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Pronounced Individual Variation in the Response to the Stimulatory Action of Exercise on Immature Hippocampal Neurons

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ABSTRACT: In the adult hippocampus, neurogenesis is influenced both by external stimuli, such as physical exercise, and by intrinsic conditions like age and disease. However, the way in which many of these external and internal cues interact in this process remains poorly understood. We have used a new, more precise, stereological cell counting method that involves confocal microscopy to analyze the effects of exercise on adult neurogenesis in the mouse. We found that treadmill exercise increases the number of differentiating neurons (doublecortin/calretinin cells) in the granule cell layer of the mouse hippocampus in a manner that is directly related to the size of the mature granule cell population. More immature neurons were found after exercise in animals that had a larger dentate gyrus (DG), while no changes were observed in those with a smaller DG. This differential response to physical exercise suggests that the pre-existing neuronal population regulates the neurogenic response in the DG to external stimuli. These data raise the possibility of anticipating an individuals' response to therapeutic interventions (like exercise) aimed at augmenting dentate neurogenesis and alleviating or preventing cognitive decline. © **2006 Wiley-Liss, Inc.**

KEY WORDS: adult neurogenesis; doublecortin; calretinin; treadmill; total mature granule cell number

INTRODUCTION

In the brain of rodents, primates, and man, new neurons are continuously being generated throughout adult life (Rakic, 2002; Kempermann et al., 2004b; Abrous et al., 2005). The precursors of these cells and their progeny often appear in clusters in the subgranular zone (SGZ) of the hippocampus, where a vascular niche exists that permits them to survive (Palmer et al., 2000). When the progeny of these precursors begin to differentiate, they extend both axons and dendrites (Hastings and Gould, 1999), although many of them die before reaching 3–4 weeks of age. Those that do not die are capable of surviving for long periods of time (Kempermann et al., 2003), during which they establish functional synapses, integrate into neural networks, and they adopt specific electrophysiological properties (Song et al., 2002b). It is now clear that adult hippocampal neurogenesis (AHN) cannot be overlooked when considering hippocampal physiology and function (Gross, 2000). Indeed, the relevance of AHN in the learning and memory of several hippocampal specific tasks has already been demonstrated (Shors et al., 2001; Shors et al., 2002).

The precursor cells involved in AHN are mostly found in the SGZ of the granule cell layer (GCL). Here, many growth factors are involved in regulating their proliferation, determination, differentiation, and maturation (i.e., IGF1, Shh, VEGF, BDNF, and many others). Complex interactions exist between many of these intrinsic factors, as well as with extrinsic factors that may also influence these processes (stress, exercise, circadian rhythms, seasonal and sexual behaviors, aging, social status, and many others). Hence, it is important to determine to what extent intrinsic factors regulate the influence of extrinsic factors on AHN. Our current understanding of how endogenous factors control AHN is fairly superficial. Endothelial VEGF in the vascular niches of the GCL (Jin et al., 2002; Fabel et al., 2003; Cao et al., 2004), as well as circulating IGF1 (Trejo et al., 2001) and BDNF (Neeper et al., 1996), are thought to influence either the proliferation or the survival of precursors. Likewise, mature granule neurons may act as a source of other factors that control AHN.

Both voluntary and treadmill exercise have been shown to significantly augment AHN (van Praag et al., 1999a,b). Since physical exercise could offer a more natural way to influence neurogenesis and a possible alternative to pharmacological treatment aimed at stimulating neurogenesis, we have studied this phenomenon further. We have focused our attention on the immature neuron population of the GCL, since there is evidence that this cell population exerts a specific influence in AHN, affecting both transient immature neurons and on prospective mature granule neurons (Leuner et al., 2004). To study the different stages in the life cycle of these cells it is possible to take advantage of different markers of immature neurons such as doublecortin, calretinin, Proxl, and NeuroD1 among others (Pleasure et al., 2000; Brandt et al., 2003; Kempermann et al., 2003; Kempermann et al., 2004a). Many in vivo manipulations may modify the rate of AHN and the expression of these mole-

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Abbreviations used: DCX, doublecortin; Calret, Calretinin; GCL, granule cell layer; AHN, adult hippocampal neurogenesis; SGL, subgranular layer.

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cules, two of the most commonly used involving an enriched environment and exercise (Nilsson et al., 1999; van Praag et al., 1999a,b; Brown et al., 2003). Such manipulations may be of therapeutic interest in situations where AHN is reported to be a putative etiopathological factor, such as in depression (Malberg et al., 2000; Duman et al., 2001; Sharp et al., 2002; Santarelli et al., 2003). Moreover, they may be relevant to combat the progress of or to aid recovery from certain symptoms of ischemia (Sharp et al., 2002), Alzheimer's disease (Wen et al., 2004), or inflammation (Ekdahl et al., 2003).

One complication in studying AHN in the GCL is the density of cells in this brain region, which often hinders the reliable determination of cell number in this layer. Hence, we have applied a new stereological method to determine both the total number of granule cells and the size of the immature neuron population in the GCL and SGZ. By using the physical dissector method with confocal microscopy, we found that the increase in the number of immature cells after exercise was directly correlated to the number of mature granule neurons. This indicates that an endogenous factor, the number of mature neurons, influences AHN. Furthermore, it reveals an aspect of the different individual sensitivity to external manipulations that may be of therapeutic interest.

MATERIALS AND METHODS

Animals

Adult C57/BL6 male mice (3 months of age) were housed at 22 ± 1°C with a 12/12 h light/dark cycle, and ad libitum access to food and water. The animals were kept under standard laboratory conditions in accordance with European Community guidelines (directive 86/609/EEC). Each experimental group consisted of nine animals.

Exercise Protocol

Animals were habituated to the treadmill (Cibertec) for 15 min on two separate days to reduce stress, and they were then divided in two groups: exercised and sedentary as described in detail in (Carro et al., 2001). The training of the exercised animals included a gradual adaptation to the running schedule for the first 2 days and from day 3 onwards, the mice ran for 1 h/day at 0.2 m/seg, 5 days/week, for 2 weeks. The control animals remained on the treadmill without running for the same amount of time.

Twenty four hours after the last exercise session, the animals were sacrificed by deep pentobarbital anesthesia and they were transcardially perfused with saline followed by 4% paraformaldehyde in phosphate buffered saline (PBS). Their brains were removed and fixed again in the same fixative overnight at 4°C. Approximately 100 serial coronal vibratome sections (50 μm) of the hippocampal formation were collected individually in 96-multiwell culture plates. Different series made up of one section from every 9th section were collected randomly (see below) and stained by immunohistochemistry or propidium iodide.

Immunohistochemistry

Slices were initially preincubated in PB with Triton X-100 0.5% and bovine serum albumin (BSA) 0.1% (PBTBSA) and then dual immunohistochemistry was performed as described previously (Trejo and Pons, 2001). The primary antibodies used were a goat antidoublecortin antibody (Santa Cruz 1:500) and a rabbit anticalretinin antibody (Swant, 1:3000). Primary antibodies were recognized with a 594 alexa-conjugated donkey antigoat, and a 488 alexa-conjugated donkey antirabbit antibody (Alexa-conjugated antibodies from Molecular Probes, 1:1000). The sections were then analyzed and photographed under an optical microscope (either a Leica DMRB or Leitz Laborlux S), and using a confocal microscope (Leitz DMIRB/TCS4D). Mature granule cells in the GCL were counted on sections stained for 15 min with 500 nM propidium iodide (Invitrogen).

Physical Disector

We have applied a physical dissector method (Pakkenberg and Gundersen, 1988), introducing some variations according to the basic principles of Howard and Reed (1998) and using the "unbiased brick" principle of the 3D dissector. We counted the cells in each pair of confocal sections using the first as a reference section and the other as a "look-up" section, and then their identities were swapped. The cells were marked on a grid superimposed on the computer screen and they were then identified in successive images of the disector to assure that each single cell was no longer counted (see Fig. 1).

We implemented the traditional optimization of the dissector by registering successive pairs of sections in the "vertical" (z) axis with the aid of a confocal microscope. The cell counts and the size of the dissector used to estimate the density, were given by the general formulae $N = \Sigma C / V_f$, where N = estimate of numerical density, ΣC = sum of cells counted, and V_f = volume of the dissector. For the immature granule neurons, the dissector was implemented as the number of cells per unit area of the SGL (see below), substituting V_f for A_f = area of the dissector. The average distance between the confocal planes throughout the study was in the range of 1.7–2 μm, as 6–9 confocal planes were recorded for all the points measured.

To ensure uniform random sampling, we considered that the supra- and infrapyramidal blades of the DG in all the sections within a series generated a continuous "line," throughout the rostro-caudal extent of the entire DG. This "line" was then measured with a digital tablet and assigned an arbitrary unit length. A random number table was then used to generate the exact points to obtain the stacks. This means that every single point of the GCL has the same chance of being selected for the confocal stack of images.

For the total number of granule cells, the physical dissector was applied to sections stained with propidium iodide so that all nuclei in the GCL were counted (excluding those nuclei that, upon observation, resembled erythrocytes). The cell density was then multiplied by the total volume of the GCL estimated by the Cavalieri method (point grid).

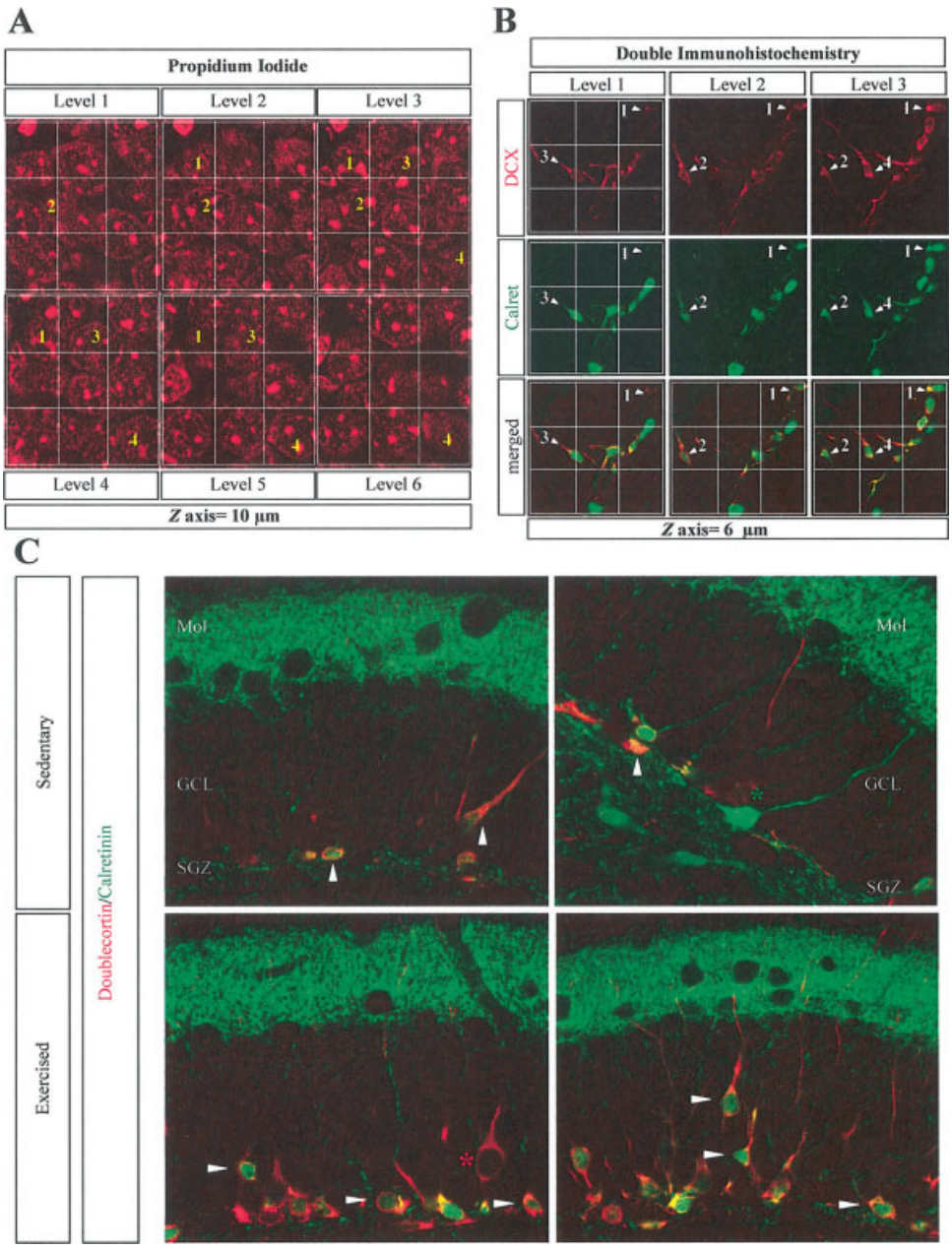

FIGURE 1. Cell counts from the GCL and SGZ of sedentary and exercised mice. A: To assess the density of granule neurons, propidium iodide staining of brain sections were analyzed using confocal microscopy. Levels 1–6 represent the stack of serial images within the width of a dentate section of the GCL, ranging from 10 µm in height and with an interimage distance of 1.6 µm. Each single cell can be identified between images using a superimposed white grid. Some of the cells are labeled with the same number throughout successive levels to illustrate the process by which they are registered on the computer screen before summing. B: To measure the density of immature neurons, cells double labeled for DCX (red)/Calret (green) were examined by confocal microscopy. Three representative levels are shown to illustrate a stack within the GCL ranging from 6 µm in height and with an interimage distance of 2 µm. Each single cell is identified within the superimposed grid and assigned with the labeling of one of the markers or as a double-labeled cell (colocalization). Some of the neurons are numbered to show the counting process as in A. C: Representative images of dual immunohistochemistry of sedentary and exercised animals at different levels of the DG. Arrowheads point to double-labeled cells in the GCL and SGZ, asterisks indicate single labeled cells in the SGZ. Note that more cells were found in the exercised animals.

A different and novel method was used for the cells expressing immature markers (doublecortin and/or calretinin). At each point of the section to be registered, we counted all the immature cells in the reference area. The reference area is a square with one side that lies on the "line" of the SGZ. By applying the simple rule of dividing the number of immature cells counted by the length of the "subgranular" line, we obtained a reliable estimate of the cell density by "unit of SGZ." The total number of immature cells was obtained by simply multiplying the cell density by the total extension of the SGZ. This total SGZ extension was measured using a semiautomatic system (ImageJ v.1.33, NIH, USA, http://rsb.info.nih.gov/ij/) with the series of images from propidium iodide-stained sections. We then drew the SGZ below the internal side of the GCL on the computer screen and measured the length of the resulting lines.

Statistical Analysis

The data regarding the cell counts was analyzed by the U-Mann-Whitney test for comparisons between sedentary and exercised animals. The categorized data was compared by two-way analysis of variance (ANOVA) for the factors "exercise" and "number of mature granule neuron numbers," and continuous data was compared by both simple and multiple linear regression analysis. The software used was either SPSS 12.0.1 (SPSS, 1989; Apache Software Foundation) or Statgraphics Plus 5.0 (Statistical Graphics, 1994).

TABLE 1.

Cell Counts of the Different Subpopulations Considered in the Study and the Volume of the Dentate Gyrus

	Sedentary	Exercised
Dentate gyrus volume (mm^3)	0.57 ± 0.02	0.57 ± 0.03
Subgranular layer area (mm^2)	6.77 ± 0.31	7.29 ± 0.20
Number of DCX^+ $Calret^-$ cells	6485.72 ± 685.97	8784.91 ± 933.13*
Number of DCX^- $Calret^+$ cells	1843.26 ± 175.20	1684.06 ± 193.68
Number of DCX^+ $Calret^+$ cells	30873.44 ± 1825.17	37799.80 ± 3685.07
Number of DCX^+ cells	38365.93 ± 2281.94	46526 ± 4012.71
Number of $Calret^+$ cells	33723.46 ± 1823.13	38327.14 ± 4054.67
Number of immature cells	40209.29 ± 2380.41	46884.91 ± 4356.04
Number of granule cells	1000832.80 ± 85560.17	925472.44 ± 61184.55
DCX^+ $Calret^-$ cells × 100/granule cells	0.72 ± 0.10	0.96 ± 0.09
DCX^- $Calret^+$ cells × 100/granule cells	0.20 ± 0.03	0.18 ± 0.016
DCX^+ $Calret^+$ cells × 100/granule cells	3.06 ± 0.29	4.10 ± 0.30*
DCX^+ cells × 100/granule cells	3.63 ± 0.21	5.06 ± 0.31*
$Calret^+$ cells × 100/granule cells	3.21 ± 0.16	4.44 ± 0.29*
Immature cells × 100/granule cells	3.99 ± 0.33	5.25 ± 0.31*

As in the rest of the study, the immature cell counts are given either as absolute numbers or as a ratio of the immature cells to the total number of granule cells. Significant differences are plotted in Figure 2. Data are group means ± SEM. $^*P < 0.05$.

Evaluation of the Errors of the Technique

The variance between the animal samples was determined by the coefficient of error of the sampling distribution (CE_s), given by $CE_s = \frac{s}{\sqrt{nM_n}}$, where s is the sample standard deviation (SD), n is the number of animals, and M_n is the sample mean.

The error coefficient of the Cavalieri estimate of the volume of brain sections (CE_{Cav}) was determined by the formula:

$$CE_{Cav} = \frac{1}{\sum P}\sqrt{\frac{1}{12}\{3a + c - 4b\} + 0.0543\frac{\bar{B}}{\sqrt{\bar{A}}}\sqrt{m\sum P}}$$

where $\sum P$ is the sum of the point counts on each section, $a = \sum_{j=1}^{m} P_i P_i$, $b = \sum_{i=1}^{m-1} P_i P_i + 1$, $c = \sum_{j=1}^{m-2} P_i P_{i+2}$, m = the number of measures or sections, $\bar{B}$ = the mean boundary length (as estimated with a digital tablet and an image processor), $\bar{A}$ = the mean transect area (as estimated from point counting), and $\frac{\bar{B}}{\sqrt{\bar{A}}}$ being the dimensionless shape coefficient.

The error coefficient of cell density with the physical dissector (CE_{cf}) can be efficiently estimated with the general formula for the calculation of the CE of a ratio estimator:

$$CE_{cf} = \sqrt{\frac{k}{k-l}\left\{\frac{\sum c_2}{\sum c \sum c} + \frac{\sum f_2}{\sum f \sum f} - 2\frac{\sum \mathrm{cf}}{\sum c \sum f}\right\}}$$

where k = number of stacks of micrographs from confocal microscope, c = number of cells counted per individual, and f = number of frames per individual.

RESULTS

We have used an improved method to measure both the total number of granule neurons and the number of immature granule cells in the DG of the hippocampus, in both sedentary and exercised adult mice. This method involved applying an adapted physical dissector method using confocal microscopy. The experimental protocol did not produce any apparent differences in the behavior of the animals, exercised or sedentary. Hence, all exercised animals used in the present study ran fairly well and none of them refused to be trained. Mice that did refuse to train were excluded from the experiment.

The new method we have applied has enabled us to determine the total number of mature granule neurons in the DG through propidium iodide staining. With the aid of a superimposed grid, it can be seen how several cells were counted in a series of disector images from the 1st to 6th (level 1–6 through the height of the disector). Indeed, it is clear how their profiles could be followed through the height of the dissector to avoid counting them more than once (Fig. 1A). Neither the cells touching the bottom image nor the cells at the left lateral and basal sides of the image were considered ("unbiased brick counting rule"). For clarity, not all cells registered in this dissector are illustrated. As a result, we found that the mean number of mature granule cells did not differ significantly between exercised and sedentary animals (see Table 1 and Fig. 2B).

The total number of immature granule neurons was also determined using this method by evaluating the number of DCX and calretinin-positive cells. With a superimposed grid, a series of dissector images from three successive levels (1–3

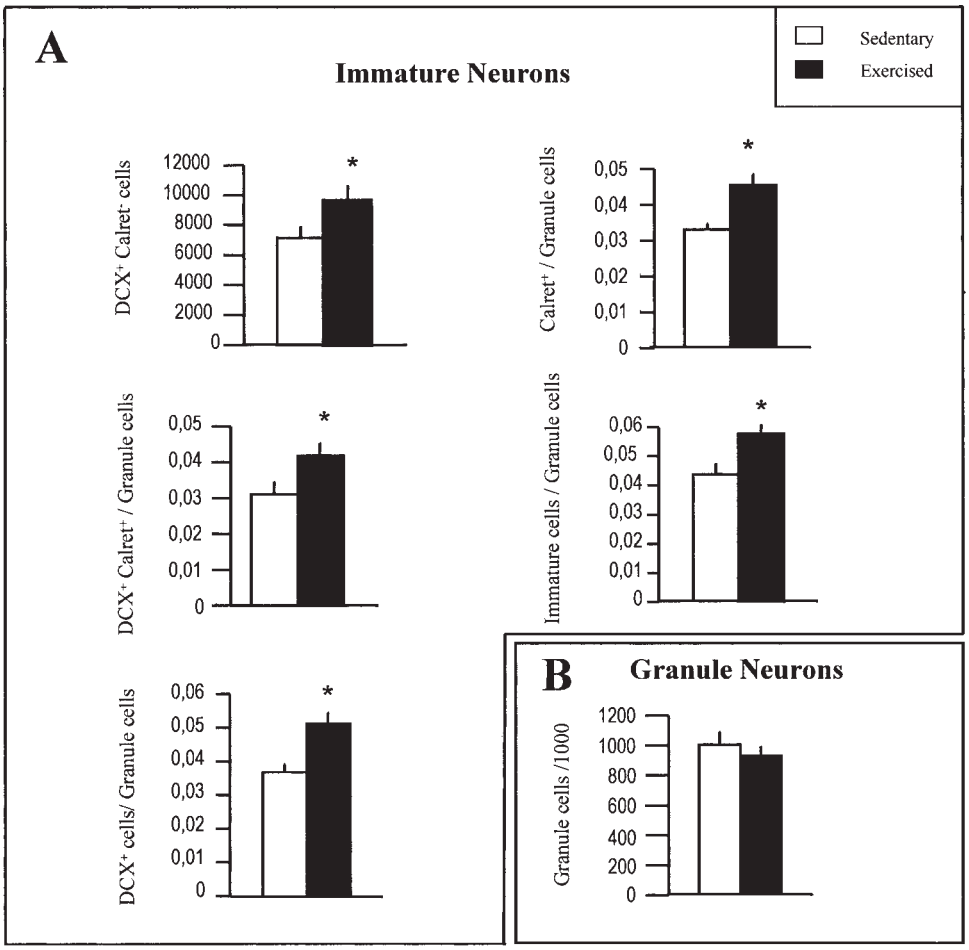

FIGURE 2. Differences in the number of immature and mature granule neurons in the DG of sedentary and exercised mice. A: The absolute numbers of immature cells were measured by dual immunohistochemistry for DCX (red)/ Calret (green), analyzing the ratio with respect to the total number of granule neurons in the GCL/SGZ of the corresponding animals. Histograms represent the statistical data from the comparisons in Table 1. B: Total number of granule neurons in sedentary and exercised animals showed no significant differences. Bars represent group means ± standard error of mean (SEM). *$P < 0.05$.

through the height of the dissector) illustrate how cells were identified as either labeled for individual marker or double stained for both. These cells could be followed through the height of the dissector thereby avoiding counting them more than once (Fig. 1B). The mean number for each combination of markers and the proportion of labeled cells with respect to the total number of mature granule neurons are shown in Table 1. Notably the total number of $DCX^+/Calret^-$ cells was significantly higher in exercised animals than in sedentary animals (Fig. 2A). Similarly, the proportions of $DCX^+/Calret^+$, total DCX^+, total $Calret^+$, and total number of immature cell with respect to the total number of mature granule neurons were also higher in exercised animals. These ratios were calculated from the number of labeled cells and the total number of mature granule cells found in each animal. Indeed, this can be seen in Figure 1C, where double-staining of the GCL for DCX and Calret is shown, illustrating the different combinations of cell labeling (either single or double stained for these markers) and the difference between the sedentary and exercised animals.

Using two-way ANOVA for categorized data, we analyzed the number of immature neurons in the DG, both in function of the total number of mature granule neuron and as a function of treatment (Table 2). With respect to the total number of mature granule neurons, we divided the animals into two groups: those above and below the median of the population (high and lower number of mature granule neurons). While this was considered as one factor, the treatment (exercise or sedentary) was considered as the second factor. Although there was no correlation between the number of mature and immature granule neurons in sedentary animals, the group of exercised animals with more mature granule neurons showed a significant increase in the numbers of immature neurons (Fig. 3). In contrast, no increase in the number of immature cells was detected in exercised mice with fewer mature granule cells. To further substantiate these results, we also performed a linear regression analysis with the three variables using a continuous scale, that is without categorizing the variable "number of mature granule neurons." ANOVA analysis confirmed that while there was no significant association between the number of mature granule neurons and immature neurons in sedentary animals, the exercised group displayed a better fit (coefficient of determination 38.7%) with a regression predictor line slope of 0.037 (Pearson correlation coefficient) with a 92.6% significance level (Fig. 3C). Multiple regression analysis produced a significant result for the interaction between the two independent variables ($F = 3{,}306$, $P = 0.052$).

The accuracy of the estimates for each individual obtained with the novel protocol employed here can be seen by the coef-

TABLE 2.

Cell Counts of Different GCL Subpopulations After Grouping the Animals Based on the Number of Granule Cells (High or Low)

	Interaction *P*-value	High number of granule cells		Low number of granule cells	
		Sedentary	Exercised	Sedentary	Exercised
Number of cases		4	4	5	5
Number of DCX^+ $Calret^-$ cells	N.S. ($P = 0.69$)	5906.23 ± 1330.83	8848.18 ± 1629.33	6834.01 ± 839.53	8734.30 ± 1330.83
Number of DCX^- $Calret^+$ cells	N.S. ($P = 0.44$)	1777.32 ± 137.96	1898.77 ± 402.19	1882.82 ± 281.41	1555.24 ± 213.18
Number of DCX^+ $Calret^+$ cells	($P = 0.0015$)***	30370.53 ± 3435.16	48414.26 ± 3251.16** ($P = 0.0088$)	31275.75 ± 2195.73	29308.14 ± 1408.95
Number of DCX^+ cells	($P = 0.0175$)*	38792.83 ± 4322.32	57262.44 ± 4661.03* ($P = 0.038$)	38109.78 ± 2988.63	37938.34 ± 1854.87
Number of $Calret^-$ cells	($P = 0.0069$)**	34664.93 ± 3320.42	50766.59 ± 4917.82* ($P = 0.053$)	33158.59 ± 2405.50	30863.48 ± 1280.63
Number of immature cells	($P = 0.0311$)*	40570.16 ± 4304.75	44272.58 ± 6000.96* ($P = 0.086$)	39992.60 ± 3205.50	39597.78 ± 1823.26
Number of granule cells	N.S. ($P = 0.27$)	1230653.25 ± 65981.88	1073113.25 ± 30798.99	816976.44 ± 68756.89	807359.8 ± 71136.65

The division was made based on the median of the whole cell population and then all the data was compared separately by two-way ANOVA for the two different factors (effect of exercise and granule cell number). Interaction *P*-values of the comparisons are given. Note that the only significant difference was an increase in the number of cells in the exercised animals with high number of mature cells.

ficient of error for the measurements from each individual (Table 3). The measures of cell density and of total volume were very precise, which implies a high degree of precision in calculating the actual cell number. The mean CE_{cf} for the number of cells per counting frame should remain below the recommended target of 0.06 (<6%). Indeed, CE_{cf} values of 0.025 (2.5%) and 0.022 (2.2%) were determined here for the sedentary and exercised animals, respectively. The final data for the corrected CE_{Cav} ranged from 0.039 to 0.049 (Table 3). The main contribution to the overall CE for the Cavalieri estimator is often found to be the "between section" contribution. Hence, point counting adds a negligible amount to the overall coefficient of error. The low error coefficients calculated imply that the estimates of cell density were precise in each individual animal. Finally, we found sampling CEs that ranged from 0.01 to 0.05, much lower than the comparable figures reported previously when estimating cell number in the DG (Table 3).

DISCUSSION

AHN is an important facet of brain physiology that has potential therapeutic applications. However, we still know comparatively little about the cellular and molecular mechanisms that regulate AHN and its main characteristics. Our aim was to analyze the response of some parameters of AHN to a two-week period of daily treadmill training, a well-known paradigm that is known to produce an increase in the rate of AHN in both rats and mice. We evaluated two important aspects of the impact of exercise: (1) its effect on the composition and number of immature granule neurons in the GCL; and (2) the existence of putative endogenous factors that might control the rate of AHN in response to exogenous stimuli, in this case treadmill exercise. To achieve these goals, it was necessary to overcome the problem presented by the high cell densities in the GCL, which makes it extremely difficult to obtain precise cell counts. Several systems have been used to resolve this problem (Kempermann et al., 1997; Abusaad et al., 1999); however, we have tried to obtain more rigorous and accurate data from this tightly packed population by using a physical dissector and confocal microscopy. The combination of these techniques permits stereological methods to be applied to cell counting, reducing the errors introduced both through under- and overestimation.

The method described here is highly reliable, accurate, and it is a more rigorous way to count populations of highly packed cells. The physical dissector is conceptually as solid as the optical dissector to ensure that every cell is counted only once, irrespective of its shape or size. Moreover, when performed on a successive series of confocal planes (stack) that are separated by a known distance, of a defined reference area and height, and when using a specifically designed grid to count the cells directly on the screen (the "unbiased brick" counting rule), it yields highly reliable data regarding cell density. It is important to note that many of the benefits offered by this technique derive from the use of confocal planes to count the cells, since confocal images have a range of 600–800 nm (depending on the objective used). Hence, the confocal microscope offers a resolution capable of distinguishing between one cell and its neighbors beyond all doubt in a single confocal plane. As for the resolution of the physical dissector itself, the optimal interplane distance must be determined experimentally in each case, to ensure that every cell appears in at least two

successive confocal images (or any other premise one wants to establish to count the cells). Furthermore, this technique permits data to be obtained from immunostained cells, either single or double labeled, irrespective of their position within the GCL. This is in part, because the cell density obtained from each stack is referred to in terms of the length of the SGZ so that the data accounts for all the immature cells in the SGZ and GCL. Besides this, it is not necessary when using this method to consider the width of the GCL. The only drawback of the technique is that it is highly time-consuming in terms of the use of a confocal microscope.

Several previous attempts to count the total cell number in the GCL of the hippocampal DG of c57BL/6J mice have used different stereological methods. The numbers obtained range from 239,000 granule cells per hemisphere using the optical dissector estimator with a semiautomatic stereology system (Stereoinvestigator 1.0) and Hoechst 33342 staining (Kempermann et al., 1997), to 433,000 granule cells per hemisphere using the optical dissector estimator and Cresyl violet staining (Abusaad et al., 1999). In both these studies, the total cell number was obtained by multiplying the neuronal density by the total volume of the dentate area under study, as estimated by the Cavalieri method (Pakkenberg and Gundersen, 1988; with Stereoinvestigator 1.0 (Kempermann et al., 1997) and with a standard cross-hatch point counting grid (Abusaad et al., 1999)). The number of mature granule cells we have obtained is four times greater that found by Kempermann et al. (1997) and 2-fold that of Abusaad et al. (1999). This is exactly what might be expected given that such highly packed populations of brain cells are likely to lead to an underestimate of the total cell number. We think that these differences are probably explained by obtaining the reference volume in which cells were counted by optical microscopy in these previous studies (numerical aperture:1.4; specifically, 40-μm sections and ×60 oil objective, (Kempermann et al., 1997) and 16-μm sections and ×100 oil objective (Abusaad et al., 1999)).

The interindividual variation found in the number of mature granule cells is not unusual. The standard error of the means from our data range between 5 and 10%, in contrast to most articles in the field where this ranges from 5 to 15%. There-

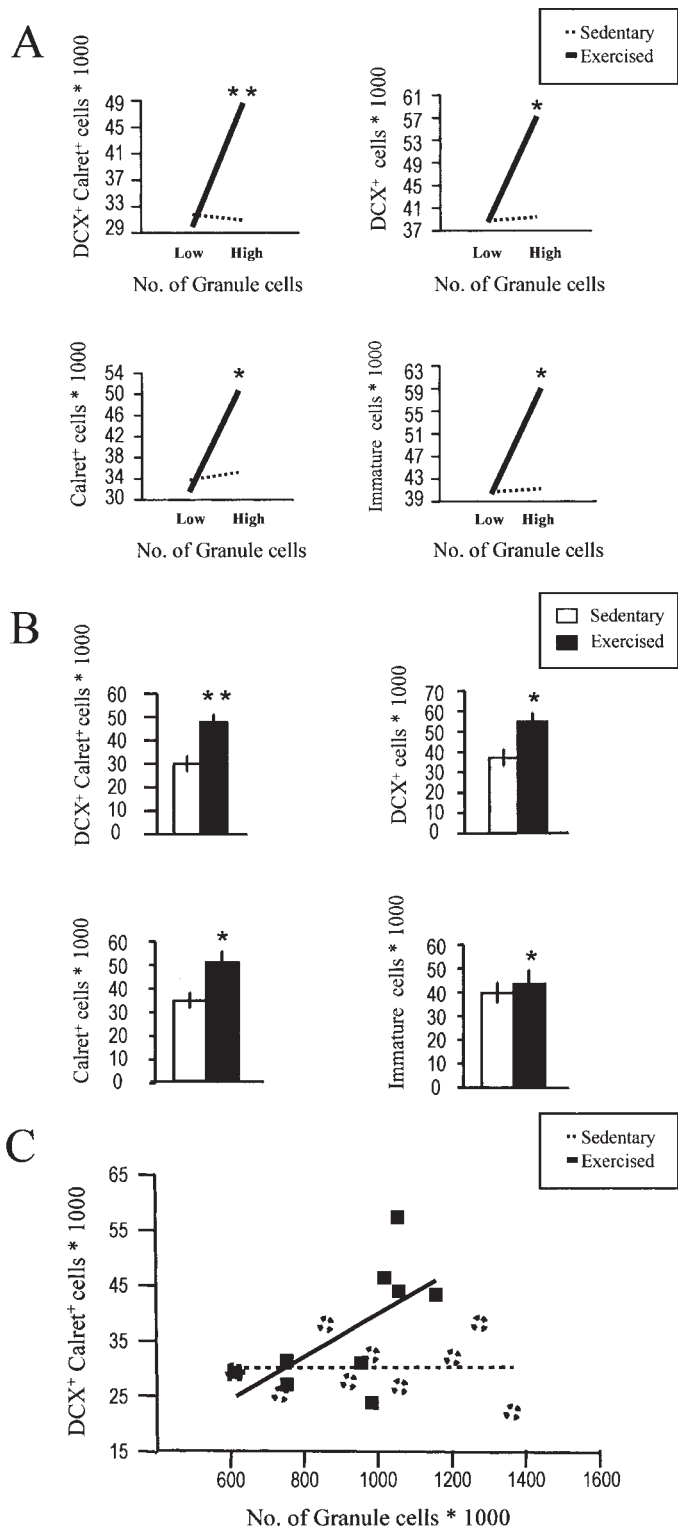

FIGURE 3. Comparison of the number of immature GCL/SGZ neurons in function of the total number of granule cells and of the experimental group. A: Variation in the number of immature cells between animals with fewer or more granule neurons. Note that no significant differences were found in the population of sedentary animals (horizontal dotted lines), in contrast to those observed in the exercised animals (bold black lines slope). The immature cells in the group of exercised animals with more cells account for all the differences in the group mean (Fig. 2). B: Significant differences were observed between sedentary and exercised animals in the absolute numbers of immature cells (dual immunohistochemistry) from those animals with more granule neurons. The charts of the significantly different cell counts are shown. Exercise increased the number of immature double-labeled neurons, as well as the total population of DCX$^+$ cells or the total population of Calret$^+$ cells. Bars represent group means ± SEM. $^*P < 0.05$; $^{**}P < 0.01$. C: Scatter diagram of the variables "number of immature neurons" (*Y* axis) and "number of mature granule neurons" (*X* axis) showing regression predictor lines for each experimental group after linear regression analysis. Immature neurons are double labeled DCX$^+$/Calret$^+$ cells (the most abundant immature subpopulation analyzed). Exercised animals show a clear association between the variables while sedentary animals have a perfect horizontal regression line (no association between variables).

TABLE 3.

Coefficients of Error for the Stereological Methods Used in this Study

Individual[a]	CE_{cf}[b]	CE_{Cav}[b]
Sedentary 1	0.03	0.04
Sedentary 2	0.03	0.04
Sedentary 3	0.02	0.04
Sedentary 4	0.01	0.04
Sedentary 5	0.04	0.03
Sedentary 6	0.02	0.04
Sedentary 7	0.02	0.04
Sedentary 8	0.02	0.03
Sedentary 9	—	0.03
Sedentary	*0.025 ± 0.003*	*0.0348 ± 0.0008*
Exercised 1	0.02	0.04
Exercised 2	0.02	0.03
Exercised 3	0.02	0.03
Exercised 4	0.02	0.03
Exercised 5	0.03	0.03
Exercised 6	0.03	0.03
Exercised 7	0.03	0.03
Exercised 8	0.03	0.04
Exercised 9	0.01	0.03
Exercised	*0.022 ± 0.002*	*0.034 ± 0.0007*
Mean CE	*0.024 ± 0.001*	*0.0364 ± 0.0005*

	CE sedentary[c]	CE exercised[c]	Mean CE[c]
Dentate gyrus volume (mm^3)	0.039	0.049	0.044
Subgranular layer area (mm^2)	0.045	0.028	0.037
Number of DCX^+ $Calret^-$ cells	0.105	0.106	0.105
Number of DCX^- $Calret^+$ cells	0.095	0.115	0.105
Number of DCX^+ $Calret^+$ cells	0.059	0.097	0.078
Number of DCX^+ cells	0.059	0.086	0.072
Number of $Calret^+$ cells	0.054	0.105	0.080
Number of immature cells	0.059	0.093	0.076
Number of granule cells	0.085	0.066	0.076

[a]Number of DCX^+cells.
[b]CE for the physical dissector and Cavalieri methods employed in the measurement of the total number of immature cells.
[c]CE of sampling distribution. Note that all of them are relatively low and homogeneous.

fore, because of the extremely high density of granule cells in the DG, the data we present has virtually the same variability as that already published, despite the maximal variation between animals with highest and lowest numbers being lower. Furthermore, no differences in behavior were found here between animals with more or less cells. This is really quite interesting, both because there was no difference in the number of mature granule neurons between exercised and sedentary animals, and also because this is a common finding in several different experimental paradigms. Specifically, we have calculated the number of mature granule neurons in males and females, both sedentary and exercised in a forced swim test, and we found the same interindividual variations in this parameter in all cases, even when counting using different double-blind evaluation. Thus, even though there was no variation after treatment and this did not translate into behavioral differences, there was an influence on the number of new neurons in the hippocampus (results submitted).

To identify immature cells, we have used doublecortin (des Portes et al., 1998; Gleeson et al., 1998) and calretinin (Brandt et al., 2003), two firmly established markers (Brandt et al., 2003; Kempermann et al., 2003). Both subpopulations of immature cells in the GCL have been previously reported to respond differently to manipulations such as an enriched environment and exercise (Kronenberg et al., 2003). While analyzing that immature neuronal markers may or may not be a suitable indicator of "net" neurogenesis (Kempermann et al., 2003; Couillard-Despres et al., 2005), we are still interested in how exercise modifies the transient population of immature neurons, since these neurons do have specific electrophysiological properties (Song et al., 2002b; van Praag et al., 2002; Ambrogini et al., 2004; Doetsch and Hen, 2005). Therefore, the response to these manipulations is not only relevant in the sense that they will become mature granule neurons, but also for their behavior during the time they exist as transient, immature, synaptically connected neurons. For this reason, we did not set out to elucidate the fate of these new neurons after long-term survival times, but rather to determine their status while they remain immature. Finally, we studied the entire population of DCX^+ and $Calret^+$ cells, rather than only those cells that might be dividing and that could be identified by injection of BrdU. In this way, we hoped to study whether the immature population as a whole is sensitive to experimental manipulations. Taking into account earlier studies (Leuner et al., 2004; Couillard-Despres et al., 2005), we believe that the present results indicate that all the immature neurons in the GCL fulfil specific roles, and not just the dividing cells. Equally, we did not analyze the $DCX^+/Calret^-$ cells further (a subpopulation that includes the Type-2b and Type 3 cells), as the majority of immature neurons in the GCL and SGZ were double labeled for $DCX^+/Calret^+$. Indeed, the population that expressed only DCX (17% of the total population of immature neurons) and those expressing only Calret (4% from the total) represented a small proportion of the total immature population.

Previous estimates of the total number of DCX^+ cells in the GCL of adult rats produced figures of 20,300 per DG. This estimate was made using the StereoInvestigator system with an optical microscope (×100 oil objective) and with the optical fractionator method (30-μm thick sections (Rao and Shetty, 2004)). However, we found a total of 40,000 DCX^+ cells per hemisphere, four times that reported in rats and possibly for the same reasons stated above for the total granule neuron number. Although the variations observed here represent 2–3% new cells from the total number of mature granule neurons, the relevance of the results can be seen in the 17.5% variation in the total number of immature, differentiating neurons, and the 21% variation in the total number of DCX^+ cells. As many others, we think that this subpopulation of immature cells has a determinant role in some kinds of hippocampal

learning and memory processes, specifically by virtue of the special immature characteristics of these cells described above. Animals in which this population of immature neurons is considerably diminished are unable to perform memory tasks as well as controls (Shors et al., 2001; Shors et al., 2002).

Effect of Two-Weeks Exercise on Adult Hippocampal Neurogenesis

We have studied the entire population of immature cells by measuring the total number of DCX^+ and $Calret^+$ cells in the hippocampal DG, as well as studying the following subpopulations of immature cells: $DCX^+/Calret^-$, $DCX^+/Calret^+$, and $DCX^-/Calret^+$ cells. The cells identified with these two markers cover the whole range of immature cells within a sequential temporal program from more immature to mature cells (for a review, see Kempermann et al., 2004a). We found that the total number of $DCX^+/Calret^-$ cells increases in exercised animals with respect to sedentary controls. According to the temporal program of maturation and the expression of immature neuronal markers, these cells are the most immature cells that have just exited the cell cycle. It would be reasonable to think that the increment in the number of new neurons as a result of performing exercise may initially be observed through an increment in the number of the most immature cells.

Indeed, it must be taken into account that the animals were performing regular exercise for just 2 weeks, which effectively provides a measure of the short-term effects of moderate exercise. Interestingly, this is the first time an increment in AHN in mice has been described after a moderate amount of daily exercise (1 h/day for 2 weeks). Exercise periods for as few as 9 days have been shown to increase dentate neurogenesis in rats (Naylor et al., 2005). However, it is also important to bear in mind that this is not a model of voluntary running and as such, all animals ran the same amount of time. In contrast to more common protocols involving voluntary (wheel) running, we demonstrate for the first time an increase in neurogenesis after forced running in mice. The net increase in neurogenesis after forced treadmill exercise (both short-term and long-term survival of newborn neurons) has previously been described in rats (Trejo et al., 2001), using a similar protocol to that used here for over 2 weeks. This indicates that our protocol can produce adequate above threshold stimulation in the animals in terms of neurogenic response.

We found that while the total number of immature DCX^+ and $Calret^+$ cells did not significantly increase following exercise, the ratio of some subpopulations of immature cells was significantly altered with respect to the total number of granule neurons. Specifically, the ratio of the following populations were all significantly greater in the exercised group: total number of immature cells, double stained cells ($DCX^+/Calret^+$), total number of DCX^+ cells, and total number of $Calret^+$ cells, with respect to the total number of granule neurons in the GCL (see Table 1 and Fig. 2). These results are in accordance with previous studies demonstrating an increase in hippocampal neurogenesis in response to exercise (van Praag et al., 1999b; Kempermann et al., 2000; Mattson, 2000; Trejo et al., 2001; Anderson et al., 2002; Cotman and Berchtold, 2002).

The data here indicate that specific effect of exercise occurs in function of the initial number of mature neurons. Hence, we analyzed why the proportion of these cell types was significantly different although the group mean of the total numbers was not. By analyzing the data in each animal one by one, we found a significant correlation between the number of cells in these subpopulations and the total number of granule neurons in the animals in the exercise group, respectively. Therefore, we consider that the total number of granule neurons is a factor that influences the sensitivity to treadmill training. Indeed, when we performed an ad hoc statistical analysis, both the experimental group and the number of granule neurons had a significant effect on the number of newborn/immature neurons. Treadmill exercise significantly increased the number of immature neurons in those animals with more mature granule cells. This finding is relevant because the total number of granule cells in the GCL did not change significantly either as a group mean, or in terms of the range of data when considering each animal individually. We believe these results indicate that treadmill exercise increased the number of immature newborn neurons in function of the number of mature granule neurons already present, without altering the survival of the mature GCL neurons. Adult hippocampal granule neurons have a well-established sensitivity to a wide range of experimental, physiological, and pathological factors. These data therefore suggest that any variation in the survival of these neurons as a result of the individual status of an animal, will affect how each animal might respond to therapeutic interventions aimed at augmenting dentate neurogenesis to alleviate or prevent cognitive symptoms. Although the influence of the principal GCL cell population (i.e., granule neurons) on the neurogenesis taking place in the region might appear obvious, the opposite results have been observed in model systems to study neurogenesis in the adult olfactory bulb (Kirschenbaum et al., 1999; Hastings and Gould, 2003; Wu et al., 2003). Indeed, the main influence controlling neurogenesis in this structure appears to arise from local astrocytes (Song et al., 2002a).

To test the possibility that the categories drawn up according to the population median did not reliably represent the population, we examined distinct categories ranging from the highest to the lowest number of granule neurons among all the animals. The population was then divided into three equally distributed groups before repeating the two-way ANOVA test. We again found a significant interaction between the two variables using this three-group categorization (not shown) and posthoc analysis revealed the same significant differences. To better substantiate these results, we also processed the data without categorizing any variable by means of a linear regression analysis. The dependent variable "number of immature neurons" was best explained (38.7% coefficient of determination) in exercised animals by the independent variable "number of mature granule neurons" with a 92.6% significance level (probability of avoiding type 1 error). No association between variables was found in the sedentary group. The ANOVA of the multiple

regression was statistically significant for the interaction between the variables "number of mature granule neurons" and "experimental group," thereby substantiating our conclusions. Finally, the error prediction formulae used in the present study for Cavalieri measurements may overestimate the CE by a factor of 10–20 when the object is quasi-ellipsoid. This overestimate becomes less as the object shape becomes more irregular. For this reason, a modified version of the formula enabled us to establish a corrected CE for the Cavalieri estimate of DG volume (Cruz-Orive and Myking, 1981). Although this is also an overestimate, it is a much better prediction than the classical CE.

This is the first time that a physical dissector implemented with a confocal microscope has been used to measure AHN and total granule cell number. In this way, we have been able to obtain more accurate data regarding both mature and immature cell numbers in this structure. We show that when both sets of data were analyzed together, animals with more granule neurons demonstrated a statistically significant increase in the number of immature neurons. Future studies will be necessary to elucidate whether this finding can be generalized to other paradigms, as for example voluntary training or more intense running protocols. Thus, our studies have revealed aspects of the regulation of adult neurogenesis that are more complex than expected. Moreover, they suggest the existence of endogenous factors in the granule cell population that may control adult neurogenesis. This possibility will now be the subject of more intense investigations.

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