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Hippocampal neurogenesis in food-storing red squirrels: the impact of age and spatial behavior

K. M. Johnson[†], R. Boonstra[†,‡] and J. M. Wojtowicz*,[†]

[†]*Department of Physiology, University of Toronto, Toronto, Ontario, Canada, and* [‡]*Department of Biological Sciences, Centre for the Neurobiology of Stress, University of Toronto Scarborough, Toronto, Ontario, Canada*
*Corresponding author: Dr J. M. Wojtowicz, Department of Physiology, University of Toronto, Toronto, Ontario, Canada M5S 1A8. E-mail: martin.wojtowicz@utoronto.ca

The adaptive significance of adult hippocampal neurogenesis remains unknown. In the laboratory, it is influenced by a variety of environmental and physiological stimuli. In the wild, it may be influenced by the reliance on spatial memory and by environmental stressors. The one common denominator in both settings is that neurogenesis declines markedly with age. Red squirrels are long-lived rodents that store food (thousands of tree cones) to permit survival under harsh winter conditions. We compared a population from the eastern North America that stores its cones singly or in small clusters with one from the west that stores its cones in large stockpiles. The reliance on spatial memory should be much greater in the east than the west, and should not decline with age, as cone storage and recovery is a yearly necessity. We found no difference between the populations in the density of young neurons and both populations showed the same decline with age. Thus, we reject the spatial memory hypothesis for adult neurogenesis in mammals in its original form. Instead, our evidence is consistent with the neurogenic reserve hypothesis in which neuronal cell production early in life leads to enhanced hippocampal function later in life according to environmental demand but without requirement for ongoing cell production as a function of site- and species-specific needs. A more general interpretation of the data leads us to consider a possible role of neurogenesis in novel, flexible episodic memories.

Keywords: Dentate gyrus, food hoarding, neurogenic reserve, population differences, spatial memory

We have known about the formation of new neurons in the dentate gyrus of the hippocampus of adult mammals for over 45 years. It has been argued that it is a universal trait in mammals (Amrein *et al*. 2008) and other vertebrates (Lindsey & Tropepe 2006). In spite of the enormous advances in our understanding, we still have no consensus on the adaptive significance of these new neurons. The hippocampus is a brain region critical for learning and memory (Squire 1992) and undeniably, when neurogenesis occurs there, it must be involved in memory and ultimately memory must serve to enhance survival and reproduction of adult animals in the natural world. However, there are three major unknowns in our understanding: first, what is the nature of the memories that require these new neurons; second, why can most mammals get along with fewer of them as they age; and third, why can some adult mammals get along without them altogether (i.e. bats – Amrein *et al*. 2007)? In this study, we tackle the first two of these unknowns and use the results to give insight into the third.

One of most consistent findings in the study of neurogenesis in the dentate gyrus has been its dramatic decline with age (Kempermann 2008a). In laboratory mice and rats, the decline in the rate of proliferation has been from 60 to 95% as the animal ages from a juvenile to middle age (Heine *et al*. 2004; Kuhn *et al*.1996; McDonald & Wojtowicz 2005). In the laboratory, animals are maintained in protected environments with unlimited access to food and water and without predators and thus the costs of reduced neurogenesis at older ages may not be apparent. Free-living animals have no such security, being faced daily with the need both to find food and to avoid becoming it. In spite of this, a similar or even more severe decline has been seen in the natural world (Barker *et al*. 2005; Bartkowska *et al*. 2008) and in humans (Manganas *et al*. 2007). In wild rats, the decline in both proliferation and young neuron survival from 35 days of age to 200 days is about 85–90% (Epp *et al*. 2009).

Hippocampal memory and neurogenesis in the dentate gyrus may be related to spatial, episodic or contextual memory (Cameron & Christie 2007; Leuner *et al*. 2006). Studies of food cache storage in free-living birds were the first to implicate the spatial functional role for neurogenesis in the natural world (Smulders 2006). In the black-capped chickadee (*Parus atricapillus*) seasonal peaks in neurogenesis in the autumn were associated with peaks in food storage (Barnea & Nottebohm 1994) and spatial memory associated with caching behavior correlates with neurogenesis (LaDage *et al*. 2010). Caching involves storage of food at one or many locations for subsequent recovery and it is found in both birds and mammals (Smith & Reichman 1984). In both classes, species are found that larderhoard (storage of large amounts of food in one or a few areas of the home range) or that scatterhoard (storage of one or a few food items in each hoarding site and distributing hundreds

or thousands of these hoards throughout the home range). In mammals, tree squirrels are amongst the best studied scatterhoarding group and evidence indicates that they use primarily spatial cues to recover their caches (Jacobs & Liman 1991; Lavenex *et al*. 1998; MacDonald 1997; McQuade *et al*. 1986). All squirrels rely on recovery of these hoards to survive winter and hence there should be strong selection pressure on the mechanisms that favor spatial memory. It is uncertain if aging squirrels relearn the locations of food caches in scatterhoarding populations and species every year or if they recall some of the long-term information from previous years. Laboratory studies on rats and mice suggest possible influence of neurogenesis during memory acquisition (Drapeau *et al*. 2003; Dupret *et al*. 2007) and/or during recall of long-term memories (Imayoshi *et al*. 2008; Snyder *et al*. 2005). Based on this evidence, we hypothesize that neurogenesis and spatial learning and memory will be related in the natural world. To test this hypothesis, we use a within species comparative approach to understand how neurogenesis changes in two populations of wild red squirrels (*Tamiasciurus hudsonicus*).

There are major geographic differences in how red squirrel populations store food. In western North America they primarily larderhoard, creating large mounds (middens) of spruce and pine cones that are used as an overwinter food storage sites and used for multiple generations; thus they do not have to relearn new storage locations (Boutin e*t al*. 2006; Gurnell 1984; Smith 1968; Streubel 1968). In contrast, in eastern North America, they primarily scatterhoard, burying their cones usually singly or in small clusters with the need to remember up to 13 000 locations (Dempsey & Keppie 1993; Hurly & Robertson 1987, 1990; Hurly & Lourie 1997). Thus, western red squirrels need to remember only a few storage locations whereas eastern squirrels need to remember large numbers of locations. Our study rests on the assumption that the red squirrels in these two areas have inherent differences with respect to their spatial abilities that are then reflected in differences in hippocampal neurogenesis. Virtually all species are collections of genetically differentiated populations that occur because they evolve to deal with unique environments (either with respect to the other species living in the different sites or climatic differences) (reviewed in Thompson 2005). For example, in chickadees along a gradient from Alaska to Kansas in western North America, Roth and Pravosudov (2009) have recently shown that hippocampal volume and neuron number are directly correlated to environmental harshness. Thus, red squirrels in eastern North America are proposed to have coevolved with their primary food to successfully exploit the mixed coniferous-deciduous forest with several cone bearing tree species whereas those in the west have coevolved with the more uniform coniferous forests with primarily one cone bearing tree species. We make two predictions if adult hippocampal neurogenesis is needed for the spatial memory of food storage locations. First, we predict that eastern red squirrels should have much higher rates of neurogenesis (proliferation and survival) than western squirrels during the late summer when cones are being clipped and stored. Second, given that red squirrels are long lived and that each year throughout life there is a need to remember where summer–autumn cones are buried, we

predict that the densities of these cell types will not decline with age in eastern squirrels that scatterhoard whereas it will decline in western squirrels that larderhoard as the location of the middens of the latter remains constant from 1 year to the next.

Material and methods

Natural history
Red squirrels are the most widespread food hoarding mammal in North America, occupying a wide range of habitats with different plant assemblages. They occupy the entire coniferous boreal forest from Alaska to the Atlantic as well as the mixed coniferous–deciduous forests stretching south along both sides of the continent – in the west along the mountains to Arizona and New Mexico and in the east along the Appalachians to North Carolina (Steele 1998). They are medium-sized (*ca*. 250 g), long-lived (up to 8 years), diurnal rodents specializing in seeds from the cones of spruce and pine. They defend individual, nonoverlapping territories year around and these protect their conifer seed stores (which are usually buried under snow) that are essential for overwinter survival (McAdam *et al*. 2007).

Collections sites and trapping methods
In western Canada, we collected squirrels east of Kluane Lake in the southwestern Yukon Territory (61°N, 138°W) from 4 to 21 August 2004. The boreal forest in this region has one coniferous species – white spruce – and one deciduous tree species. The climate is cold continental with a short growing season (mid-May through mid-August) and snow cover from October through early May. The mean monthly temperatures for the warmest (July) and coldest (January) are 12.8 and −21.9°C, respectively, and total annual precipitation averages 28.4 cm/year (mostly snow) (Boonstra *et al*. 2008). In eastern Canada about 4300 km away, we collected 15 squirrels in Algonquin Provincial Park, Ontario (48°N, 78°W) from 4 to 9 August 2006. The park is between the Great Lakes and St Lawrence Forest Region, a mixed forest with at least 6 coniferous species (white and black spruce, eastern white pine, red pine, balsam fir and eastern hemlock) and 10 deciduous tree species. The area is snow covered from mid-November to April. The mean monthly temperatures for the warmest (July) and coldest (January) are 17.9 and −12.5°C, respectively, and total annual precipitation averages 79.9 cm/year (evenly distributed throughout the year) (Falls *et al*. 2007). Both sites have up to a meter or more of standing snow in winter. Our collection of red squirrels at both sites occurred when they were beginning to cut the cones from the coniferous trees and store them before they opened up and shed their seeds.

In the west, 6 juveniles (three males: three females) and 9 adults (4:5) were captured and in the east, 10 juveniles (7:3) and 5 adults (3:2) were captured. They were captured in Tomahawk live-traps (14 × 14 × 40 cm or 16.5 × 16.5 × 48 cm; Tomahawk Live Trap Company, Tomahawk, Wisconsin, USA) baited with peanut butter. In the west, traps were set on or near each squirrel's midden between 0730 and 0930 h and checked every 1–1.5 h until 1200 h. In the east, traps were set on the ground near stands of coniferous trees between 0600 and 0800 h and checked before 1200 h. At both sites, the animals were naïve and had not previously been trapped. Trapped squirrels were covered with a burlap bag, transported to a central site and allowed 1 h to habituate prior to processing. Squirrels from both populations were removed from the traps into a mesh handling bag, weighed to the nearest 5 g using a 500 g Pesola scale and subjected to a hormonal stimulation test to assess the responsiveness of the hypothalamic–pituitary–adrenal axis (Boonstra and McColl 2000). This research was approved by the University of Toronto Animal Care Committee in accordance with the guidelines of the Canadian Council for Animal Care.

Animal processing
At completion of the stimulation test, animals were sacrificed by an overdose of halothane (Halocarbon Laboratories, River Edge,

NJ, USA) and brain tissue collected rapidly. The left hemisphere was immersed in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffered saline (PBS) for 48 h and then transferred into 0.1 M PBS with 0.2% (w/v) sodium azide and stored at 4°C until analysis of neurogenesis. An autopsy was performed on all animals to assess age. We distinguished adult squirrels (those that had bred) from juveniles (prereproductives) by testes weight in the males (juveniles < 100 mg, adult > 100 mg) and by the presence of uterine morphology and uterine placental scars in females. Juvenile females had a stringy, thin uterus (1–2 mm diameter) and no placental scars; adults had larger, flaccid uterus (>2 mm diameter) and evidence indicative of previous pregnancy such as scars, corpora lutea and/or its breakdown product corpora albicantia in the ovary. Juvenile females usually, but not always, mature in their second summer at the age of 10–12 months old. In the Yukon, some delay until their third summer and thus one of the juveniles may have been older than 10–12 months.

Histological analysis
Immunohistochemistry
The hippocampus of each brain was dissected and cut into three roughly equivalent sized blocks, along the dorso-ventral axis. The blocks were sectioned exhaustively into 30-μm thick sections, which were stored at 4°C in 0.1 M PBS with 0.2% (w/v) sodium azide until processed. Proliferating cells were detected using Ki-67, a protein expressed in cells during mitosis. Immature neurons were detected using doublecortin (DCX) and post-mitotic, mature neurons using NeuN (Brown *et al*. 2003; Wojtowicz & Kee 2006). Immunohistochemical detection was performed on approximately every 24th tissue section from the dorsal third of the hippocampus, for a total of five sections per animal. This sampling was sufficient according to the criteria of West *et al*. 1991. According to this procedure, the variability, as measured by coefficient of error (CE), of the cell number among sections should be less than half the variability of average cells number among the animals. Our sampling scheme satisfied this criterion. We further verified validity of such sampling by doubling the number of sections (every 12th section) in a subset of animals (n = 3) in each group. Although CE was further reduced by increased sampling the means did not change significantly. Data with such increased sampling (n = 9–10 sections per animal) are included in the graph in Fig. 4 (see Supporting Information for details).

We focused our evaluation on the dorsal third of the hippocampus as previous experiments have suggested that the hippocampus is functionally subdivided along the septo-temporal axis into two distinct regions; the dorsal being preferentially involved in certain forms of learning and memory, including spatial memory, and the ventral region is involved in anxiety-related behaviors (Bannerman *et al*. 2004; McDonald *et al*. 2006; Moser *et al*. 1995).

All immunohistochemistry was performed on free-floating sections. Initially sections were washed for 3 × 10 min with 0.1 M PBS (pH 7.4). Tissue was then transferred to the primary antibody solution for an 18-h incubation at room temperature, on a rotating table. Following incubation, sections were again washed in 0.1 M PBS (pH 7.4) for 3 × 10 min. To visualize the immunoreactivity, sections were then transferred into secondary antibody solution and incubated in the dark for 2 h at room temperature on a rotating table. Sections were then washed in 0.1 M PBS (pH 7.4) for 3 × 10 min, rinsed with distilled water, mounted on slides with Shandon Permaflour. Primary antibodies were diluted 1:200 and directly conjugated secondary antibodies were diluted 1:200, using 0.1 M PBS (pH 7.4) with 0.3% (v/v) Triton X-100 as a diluent in all cases. For Ki-67 staining, sections were labeled with a rabbit anti-Ki-67 primary antibody (Vector Laboratories Inc.; Burlington, ON, Canada) and an Alexa-568 goat anti-rabbit IgG secondary antibody (Molecular Probes; Eugene, OR, USA). For DCX staining, sections were labeled with a goat anti-DCX primary antibody (Santa Cruz Biotechnology; Santa Cruz, CA, USA) and an Alexa-488 donkey anti-goat IgG secondary antibody (Molecular Probes). For NeuN staining, sections were labeled with a mouse anti-NeuN primary antibody (Chemicon; Temecula, CA, USA) and an Alexa-488 goat anti-mouse IgG secondary antibody (Molecular Probes).

Additional technical details on immunohistochemistry, cell counting and estimation of the area of the granule cell layer (GCL) and

CA1 can be found in Supporting Information. Typical images contrasting neurogenesis in red squirrels from these two populations with these immunocytochemical markers are also placed in the Supporting Information repository.

Statistical analysis
All data are presented as mean ± 1 SE, unless otherwise stated. All data were analyzed with the software package SIGMASTAT 3.0 (Systat Software Inc.; San Jose, California, USA), except for the three-way analysis of variance (ANOVA) where we used STATVIEW 5.0.1 (SAS Institute, Cary, NC). All data were normally distributed (Kolmogorov–Smirnov test). All data were initially analyzed with three-way ANOVA for factors sex, age (adult vs. juvenile) and population (east vs. west). As there was no effect of sex on any of the measures, data from the two sexes were pooled and analyzed with two-way ANOVA (age × population). In the experiments comparing dorsal and ventral hippocampal regions, three-way ANOVA (age × population × region) was used. Post hoc *t*-tests on significant effects and interactions were performed using the Holm–Sidak method. All cell numbers (Ki-67 and DCX) were expressed as numbers per volume of the GCL and numbers per length of the subgranular zone (SGZ). The SGZ is a narrow tissue zone underneath the GCL in which precursor stem cells divide and differentiate prior to incipient neurons migrating toward the GCL. We give P values between 0.10 and 0.05 and infer that these may be biologically, although not statistically, significant, possibly because of reduced power because of sample sizes in some of our results (Yoccoz 1990).

Results

Eastern and western animals differed with regard to their body mass. The western animals were larger and both populations showed significant differences between the age groups. A two-way ANOVA on body mass (age × population) indicated population effect ($F_{1,26} = 167.7$, $P < 0.001$), an age effect ($F_{1,26} = 70.36$, $P < 0.001$) and no interaction effect ($F_{1,26} = 0.34$, $P = 0.56$). Overall, the western squirrels weighed 59% more than the eastern squirrels (western = 227 ± 7.4 g, eastern = 143 ± 6.5 g) and adults weighed 42% more than juveniles (adults = 219.9 ± 36, juveniles = 154.5 ± 10.1 g). This pattern was similar in both age groups (adults: western = 244.2 ± 4.9, n = 9, eastern = 176.0 ± 2.9, n = 5; juveniles: western = 201.2 ± 9.9, n = 6, eastern = 126.5 ± 2.6, n = 10). Each group included approximately equal number of males and females. Initial analysis showed no significant effects of gender in any of the measures used in this study so males and females were combined in the reported data.

Two essential measures of adult neurogenesis are the rate of proliferation and the rate of cell differentiation into neurons. Because this is the first report of neurogenesis in red squirrels, we used densities of cells per volume of the GCL to make our results comparable to other species (Amrein *et al*. 2008). A two-way ANOVA (age × population) on proliferating cell densities (Ki-67) indicated an age effect ($F_{1,26} = 69.9$, $P < 0.001$), no population effect ($F_{1,26} = 0.13$, $P = 0.73$) and no interaction effect ($F_{1,26} = 2.56$, $P = 0.12$) (Fig. 1a). The age effect occurred because adults had 80% fewer cells per unit volume than juveniles (adults = 4474 ± 785 mm³, juveniles = 22 151 ± 1785 mm³). A two-way ANOVA on differentiating cell densities (DCX) indicated an age effect ($F_{1,26} = 60.12$, $P < 0.001$), no population effect ($F_{1,26} = 0.31$, $P = 0.58$) and no interaction
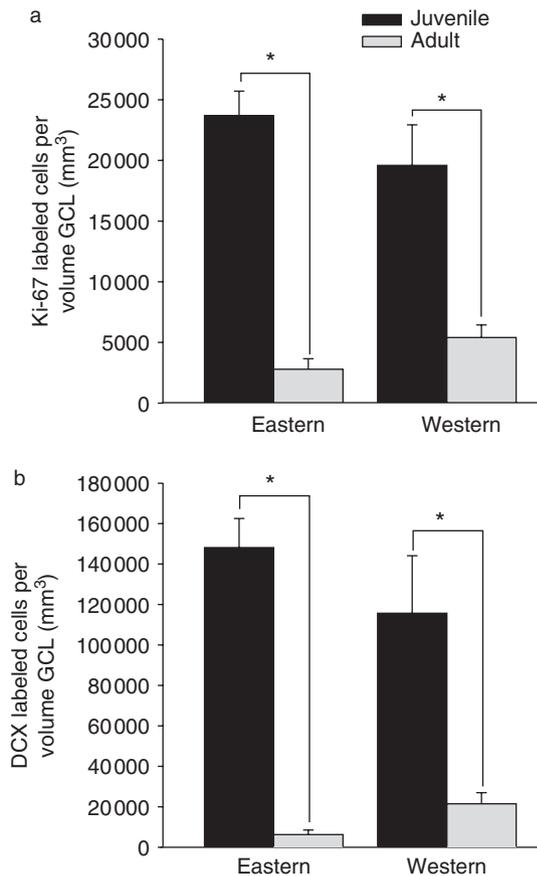
Figure 1: Effect of age (juvenile versus adult) and area (eastern versus western North America) on proliferating cell densities (Ki-67) (panel a) and on differentiating cell densities (DCX) (panel b) in the granule cell layer (GCL) of the dentate gyrus of the hippocampus of red squirrels. Asterisks indicate statistically significant differences as explained in the text.

effect ($F_{1,26} = 2.41$, $P = 0.13$) (Figs 1b and 2). The age effect occurred because adults had 88% fewer cells per until volume than juveniles (adult = 16 032 mm^3 ± 4087 mm^3, juveniles = 135 672 ± 13 399 mm^3). Neither Ki-67 nor DCX densities differed between east and west populations (see Supporting Information for representative Ki-67 images).

As an approximate measure of mature cell density, we used the GCL volume obtained by staining with NeuN, a marker of mature neurons (Wojtowicz & Kee 2006). An increase in mature neurons is expected to be most pronounced in adults because of the accumulation of cells over time. A two-way ANOVA on GCL area (age × population) indicates a marginal age effect ($F_{1,26} = 3.59$, $P = 0.07$), a population effect ($F_{1,26} = 4.44$, $P < 0.05$) and no interaction effect ($F_{1,26} = 1.27$, $P = 0.27$). The population effect was due exclusively to eastern adults having 31% larger area than western adults, with juveniles in the two populations being similar (Fig. 3a). Moreover, eastern adults had 29% larger

areas than eastern juveniles (Fig. 3a, Supporting Information, Fig. S2), whereas both age groups were similar in western animals. In contrast, the length of the SGZ, which contains the region giving rise to new neurons in dentate gyrus adjacent to GCL, was unchanged (Fig. 3b). Consequently, the change in GCL was because of its increased width. Indeed, analysis on widths of the GCL (age × population) indicated no age effect ($F_{1,26} = 1.57$, $P = 0.22$), a population effect ($F_{1,26} = 10.5$, $P < 0.01$) and a marginal interaction effect ($F_{1,26} = 3.87$, $P = 0.06$). A post hoc analysis showed that eastern adults had 25% larger GCL width than western adults ($t_{12} = 3.55$, $P < 0.01$), but the juveniles were similar in the two populations ($t_{14} = 0.94$, $P = 0.36$). In addition, eastern adults had 14% larger width than eastern juveniles ($t_{13} = 2.10$, $P < 0.05$), but western adults and juveniles were similar ($t_{13} = 0.52$, $P = 0.61$). These effects are illustrated in Fig. 3c.

If enhanced GCL volume is the ultimate result of enhanced neuronal recruitment in the eastern population why is this recruitment not reflected in higher rates of proliferation and or neurogenesis? We suggest that the differences are masked by a common method of data standardization per volume of GCL. Although the norm in the literature, such expression of densities per GCL volume, can be misleading if GCL grows or shrinks during animal's life. To correct this, we offer an alternative method of expressing cell densities per length of SGZ, a narrow tissue region underneath the GCL, where cells divide and differentiate prior to migrating toward the GCL. In fact, the number of immature neurons (DCX) calculated per length of SGZ was 38% larger in eastern than western juveniles ($t_{14} = 2.45$, $P = 0.02$). Similarly, eastern juveniles had 29% more proliferating cells per length than western juveniles, $t_{14} = 2.09$, $P < 0.05$. All values for GCL areas and SGZ lengths were obtained in dorsal hippocampal subregion except for data reported in Fig. 4.

In contrast to the dentate gyrus, the CA1 field (an area with no adult neurogenesis) showed no significant changes in cell layer areas stained for NeuN with age (Fig. 3d). A two-way ANOVA on area of the CA1 (age × population) indicated no age effect ($F_{1,26} = 1.04$, $P = 0.32$), a population effect ($F_{1,26} = 4.6$, $P = 0.04$) and no interaction effect ($F_{1,26} = 0.07$, $P = 0.80$). However, when t-tests were deployed on each age group independently, the populations were found to be similar both between the juveniles $t_{13} = 1.8$, $P = 0.08$) and the adults ($t_{10} = 1.26$, $P = 0.22$). A trend toward western population having larger CA1 field was expected given their larger size.

Our data support the concept that adult neurogenesis is a species-specific phenomenon. The numbers of proliferating (Ki-67) and immature DCX-labeled neuronal cells are several-fold higher in squirrels than in rats of the same age. Squirrel juveniles were estimated to be about 5–6 months old when they were trapped. In the combined (east and west) population, the numbers of Ki-67+ cells per mm^3 GCL were 22 151 ± 1785 and those of DCX+ young neurons were 135 672 ± 13 399. These values compare to 3100 and 6085 cells reported for 5-month old rats (Epp et al. 2009). The exact age of adult squirrels is not known but the animals must have been at least 18 months old. The Ki-67 and DCX densities in adult squirrels were 4474 ± 785 and 16 032 ± 4087, respectively. Although wild rats do not generally survive this
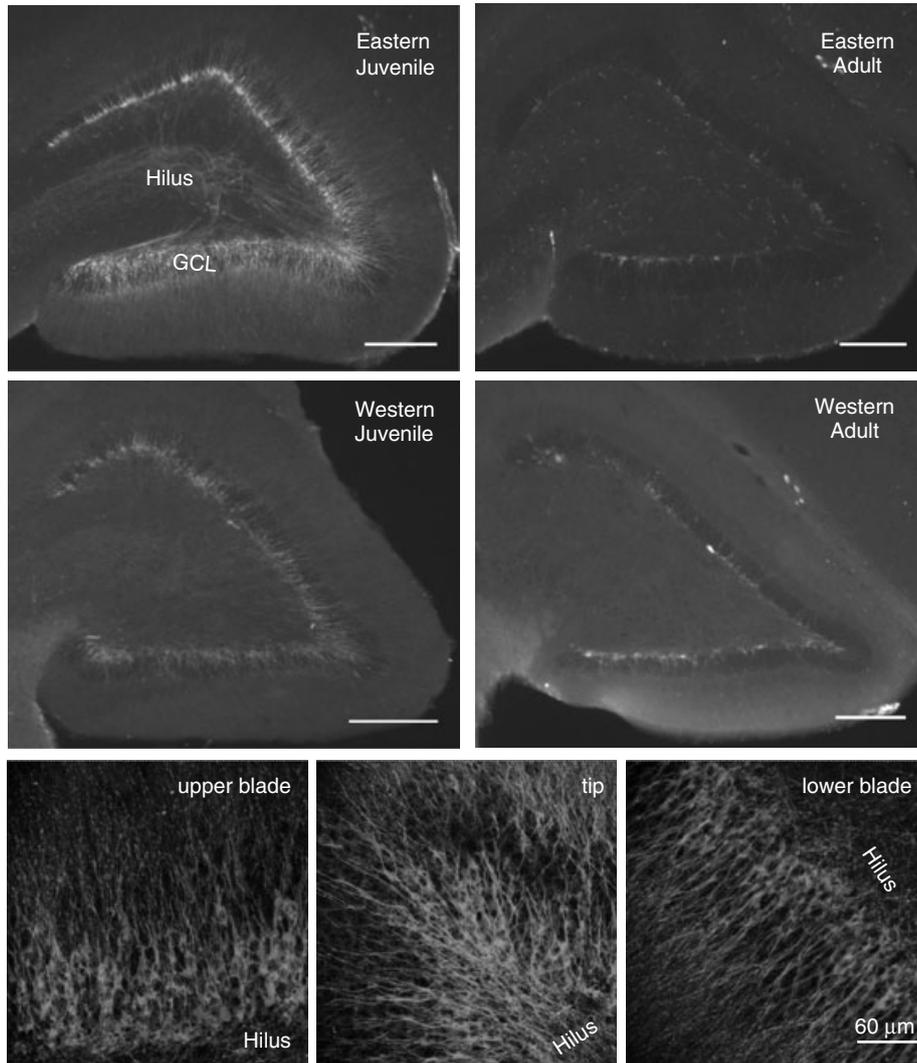
Figure 2: Typical images representing sections stained for differentiating cells (DCX) in red squirrels. Note DCX-positive cells lining the inner edge of granule cell layer (GCL) with dendrites projecting towards the molecular layer and axons streaming towards the hilus. There were typically over 1000 cells/section in the juveniles and 100 cells/section in the adults. Scale bar: 0.25 mm. Confocal images in the bottom row are taken from the upper blade, the tip and the lower blade of the dentate gyrus in the eastern juvenile animal illustrated above. Typically, the DCX-positive neurons occupied the bottom half of the granule cell layer.

long (Davis 1948), the values seen in 200-day-old animals kept in the laboratory are only 2000 and 3000, respectively (J.M.W. unpublished data). Another way to analyze the age effects in the two species is to compare squirrel juveniles to rat juveniles. The rat juveniles, at 35 days after birth and just prior to sexual maturity, express 11 800 Ki-67+ cells/mm^3 and 33 000 DCX+ cells/mm^3 (Epp *et al*. 2009).

Our study concentrated on dorsal hippocampus according to dominant evidence for preferential involvement of this hippocampal subregion in spatial behaviors (Bannerman *et al*. 2004). A few studies suggest that ventral hippocampus contributes to some aspects of spatial memory such as processing of large spatial environments (Kjelstrup *et al*.

2008). We compared key findings of the present study on a representative sample from all four groups of animals to assess relative effects in dorsal and ventral hippocampus in the same individuals. Three-way ANOVA (hippocampal region × population × age) showed a significant effect of hippocampal region ($F_{1,20} = 9.03$, $P < 0.008$), an effect of age ($F_{1,20} = 53.62$, $P < 0.001$) but no effect of population ($F_{1,20} = 1.43$, $P = 0.25$). Thus the results suggest that spatial behavior of the animals had no effect on neurogenesis in the dorsal or ventral hippocampus. Qualitative assessment of the numbers of DCX-label suggested similar conclusions (not shown). There were no interactions between hippocampal region and population, or between age and population,
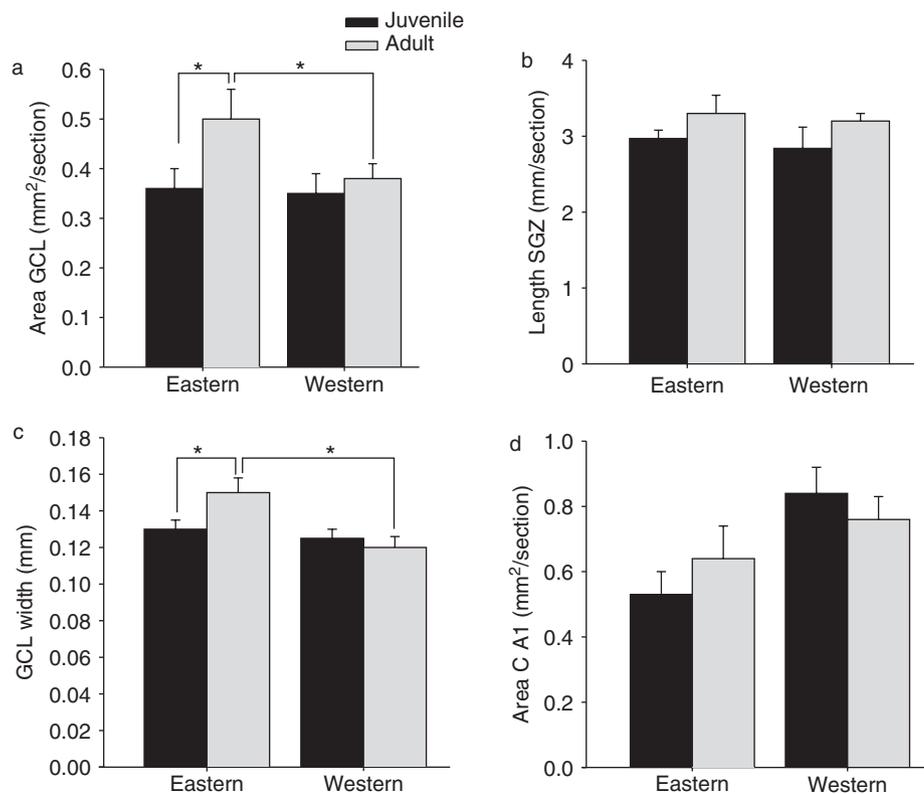
Figure 3: Effect of age (juvenile versus adult) and population (eastern versus western North America) on several parameters in dentate gyrus sections stained for a neuronal marker of post-mitotic, mature neurons (NeuN) in the granule cell layer (GCL). (a) GCL area; (b) Subgranular zone (SGZ) length; (c) GCL width; (d) CA1 area. Asterisks indicate statistically significant differences as explained in the text.

but there was a significant interaction between age and hippocampal region ($F_{1,20} = 9.17$, $P < 0.008$). In accordance with the data obtained in rats (Snyder *et al*. 2009b) the dorso-ventral gradient of neurogenesis is greatly diminished in adult squirrels in comparison to juveniles (hippocampal region × age interaction was significant). This may suggest reduced functional differentiation between these two hippocampal subregions with aging (Snyder *et al*. 2009b). To deal with the interaction, we pooled populations and did a two-way ANOVA on age × hippocampal region. With this approach, there was a significant effect of age ($F_{1,20} = 55.92$, $P < 0.006$), of region ($F_{1,20} = 9.41$, $P = 0.006$) and of the interaction between the two ($F_{1,20} = 9.56$, $P = 0.006$). As shown in Fig. 4, neurogenesis in ventral region of squirrel hippocampus was less robust and this is in agreement with similar observations on rats (Snyder *et al*. 2008).

Discussion

Our evidence on red squirrels provides no support for the hypothesis that the function of adult neurogenesis is spatial memory (Figs 1–3). The field evidence that the eastern red squirrels scatterhoard whereas the western ones larderhoard is clear. How they actually find their food is not known, and

we are assuming that some form of spatial memory must be occurring as it does in other squirrel species. We did not find significantly higher densities of either proliferating cells (Ki-67 labeling) or immature neurons (DCX labeling) in eastern squirrels than western ones in spite of higher spatial memory demands of the eastern squirrels. Thus, adult red squirrels must be able to meet the spatial demand of their environment without relying on the birth of new neurons from the dentate gyrus. Moreover, contrary to our prediction, there was an equivalent decline of neurogenesis with age in both eastern and western squirrels. These results lead us to suggest that adult animals can locate food caches on the basis of long-term memories from past experiences which can be recalled without ongoing neurogenesis. The sharp decline of neurogenesis with age was a surprise as we previously found a more gradual decline in gray squirrels and chipmunks (Barker *et al*. 2005). Our results do not preclude a role of neurogenesis in some aspects of spatial learning. Detailed laboratory studies provide support for subtle contribution of new neurons in flexible learning strategies and in contextual learning (Garthe *et al*. 2009; Wojtowicz *et al*. 2008). These aspects could provide natural advantage to young animals, especially in eastern population.

The net increase in the size of GCL layer in eastern adults supports the idea of neurogenic reserve (Kempermann
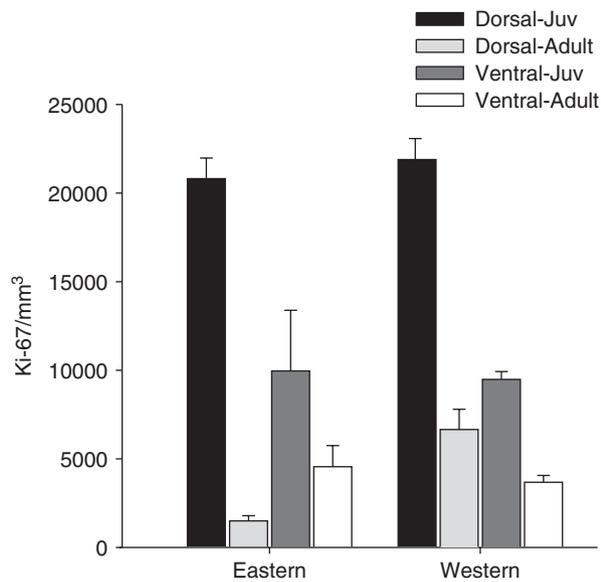
Figure 4: Comparison of Ki67 cell densities in a representative sample from dorsal and ventral hippocampal subregions. There were significant effects of the hippocampal subregion and age but no effect of population.

2008b). Functionally, more granule neurons could provide greater capacity for dealing with and learning of many similar events for animals in the east. According to experimental and modeling data, the accepted understanding of the role that dentate granule neurons play in learning is to acquire distinct memories of similar events (Becker 2005). This function is believed to deteriorate with aging (Wilson *et al.* 2006) and the enhanced neurogenic reserve in eastern adults may be adaptive in countering such age-related decline.

Thus, in light of our evidence, we propose an alternative hypothesis, which states that neurogenesis plays a long-term role in recruitment and accumulation of granule cells during adulthood. Accumulated neurons would constitute a neurogenic reserve that could be used in recovery from injury or by providing a cognitive reserve analogous to cognitive reserve in aging human populations (Nithianantharajah & Hannan 2006; Valenzuela & Sachdev 2006). This pool of accumulated neurons may also explain the total lack of neurogenesis in bats (Amrein *et al.* 2007), with all neurons originating prior to birth, and presence of neurogenesis only in juveniles in shrews (Bartkowska *et al.* 2008), with the pool originating just after or prior to birth. Thus, in red squirrels, we predict that the population demonstrating higher rate of neurogenesis early in life would show larger overall neuronal numbers in adulthood. The larger neuronal numbers are expected to be manifest as an increase in GCL width because of radial translocation of newly generated neurons during adult neurogenesis (Crespo *et al.* 1986; Snyder *et al.* 2008).

Could we have missed enhancement of neurogenesis because of incorrect time of capturing the animals? Assuming that learning behavior stimulates neurogenesis over a time course of several weeks we anticipated that

cell proliferation (Ki67) and cell maturation (DCX) would be enhanced during caching which takes place during the course of August to September. Only a 1–2 week delay between cell birth and their stimulation by learning is expected (Gould *et al.* 1999). Thus, by trapping the animals during a 2 month period, we should observe changes in cell proliferation or maturation. However, we cannot exclude a possibility that there is an outburst of proliferation in the end of July that would be missed in our study.

It is also informative to compare ratios of immature neurons to proliferating precursors, i.e. DCX/Ki-67 ratios. Using this measure of neurogenesis, we obtained values of six for juveniles and four for adult squirrels from both eastern and western populations. These ratios are much higher than 1.7–2.8 ratios estimated in laboratory or wild rats (Epp *et al.* 2009). These data suggest a much higher yield of young neurons generated by relatively small pool of precursors in the squirrels than rats. Future studies should compare such ratios along with cell densities per SGZ length to emphasize differences among species. Alternative interpretation of the ratios is to consider them as measures of cell turnover. Larger DCX/Ki-67 may be an indication of relatively slower turnover rate of DCX-positive young neurons. This would indicate the increased availability of the young neurons in squirrels in comparison to rats. To facilitate future comparisons with other species, we present a table in Supporting Information (Table S1) with a summary of squirrel neurogenesis data. Absolute values of proliferation seen in our study may have been influenced by stressful procedures required to capture and handle the animals prior to decapitation (see section *Material and methods*). This in turn could have an effect on the DCX/Ki-67 ratios because DCX counts are not expected to be influenced by stress to the same extent (but see Snyder *et al.* 2009a). Nevertheless, the absolute values of Ki67 densities were much higher in squirrels in comparison to commonly reported values of cell proliferation in wild or laboratory rats (Epp *et al.* 2009; Heine *et al.* 2004). Thus, clearly, neurogenesis is more robust by all measures in squirrels in comparison to rats.

What could be the reasons for such large differences among species? Epp *et al.* (2009) suggest that in wild rats the levels of neurogenesis may reflect balance between the need for new neurons for behaviors such as spatial navigation and the suppression of neurogenesis by stress. Juvenile red squirrels should be under intense pressure during their first 6 months of life as they try to secure the critical territory that will permit overwinter survival. They are competing with adult territorial holders, who occupy the majority of the forest. Three-quarters of the juveniles in the Yukon fail to obtain a territory and die (McAdam *et al.* 2007). Thus, the higher level of neurogenesis in spite of stress may reflect a relatively higher demand for new neurons in the squirrels. Measurements of the stress response and its changes with age in natural populations in comparison to laboratory rodents await further studies (Heine *et al.* 2004).

In summary, we provide evidence for correlation between high spatial demands in eastern population of squirrels and the size of GCL within the hippocampal dentate gyrus. This relationship was robust in adults but not in juveniles. Considering high rate of new cell production in

eastern juveniles the larger GCL in eastern adults may be because of cell recruitment and accumulation during life span. Overall, the data are consistent and provide support for the neurogenic reserve theory proposed recently by Kempermann (2008b).

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Acknowledgments

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1

Figure S1: Typical images representing sections of dorsal hippocampus in red squirrels. Note that proliferating cell (Ki-67-expressing nuclei) line the subgranular zone (SGZ) indicated by dashed line. Larger puncta represent clusters of labeled nuclei. Granule cell layer (GCL) and hilus are indicated. Scale bar 0.25 mm.

Figure S2: Typical images of dentate gyrus stained for a mature neuronal marker (NeuN). The granule cell layer (GCL) is clearly delineated and shows characteristic 'bulges' in an eastern adult. Scale bar 0.25 mm.

Table S1: Summary of neurogenesis data

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Appendix to be included in supplementary materials repository (ESM)

Immunohistochemistry

All immunohistochemistry was performed on free-floating sections. Initially sections were washed for 3 x 10 minutes with 0.1M PBS (pH 7.4). Tissue was then transferred to the primary antibody solution for an 18-hour incubation at room temperature, on a rotating table. Following incubation, sections were again washed in 0.1M PBS (pH 7.4) for 3 X 10 minutes. To visualize the immunoreactivity, sections were then transferred into secondary antibody solution and incubated in the dark for 2 hours at room temperature on a rotating table. Sections were then washed in 0.1M PBS (pH 7.4) for 3 x 10 minutes, rinsed with distilled water, mounted on slides with Shandon Permaflour. Primary antibodies were diluted 1:200 and directly conjugated secondary antibodies were diluted 1:200, using 0.1M PBS (pH 7.4) with 0.3% (v/v) Triton X-100 as a diluent in all cases. For Ki-67 staining, sections were labeled with a rabbit anti-Ki-67 primary antibody (cat# VP-K451, lot 30113, Vector Laboratories Inc., Burlington, ON, Canada) and an Alexa-594 goat anti-rabbit IgG secondary antibody (cat# A11011, lot 40885A, Molecular Probes; Eugene, OR, USA). For DCX staining, sections were labeled with a goat anti-doublecortin primary antibody (cat# SC-8066, lotH1506, lot H1506, Santa Cruz Biotechnology; Santa Cruz, CA, USA) and an Alexa-488 donkey anti-goat IgG secondary antibody (cat# A11055, lot 43441A, Molecular Probes; Eugene, OR, USA). For NeuN staining, sections were labeled with a mouse anti-NeuN primary antibody (cat# MAB377, lot 24030356, Chemicon; Temecula, CA, USA) and an Alexa-488 goat anti-mouse IgG secondary antibody (cat# A11001, lot 45498A, Molecular Probes; Eugene, OR, USA).

Validity of sampling

We sampled tissue from dorsal one third of the hippocampus by taking 5 sections selected according to systematic random scheme (West et al., 1991, West, 1999). This represented 1 in 24 sections. To verify the validity of such sampling we compared coefficient of error (CE) among animals within each group with CE from 5 sections vs. 9-10 sections. Taking randomly chosen animals, (also included in Figure 4 in Results) we obtained the following values for intra- and inter-animal variations. Animal # 121 (Eastern Juvenile), CE=0.017 (n=5 sections), CE=0.019 (n=9 sections), CE=0.10 (n=10 animals); #122 (Eastern Adult), CE=0.10 (n=5 sections), CE=0.05 (n=9 sections), CE=0.36 (n=5 animals); animal# 61 (Western Juvenile), CE=0.06 (n=5 sections), CE=0.03 (n=10 sections), CE=0.12 (n=6 animals); animal #111 (Western adult), CE=0.04 (n=5 sections), CE=0.017 (n=9 sections), CE=0.19 (n=9 animals).
These measurements demonstrate that increasing number of sections usually, but not always, reduces values of CE, but 5 sections are well within allowable

limits. We conclude that intra-animal CE is much lower than inter-animal CE justifying the use of 5 sections per animal.

Note the same arguments apply to measurements of GCL areas.

Cell counting

The number of Ki-67 labeled cells was counted directly, under a fluorescence miscroscope with a 40X objective, from each of five Ki-67 stained sections from all thirty animals. Specifically, cells were counted if they resided in the granule cell layer (GCL) and/or the subgranular zone (SGZ) of the dentate gyrus of the hippocampus. The SGZ was defined as a two-cell width wide (approximately 20 µm) region just below the GCL. Although not strictly stereological, this method is not significantly biased by counting extra profiles at the cut edges of the sections. This is probably due to relatively low density of the cells within the sections and a procedure of avoiding counting at the edges of sections. To further reduce any possibility of bias we measured profile diameters in all 4 groups of animals and found no differences (data not illustrated). The number of DCX labeled cells was counted stereologically from each of five DCX-stained sections from all thirty animals using the optical dissector technique with a fractionator sampling method (West *et al.* 1991). Analysis was performed using a Leica DMR8 epifluorescence microscope equipped with a DVC colour digital camera, motorized x-, y-stage, motorized focus control and StereoInvestigator software (MicroBrightField Inc.; Williston, VT, USA) and cells were counted with a 63X oil immersion objective lens. The movements made by the stage were 100 µm along the x-axis and 100 µm along the y-axis for an x, y step area of 10,000 µm2. The x, y dimensions of the counting frame were 25 µm x 25 µm for area of 625 µm^2. Thus, the areal sampling fraction, a(frame) / a(x, y step), was set to 1/16. The dissector height (h) was set at 10 µm and cells within the first 5 µm of the section (guard zone) were not counted. The section thickness (t) was measured for each section on a Leica TCS-SL confocal microscope (Leica Microsystems (Canada) Inc.; Richmond Hill, ON, Canada) with a 63X objective lens and an average section thickness obtained for each animal. Thickness measurements were made roughly at the middle of each of the two GCL blades as well as at the apex, using NeuN stained sections. The number of DCX labeled cells was calculated per section by multiplying the total number of DCX labeled cells actually counted multiplied by the fraction of the thickness of the section sampled multiplied by the reciprocal of the area of the dentate gyrus sampled.

Dimensions of GCL and CA1

NeuN labeled sections were used to measure the dimensions of the GCL of the DG and the area of CA1 of the hippocampus. The area of the GCL and the length of the SGZ were measured from photomicrographs taken with a 4X objective lens, of each of five NeuN stained sections from all thirty animals, using

Image J Software. For measurements of the area of the GCL, the polygon selections tool was used to outline the outer edges of the granule cell layer, such that all NeuN labeled cells within the granule cell layer were contained within the borders outlined. For measurements of the length of the SGZ, the segmented line selections tool was used to outline the innermost border of the GCL, such that the line just touched the NeuN labeled cells in the GCL that border the hilus. The volume of the GCL was calculated for each section by multiplying the measured area of the GCL by the cut section thickness of 30 µm. The average width of the GCL was determined by dividing the measured area of the GCL by the measured length of SGZ for each section. The volume of the GCL and length of the SGZ measurements were also used to standardize the per section cell counts, accounting for any size differences among animals.

Five sections appear to provide sufficiently precise values for area measurements as shown by comparison of measurements on 5 vs. 10 sections and calculating coefficient of error. Performing such measurements on a subgroup of animals showed that CE among sections was much smaller than CE among animals.

To attribute size differences in the dentate gyrus to neurogenesis, we needed to determine if there were any size differences in the hippocampus between the populations and the age groups. Measurements of the regio superior (CA1) area of the hippocampus were chosen since this area is within the hippocampus itself and is non-neurogenic. The areas of the pyramidal cell layer of CA1 were measured from the photomicrographs, taken using a 4X objective lens, in each of five stained sections from each animal. CA1 area measurements were not obtained for 3 of the 30 animals (one eastern adult, one western juvenile and one western adult) due to tissue damage during hippocampal extraction and sectioning. Where multiple images were required to cover the entire CA1 area, images were superimposed to create a single complete image. For the purpose of this study the division between the regio inferior (CA3) and CA1 was identified based on the fact that perikarya and nuclei of the pyramidal cells of the CA1 are smaller than those of CA3, creating a pinching in the cellular layer (West *et al.* 1991). The division between CA1 and the subiculum was defined as the point in which the deeper cells of the layer of CA1 are no longer contiguous. For measurements of the area of CA1, the polygon selections tool was used to outline all of the NeuN labeled pyramidal cells of CA1 and a line perpendicular to the cellular layer was used as a division at the borders with CA3 and the subiculum.

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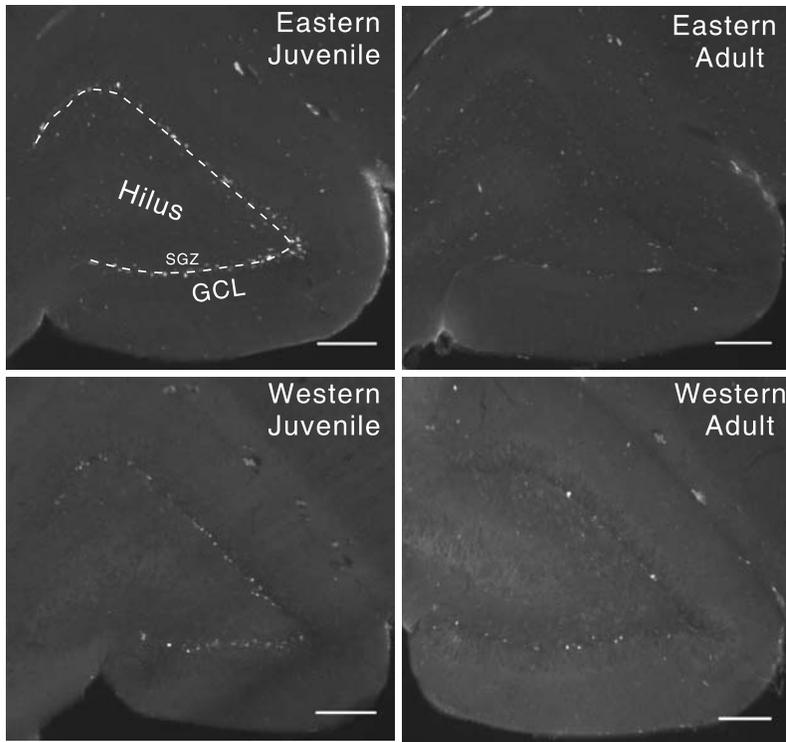
Supplementary Figure 1. Typical images representing sections of dorsal hippocampus in red squirrels. Note that proliferating cell (Ki-67-expressing nuclei) line the subgranular zone indicated by dashed line (SGZ). Larger puncta represent clusters of labeled nuclei. Granule cell layer (GCL) and hilus are indicated. Scale bar 0.25mm.
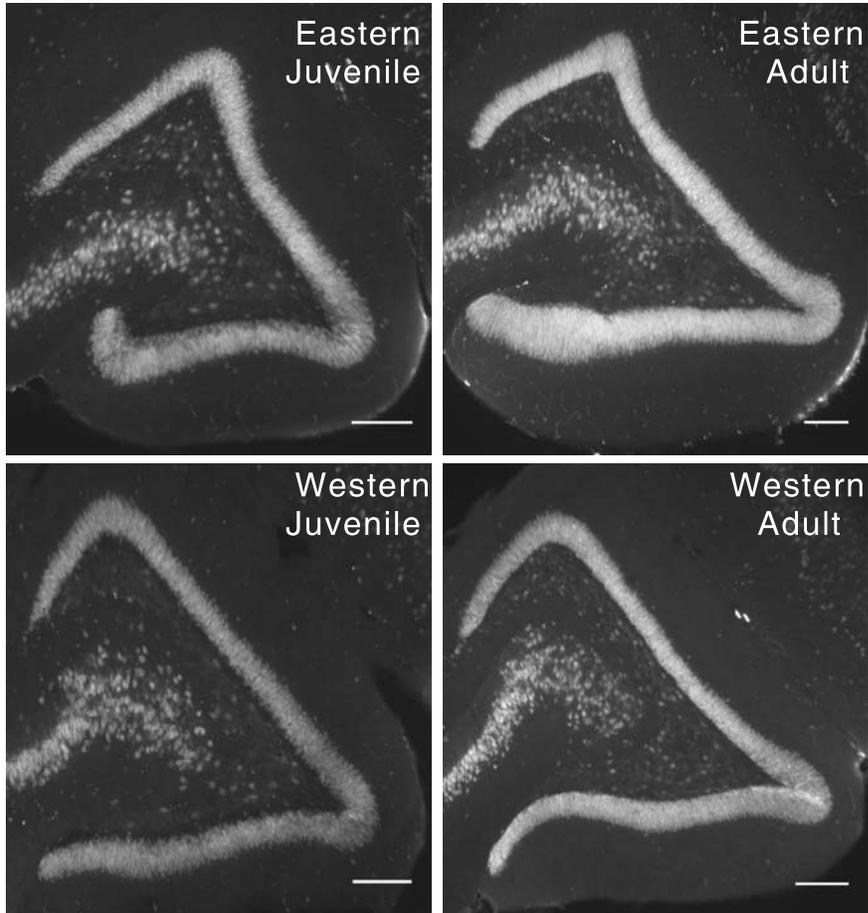
Supplementary Figure 2. Typical images of dentate gyrus stained for a mature neuronal marker (NeuN). The granule cell layer (GCL) is clearly delineated and shows characteristic "bulges" in an eastern adult. Scale bar 0.25mm.

Table 1 Summary of neurogenesis data

Animal group	weight (g)	HPC region	sampling	Ki-67	DCX
				(cells/mm^3)	
East juvenile	126.5 ±2.6	dorsal	1/24	23,689 ±6,386	148,157 ±43,197
		ventral	1/12	9,963 ±8,374	
East adult	176.0 ±2.9	dorsal	1/24	2,792 ±1,933	6,227 ±5,070
		ventral	1/12	4,552 ±2,927	
West juvenile	201.2 ±9.9	dorsal	1/24	19,589 ±8,183	115,703 ±69,581
		ventral	1/12	9,479 ±1,091	
West adult	244.2 ±4.9	dorsal	1/24	4,071 ±3,399	21,452 ±16,598
		ventral	1/12	3,677 ±932	

Means and standard deviations are given. Cell densities are expressed per mm³ of granule cell layer. Sampling refers to number of sections. All values for dorsal region are taken from figure 1 and the ventral region from figure 4.