalling the large numbers of food cache locations on which squirrels rely for survival.

It is not presently possible to determine an individual's neurogenesis rate without sacrificing the animal, so it is not possible to determine future survival rates of animals with intrinsically different neurogenesis rates. It is possible with laboratory animals to use various cognitive tasks to test individuals on various abilities and attempt to relate their performance to subsequent neurogenesis rates (Bizon & Gallagher 2003; Drapeau *et al.* 2003; Merrill *et al.* 2003). However, because learning affects hippocampal neurogenesis (Ambrogini *et al.* 2000; Gould *et al.* 1999b), testing itself may affect subsequent neurogenesis rates.

In summary, this study provides evidence for the presence of adult neurogenesis in wild species of rodents. The complexities of the natural environment, combined with the shortcomings of the available methodologies, preclude definitive conclusions with respect to functional significance of neurogenesis in the behaviors relying on spatial memory. However, the emerging picture points to significant differences in certain parameters of neurogenesis among species that differ in food-storing strategies.

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