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Calorie restriction alleviates the age-related decrease in neural progenitor cell division in the aging brain

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Abstract

Production of new neurons from stem cells is important for cognitive function, and the reduction of neurogenesis in the aging brain may contribute to the accumulation of age-related cognitive deficits. Restriction of calorie intake and prolonged treatment with rapamycin have been shown to extend the lifespan of animals and delay the onset of the age-related decline in tissue and organ function. Using a reporter line in which neural stem and progenitor cells are marked by the expression of green fluorescent protein (GFP), we examined the effect of prolonged exposure to calorie restriction (CR) or rapamycin on hippocampal neural stem and progenitor cell proliferation in aging mice. We showed that CR increased the number of dividing cells in the dentate gyrus of female mice. The majority of these cells corresponded to nestin–GFP-expressing neural stem or progenitor cells; however, this increased proliferative activity of stem and progenitor cells did not result in a significant increase in the number of doublecortin-positive newborn neurons. Our results suggest that restricted calorie intake may increase the number of divisions that neural stem and progenitor cells undergo in the aging brain of females.

Introduction

Adult hippocampal neurogenesis is linked to behavior (e.g. facilitating pattern separation in similar contexts), response to therapy (e.g. mediating the response to antidepressants), and neural tissue repair (Zhao *et al.*, 2008; Deng *et al.*, 2010; Aimone *et al.*, 2011; Ming & Song, 2011; Sahay *et al.*, 2011; Samuels & Hen, 2011). Production of new neurons diminishes dramatically with age, and this decline in neurogenesis may underlie age-related cognitive impairment (Kuhn *et al.*, 1996; Cameron & McKay, 1999; Leuner *et al.*, 2007). The decrease in production of new neurons is, to a large degree, driven by the decrease in the number of neural stem cells (Encinas *et al.*, 2011b; Encinas & Sierra, 2012), which may be further exacerbated by their decreased propensity to divide (Hattiangady & Shetty, 2008). This decrease may potentially be attenuated by symmetric divisions of stem cells (Bonaguidi *et al.*, 2011) or through the proliferative activity of specific subpopulations of stem cells (Lugert *et al.*, 2010). Radial glia-like hippocampal neural stem cells are largely quiescent in adulthood. Our results suggest that, upon activation, they undergo a rapid series of divisions followed by astrocytic differentiation, and, although physically still present in the dentate gyrus (DG), leave the pool of functional stem cells (Encinas *et al.*, 2011b). Given the link between hippocampal neurogenesis and cognitive function, it is plausible that slowing down the loss in neural stem cells or increasing their productivity in giving rise to new neurons may help to ameliorate age-related cognitive deficits.

Restriction of calorie intake can increase the lifespan of a diverse range of species, including mammals (Anderson & Weindruch, 2010, 2012; Houtkooper *et al.*, 2010; Mercken *et al.*, 2012). Lifespan can also be significantly extended by prolonged treatment with the mammalian target of rapamycin (mTOR) pathway inhibitor rapamycin, even when treatment is started late in life (Harrison *et al.*, 2009; Houtkooper *et al.*, 2010; Miller *et al.*, 2011). Both calorie restriction (CR) and rapamycin, usually applied for a limited period of time, have been shown to affect adult neurogenesis (Bondolfi *et al.*, 2004; Stangl & Thuret, 2009; Paliouras *et al.*, 2012). However, it is not clear whether prolonged treatment with CR or rapamycin has similar benefits, and whether such benefits are observed in old animals. It is also not clear which specific subclasses of neural stem and progenitor cells and which steps of the neuronal differentiation cascade are targeted by CR or rapamycin. Here, we used a reporter transgenic mouse line in which different subclasses of progenitor cells can be distinguished on the basis of expression of the green fluorescent protein (GFP) transgene, morphology, and mitotic behavior (Mignone *et al.*, 2004; Enikolopov & Overstreet-Wadiche, 2008) to investigate the effects of CR and rapamycin on hippocampal stem and progenitor cells in aging animals.

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Materials and methods

Animals

The nestin–GFP reporter mouse line is described in Mignone *et al.* (2004). Nestin–GFP homozygous transgenic mice, previously backcrossed to C57BL/6 mice for > 10 generations, were bred and initially housed in the animal facility of Cold Spring Harbor Laboratory in a standard light-controlled and temperature-controlled environment (12-h light/dark cycle; light on at 07 : 00 h; 21 °C) with access to food and water *ad libitum*. Mice were then shipped to the Barshop Institute for Longevity and Aging Studies at UT Health Science Center, and were maintained there during the entire treatment period. Before 6 months of age, all mice were maintained with access to standard Purina Mills Test diet *ad libitum*. Starting at 6 months, mice were switched to the experimental diet treatments. The mice were randomly divided into three groups: control (n = 28; 14 females and 14 males), CR (n = 26; 12 females and 14 males), and rapamycin (n = 28; 14 females and 14 males). The control group received standard diet *ad libitum*. Calorie restriction was established by feeding mice 80% of *ad libitum* consumption for 1 week, and then lowering it to 60% for the remainder of the study (Fok *et al.*, 2012). Mice in the rapamycin group were fed *ad libitum* the Purina Mills chow containing 14 p.p.m. of encapsulated rapamycin in the diet, as previously described (Harrison *et al.*, 2009; Miller *et al.*, 2011; Fok *et al.*, 2012). Empty capsules were added to the *ad libitum* control diet. After 12 months of treatment, half of the mice were randomly selected to be euthanised for analysis (control, n = 12, five females and seven males; CR, n = 12, five females and seven males; rapamycin, n = 12, six females and six males). Diet treatment on the remaining mice was continued for an additional 10 months, at which point all remaining mice were euthanised for analysis.

All mice were monitored daily for general health and lifespan assessment during the diet period. Their body weight and age-related illness were recorded at least once per month. The symptoms of age-related illness that were monitored included tumors, hair loss, aberrant postures (hunchbacked), cataracts, and other eyes problems such as bulging, discoloration, and conjunctivitis. The mice that were judged to be moribund were killed humanely.

Mouse maintenance was according to the guidelines for the use and treatment of laboratory animals from the National Institutes of Health, and all procedures were approved by the Animal Care and Use Committees of Cold Spring Harbor Laboratory and the University of Texas Health Science Center.

Double S-phase pulse labeling of dividing cells and immunohistochemistry

For labeling of proliferating cells, all mice received single intravenous injections of 5-chloro-2′-deoxyuridine (CldU) (128 mg/kg; Sigma) and 5-iodo-2′-deoxyuridine (IdU) (173 mg/kg; Sigma) 24 h and 2 h, respectively, prior to being killed. After being given an overdose of anesthetic, the mice were transcardially perfused with 30 mL of phosphate buffered saline (PBS) and 30 mL of 4% paraformaldehyde in PBS (pH 7.4). The extracted brains were further post-fixed in 4% paraformaldehyde in PBS overnight at 4 °C, and stored in PBS with 0.1% sodium azide at 4 °C until sectioning. The brains were sagittally sectioned at a thickness of 50 μm with a vibratome (Vibratome). The brain sections were sequentially collected, and subsets of sections at 100-μm intervals were taken for immunohistochemistry and analysis. The brains of four mice (of the total of 36) were removed from analysis because of damage or malformations. Approximately 40 brain sections per mouse were processed in total. After rinses with PBS, the sections were denatured in 2 M HCl at 37 °C for 1 h for detection of CldU-incorporating and/or IdU-incorporating stem and progenitor cells. The denatured sections were neutralised with 0.1 M borate (pH 8.0) twice for 20 min each. The sections were rinsed with washing solution (PBS with 0.2% Triton X-100), and incubated for blocking and permeabilisation in PBS with 2% Triton X-100 and 5% goat serum at room temperature for 2 h. Particularly for the double labeling of CldU and IdU, the brain sections were pre-incubated with rat anti-CldU (1 : 500 dilution; OBT-0030, Accurate Chemicals) for 12 h at room temperature and overnight at 4 °C prior to exposure to other antibodies, in order to lessen cross-reactivity of CldU antigens with the anti-IdU antibody. After rinses with washing solution, the sections were incubated at 4 °C overnight in antibody solution (PBS with 0.2% Triton X-100 and 3% goat serum) containing primary antibodies: chicken anti-GFP (1 : 400 dilution; GFP-1020, Aves Labs), rat anti-CldU, mouse anti-IdU (1 : 300 dilution; 347580, BD Bioscience), rabbit anti-glial fibrillary acidic protein (1 : 500 dilution; Z-0034, Dako), and guinea pig anti-doublecortin (DCX) (1 : 500 dilution; AB2253, Millipore). The primary antibody reaction was extended for 2 h at room temperature, and the sections were rinsed with washing solution. The sections were then incubated for 2 h at room temperature with Alexa Fluor (AF) fluorescent dye-conjugated goat secondary antibody AF-405, AF-488, AF-568, or AF-633 (1 : 400 dilution; Invitrogen). After rinses with washing solution, the sections, mounted on gelatin-coated slide glasses, were cover-slipped over fluorescence mounting media (Dako) for confocal microscopy.

Confocal microscopy and quantification

Quantitative analysis of cell populations was performed by means of design-based stereology, as previously described (Encinas & Enikolopov, 2008; Park & Enikolopov, 2010; Encinas *et al.*, 2011a, b). For quantification of proliferating stem and progenitor cells, we performed confocal microscopy by using a spinning disk confocal microscope (PerkinElmer UltraVIEW Vox; PerkinElmer). The images were taken under × 4 pixel binning, with a 12-bit image depth and a × 40 objective air lens. Optical serial *Z*-stacks were sliced at 1.8-μm intervals, directed from the bottom to the top. To diminish the potent crosstalk that frequently occurs from multiple fluorophore labeling, the entire optical *Z*-stack was imaged for each channel sequentially. The laser power was set to 30% for 3–20 ms of exposure time. Stitched composite images were created with 10% overlap. The same scan conditions were applied to the entire set of sections. The confocal images were further analysed for quantification by using VOLOCITY 6.0.1 (PerkinElmer). Quantification of immunolabeled cells was achieved with several image view options, including extended focus, *Z*-planes, and three-dimensional opacity-elicited rotation. Representative images were collected with an LSM 710 laser scanning microscope (Carl Zeiss). Images were imported into ADOBE PHOTOSHOP 13.0 (Adobe Systems), and were minimally processed to adjust the brightness, contrast, and background.

Statistical analysis

Data are presented as the mean and standard error of the mean. We used Bartlett's test and the Brown–Forsythe test to evaluate the normality of the data distribution, in order to determine whether a parametric (ANOVA) or non-parametric [Kruskal–Wallis (KW) test] test,

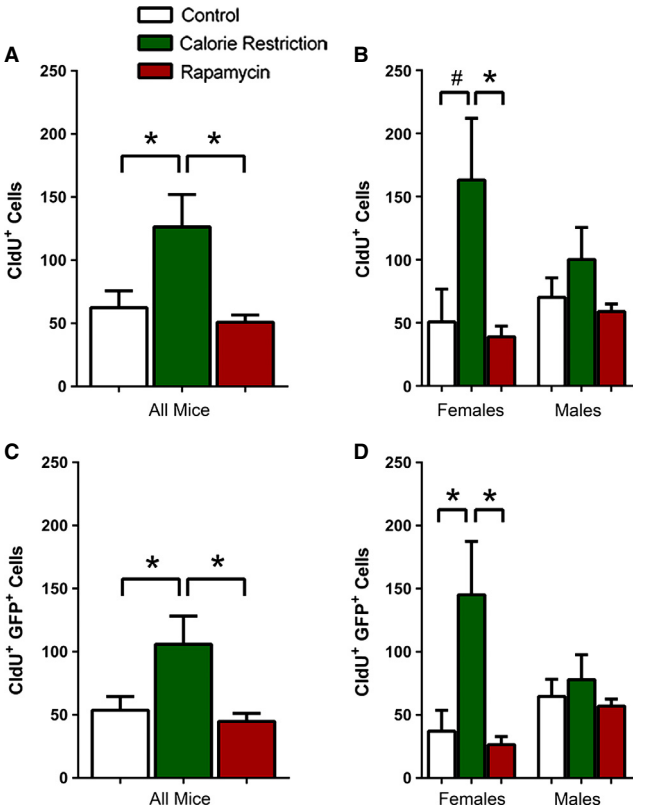

FIG. 1. (A and B) $CldU^+$ cells in all mice (A) and separately in females and males (B) after 12 months of CR or rapamycin treatment. (C and D) $CldU^+$ nestin–GFP-positive cells in all mice (C) and separately in females and males (D) after 12 months of CR or rapamycin treatment. ANOVA and KW tests indicate significance of the changes in all groups except males. #$P < 0.1$ for the difference between the control and the CR groups in B. *$P \leq 0.05$.

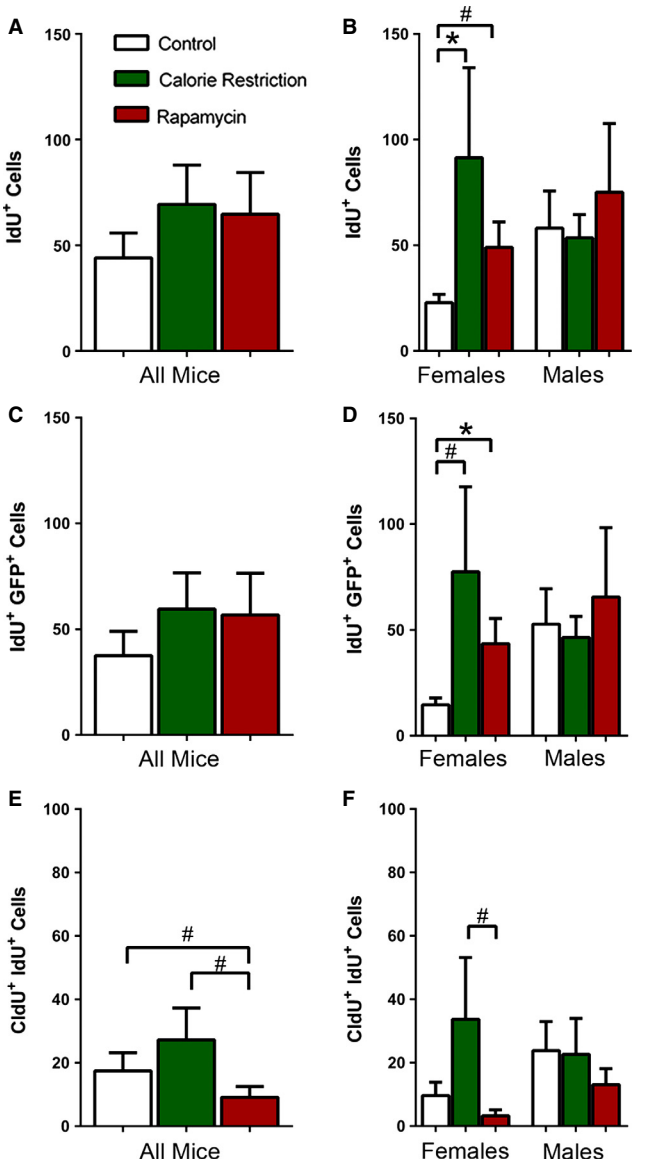

FIG. 2. (A and B) IdU^+ cells in all mice (A) and separately in females and males (B) after 12 months of CR or rapamycin trearment. (C and D) IdU^+ nestin–GFP-positive cells in all mice (C) and separately in females and males (D) after 12 months of CR or rapamycin treatment. (E and F) $CldU^+IdU^+$ cells in all mice (E) and in females and males (F) after 12 months of CR or rapamycin treatment. #$P < 0.1$. *$P \leq 0.05$.

followed by *post hoc* comparisons by the use of *t*-tests or Mann–Whitney tests, was appropriate. Statistical analysis was performed with PRISM6 software (GraphPad Software).

Results

We exposed nestin–GFP mice to standard diet *ad libitum* (control group), a calorie-restricted diet (CR group), or standard diet supplemented with microencapsulated rapamycin (rapamycin group). The treatments started at 6 months, and were carried out for 12 or 22 months. All mice in the three experimental groups survived to 18 months of age (12 months of treatment). We did not see any reliable differences in the lifespans of those mice that died between 18 and 28 months (i.e. between 12 and 22 months of treatment). This is compatible with other lifespan studies, in which 30–40% mice survived to that age (Harrison *et al.*, 2009; Miller *et al.*, 2011) (that is, the timespan and sample size of our experiment would prevent us from observing reliable differences in longevity between the treatment groups). However, we noticed that CR-treated females had a lower incidence of age-related signs and illnesses: whereas most of the mice (31) showed loss of hair, cataracts, tumors, hunchback, dermatitis, or protruding xyphoid, only one of the CR-treated females showed such signs.

We next examined whether long-term (12 months) exposure of adult mice to CR or rapamycin changed the level of hippocampal neurogenesis as judged by the incorporation of the nucleotide analogs CldU and IdU. We injected CldU, and 24 h later determined the total number of $CldU^+$ cells. The number of $CldU^+$ cells in the subgranular zone of the DG was increased in CR mice as compared with both control and rapamycin mice [$KW_{2,29} = 6.498$, $P = 0.039$; CR 2.0-fold increase over control, two-tailed Mann–Whitney test (MW_2), $P = 0.042$; CR 2.5-fold increase over rapamycin, MW_2, $P = 0.025$]. There was no difference between the control and the rapamycin-treated mice (Fig. 1A). However, when we analysed the results for males and females separately, we found that CR increased the number of $CldU^+$ cells in females, but not in males, in relation to the control and rapamycin groups [$KW_{2,10} = 6.267$, $P = 0.032$; CR 3.2-fold increase over control, one-tailed Mann–Whitney test (MW_1), $P = 0.056$; CR 4.2-fold increase over rapamycin, MW_2, $P = 0.016$]. Rapamycin did not affect the $CldU^+$ cell count in females or males (Fig. 1B). Note that, in this and the following series of experiments, the low number of labeled cells

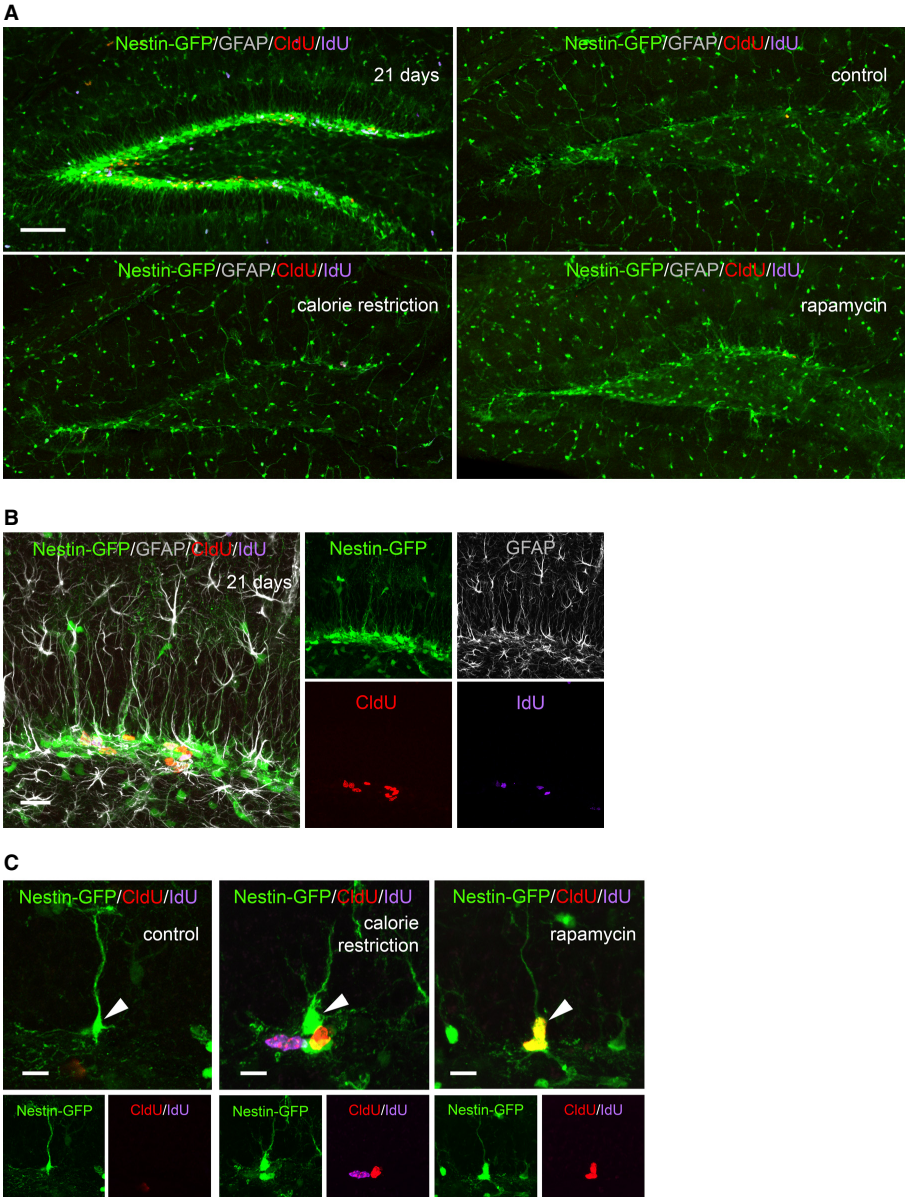

FIG. 3. (A) Dividing stem and progenitor cells after 12 months of CR or rapamycin treatment. The upper left panel shows the DG of young (21 days) nestin–GFP mice for comparison. Note the large numbers of nestin–GFP-positive cells and of dividing $CldU^+$, IdU^+ and $CldU^+IdU^+$ cells in the young, but not the old, brain. (B and C) QNPs change their morphology and division in the DG of old mice (C) as compared with young mice (B; shown here for comparison purposes). Note the large numbers of nestin–GFP-positive cells and of dividing $CldU^+$, IdU^+ and $CldU^+IdU^+$ cells in the young brain. Brain sections were also probed with anti-glial fibrillary acidic protein (GFAP) antibody to reveal astrocytes and the $GFAP^+$ radial processes in QNPs; note the correspondence between the GFP^+ and $GFAP^+$ radial processes. (C) Examples of dividing cells in the vicinity of nestin–GFP-positive QNPs; note the changed morphology of QNPs (arrows) in old mice (thicker processes with increased branching and ramifications). Also, note the $CldU^+IdU^+$ stem and progenitor cells. Markers (nestin–GFP, GFAP, CldU, and IdU) are indicated on the individual panels. Scale bars: 100 μm in A, 20 μm in B, and 10 μm in C.

reflects the advanced age of the analysed mice, with the number of dividing cells in the old rodent brain being over 100 times lower than in young (e.g. 1 month) animals (Kuhn *et al.*, 1996; Cameron & McKay, 1999; Olariu *et al.*, 2007; Encinas *et al.*, 2011b). Such experiments were also carried out after 22 months of treatment (i.e. at 28 months of age), but there were too few dividing cells for reliable estimates to be obtained (not shown).

Incorporation of nucleotide analogs, such as bromodeoxyuridine, CldU, or IdU, marks all cells that were in S-phase of the cell cycle at the time of the nucleotide injection. These include dividing stem cells, transit amplifying progenitor cells, postmitotic neuroblasts, cells undergoing early stages of apoptosis, oligodendrocyte progenitor cells, microglia, pericytes, and endothelial cells of the blood vessels (however, the vast majority of labeled cells correspond to neural progenitors). The nestin–GFP reporter mouse line, in which expression of GFP is controlled by the regulatory elements of the nestin gene, can be used to distinguish neural stem and progenitor cells from other cell types on the basis of their expression of GFP (Mignone *et al.*, 2004; Enikolopov & Overstreet-Wadiche, 2008). Therefore, we next analysed the number of GFP^+ cells that were labeled with CldU (i.e. dividing neural progenitors) in the subgranular zone. The number of $CldU^+GFP^+$ cells was increased in CR-treated

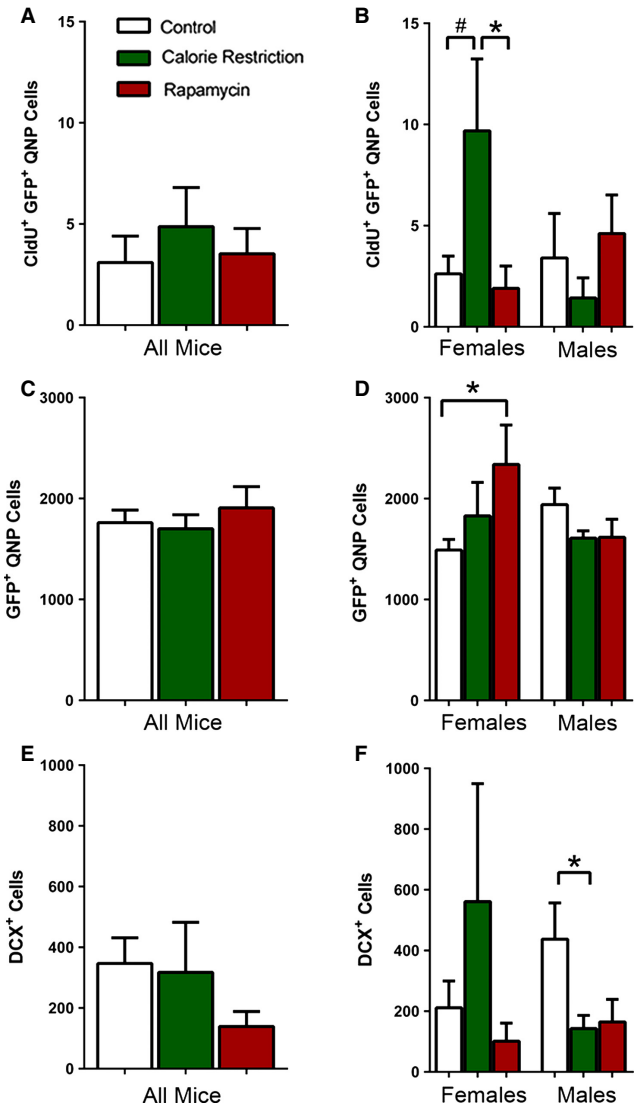

FIG. 4. (A and B) $CldU^+$ nestin–GFP-positive stem cells (QNPs) in all mice (A) and separately in females and males (B) after 12 months of CR or rapamycin treatment. (C and D) Total numbers of nestin–GFP-positive QNPs in all mice (C) and separately in females and males (D) after 12 months of CR or rapamycin treatment. (E and F) DCX^+ neuroblasts and newborn immature neurons in all mice (A) and separately in females and males (B) after 12 months of CR or rapamycin treatment. #$P = 0.06$ in B. *$P \leq 0.05$.

mice as compared with both the control and the rapamycin groups ($KW_{2,29} = 6.702$, $P = 0.035$; CR 2.0-fold over control, MW_1, $P = 0.029$; CR 2.4-fold over rapamycin, MW_2, $P = 0.019$) (Fig. 1C). Characteristically, the number of $CldU^+GFP^+$ cells in all groups was somewhat lower than the total number of $CldU^+$ cells, reflecting the fact that some of the $CldU^+$ cells do not correspond to neural progenitors. As with the total $CldU^+$ cells, the distribution of counts was different when the results in females and males were considered separately. The number of dividing neural progenitors in CR-treated females was increased 3.9-fold as compared with females maintained on the normal diet, and increased 5.6-fold as compared with rapamycin-treated females ($KW_{2,10} = 7.810$, $P = 0.009$; CR 3.9-fold over control, MW_2, $P = 0.032$; CR 5.6-fold over rapamycin, MW_2, $P = 0.016$); there was no difference between control and rapamycin-treated females (Fig. 1D). In contrast, there were no statistically significant differences between the counts from all three groups when only male mice were considered (Fig. 1D). Thus, CR, but not rapamycin, increases the number of dividing neural progenitors in female, but not male, mice after 12 months of treatment.

Cell division can be analysed with higher temporal resolution when a nucleotide label of one type is followed by a label of another type (double S-phase labeling) (Hayes & Nowakowski, 2002; Encinas *et al.*, 2011b). We combined labeling with CldU with subsequent labeling with IdU (22 h after CldU; 2 h before the analysis). Whereas the numbers of IdU^+ cells were lower than the numbers of $CldU^+$ cells (because $CldU^+$ cells had a chance to undergo mitosis and thus increase their number), the general trend for IdU^+ cells matched that of $CldU^+$ cells, with an increase in the number of labeled cells in female, but not male, mice treated with CR (female CR 4.0-fold increase over control, MW_2, $P = 0.032$) (Fig. 2A and B). Likewise, the trend towards an increase in response to CR in female mice was observed when only the GFP^+IdU^+ progenitors were analysed (female CR 5.3-fold increase over control, MW_1, $P = 0.087$); again, there was no change in male mice in response to CR or rapamycin (Fig. 2C and D).

Among $CldU^+$ and IdU^+ cells, a fraction are labeled with both thymidine analogs. These cells correspond to progenitor cells that have undergone a round of division after the first (CldU) labeling and have entered S-phase for the second time at the time of the second label (IdU) injection. For the $CldU^+IdU^+$ cells, the trend was again similar, with an increased number of double-labeled cells in females treated with CR (Fig. 2E and F). These results suggest that 20–40% of cells that were in S-phase at the time of CldU injection underwent another round of DNA synthesis at the time of IdU injection (note, however, that the total number of labeled progenitor cells in the subgranular zone is very low at this age, and the absolute number of double-labeled cells is even lower, complicating the precise analysis and comparison of the fraction of the double-labeled cells between experimental groups).

The majority of GFP^+ cells in the reporter line DG correspond to the amplifying neural progenitors, whereas a smaller fraction correspond to radial glia-like stem cells. These cells are largely quiescent under normal conditions, with only a small fraction of these cells being labeled by thymidine analogs (Encinas *et al.*, 2006, 2011b). These quiescent neural progenitors (QNPs) can be distinguished from the rest of the GFP^+ cells by their morphology and expression of astrocytic markers, even though their morphology changes in the aging mice: the apical process becomes thicker and more branched, with additional ramifications at the top of the process; we present the view of the DG and of individual stem cells in the young brain for comparison (Fig. 3A–C). Whereas the overall number of labeled QNPs was, as expected, very low in all groups, the number was higher in CR-treated females (female CR 3.7-fold increase over control, *t*-test, $P = 0.06$; CR 5.1-fold increase over rapamycin, *t*-test, $P = 0.05$) (Fig. 4A and B). As this increase may reflect a change in either the total number of QNPs or a change in the proliferating fraction of the QNP population, we determined the total number of QNPs. There was no detectable difference in the number of QNPs between genders or types of treatment, except for rapamycin-treated females (however, the fraction of dividing QNPs did not differ from that in the control group) (Fig. 4C and D). This suggests that prolonged exposure to CR does not change the overall number of hippocampal neural stem cells, but that the fraction of dividing stem cells increases in females exposed to CR.

To examine whether the CR-induced increase in division of neural progenitors may later be translated into an increased number of young neurons, we determined the number of DCX^+ cells in the DG of the experimental groups. Although the trend was similar to that

seen with dividing progenitors, there was no significant difference in the number of DCX^+ cells (except for a decrease in the CR-treated males) (Fig. 4E and F).

Discussion

Our results indicate that prolonged exposure to CR increases both the total number of dividing cells and the number of dividing neural stem and progenitor cells in the DG of adult female mice. Our study also support the notion that the age-dependent decrease in progenitor cell division is, to a large degree, attributable to the loss of stem cells rather than solely a decrease in their ability to divide and produce progeny (Olariu *et al.*, 2007; Encinas *et al.*, 2011b). Interestingly, whereas the number of dividing stem cells drastically increased in response to CR, the overall number of stem cells did not change. This may suggest that a larger fraction of the stem cell pool is recruited to division in the CR-treated female brain; however, with a series of divisions acting as a signal for a stem cell to convert into an astrocyte, this mechanism would imply rapid exhaustion of the total stem cell pool specifically in the CR group (Encinas *et al.*, 2011b; Encinas & Sierra, 2012). Another possibility is that CR induces symmetric divisions of stem cells; however, if this happened during the entire period of CR treatment, one would expect an increase in the total number of stem cells, which was not the case (unless the resulting increase in stem cells is perfectly balanced by an increased loss of these or other stem cells). A model that is more compatible with our experimental results is that the fraction of the stem cell pool involved in division is the same, but each stem cell undergoes more asymmetric divisions; in this case, a larger number of stem cells would be labeled after a single injection of the label, but the total number of stem cells would not change. This mechanism could also explain other situations in which an increased number of dividing cells is not matched by the changes in the stem cell pool.

The increase in the number of dividing stem and progenitor cells in CR-treated females was not observed in the population of advanced progenitors, neuroblasts and immature neurons that express DCX (although the findings were compatible with the general trend of an increase). This suggests that the CR-induced increase in progenitor cell proliferation is not fully translated into the generation of mature new neurons. However, it should be noted that the maturation of young neurons is controlled independently of progenitor cell proliferation (Plumpe *et al.*, 2006). Further studies, perhaps by using a more targeted immunohistochemical approach, fate-mapping, or behavioral analysis, will help to clarify this issue.

Calorie restriction and rapamycin have both been shown to increase the lifespan of a variety of species (Harrison *et al.*, 2009; Anderson & Weindruch, 2010; Houtkooper *et al.*, 2010). In addition, rapamycin is an effector of the mTOR signaling pathway, the same pathway through which CR affects tissue maintenance and remodeling (Anderson & Weindruch, 2010; Houtkooper *et al.*, 2010). Thus, it may be surprising that we did not observe obvious effects of rapamycin either on the basal level of progenitor cell division or on the size of the stem cell pool in female or male mice (except for an increase in the total number of QNPs in rapamycin-treated females; however, this increase was not translated into an increased number of dividing progenitors or DCX^+ immature neurons). Our results may indicate that CR relies on some additional signaling pathway(s) besides the mTOR pathway to support higher levels of hippocampal neurogenesis, and that this pathway(s) is not shared with rapamycin.

Importantly, the CR-induced increase in the number of dividing cells, including dividing neural stem cells, was observed only in female mice. Although there is no evident gender difference in the basal level of hippocampal neurogenesis in young adult mice (Lagace *et al.*, 2007), the situation may be less clear in old animals, where changes in hormonal levels may affect neurogenesis in males and females to different degrees. Our results concerning the gender difference in response to CR may reflect the fact that the gonadal hormones have profound effects on neurogenesis (Tanapat *et al.*, 1999; Galea, 2008), and that reproductive function and sex hormone levels decrease earlier in females than in males. As CR reduces the manifestation of physiological age-related changes (Harrison *et al.*, 2009; Anderson & Weindruch, 2010, 2012), it is possible that slower impairment of the female hormone status acts to support a higher level of hippocampal neurogenesis even in older CR females. Interestingly, although the number of analysed animals was not sufficient for statistically significant estimates to be made, females exposed to CR not only had more robust neurogenesis, but also showed fewer signs of age-related pathologies (alopecia, dermatitis, cataracts, or tumors).

The neural stem cell pool may not be the only target of CR: for instance, even a shorter exposure to CR increases the availability and activity of skeletal muscle stem cells in old animals (Cerletti *et al.*, 2012). Notably, the GFP signal in the nestin–GFP mouse line, while faithfully reporting the stem and progenitor status of cells of the neural lineage, also highlights stem and progenitor cells of a number of other lineages, e.g. in the anterior pituitary, liver, hair follicle, muscle, and bone marrow (Gleiberman *et al.*, 2005, 2008; Day *et al.*, 2007; Mignone *et al.*, 2007; Mendez-Ferrer *et al.*, 2010); this would allow a parallel analysis of the effects of CR and rapamycin on stem and progenitor populations in several tissues of the same animal.

Changes in hippocampal neurogenesis are linked to changes in cognitive function, with increased levels of neurogenesis usually corresponding to improved performance in learning and memory tests and reduced anxiety (Sahay & Hen, 2007; Aimone *et al.*, 2011; Ming & Song, 2011; Sahay *et al.*, 2011). In the future, it will be interesting to examine a possible correlation between the number of dividing stem cells and the number of young neurons in individual mice after different periods of CR, and their performance in behavioral tasks, to determine whether life-long or even short-term CR may benefit the cognitive function of aging animals.

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Abbreviations

AF, Alexa Fluor; CldU, 5-chloro-2-deoxyuridine; CR, calorie restriction; DCX, doublecortin; DG, dentate gyrus; GFP, green fluorescent protein; IdU, 5-iodo-2-deoxyuridine; KW, Kruskal–Wallis; mTOR, mammalian target of rapamycin; MW_1, one-tailed Mann–Whitney test; MW_2, two-tailed Mann–Whitney test; PBS, phosphate-buffered saline; QNP, quiescent neural progenitor.

References

Aimone, J.B., Deng, W. & Gage, F.H. (2011) Resolving new memories: a critical look at the dentate gyrus, adult neurogenesis, and pattern separation. *Neuron*, **70**, 589–596.

Anderson, R.M. & Weindruch, R. (2010) Metabolic reprogramming, caloric restriction and aging. *Trends Endocrin. Met.*, **21**, 134–141.

Anderson, R.M. & Weindruch, R. (2012) The caloric restriction paradigm: implications for healthy human aging. *Am. J. Hum. Biol.*, **24**, 101–106.
Bonaguidi, M.A., Wheeler, M.A., Shapiro, J.S., Stadel, R.P., Sun, G.J., Ming, G.L. & Song, H. (2011) In vivo clonal analysis reveals self-renewing and multipotent adult neural stem cell characteristics. *Cell*, **145**, 1142–1155.
Bondolfi, L., Ermini, F., Long, J.M., Ingram, D.K. & Jucker, M. (2004) Impact of age and caloric restriction on neurogenesis in the dentate gyrus of C57BL/6 mice. *Neurobiol. Aging*, **25**, 333–340.
Cameron, H.A. & McKay, R.D. (1999) Restoring production of hippocampal neurons in old age. *Nat. Neurosci.*, **2**, 894–897.
Cerletti, M., Jang, Y.C., Finley, L.W., Haigis, M.C. & Wagers, A.J. (2012) Short-term calorie restriction enhances skeletal muscle stem cell function. *Cell Stem Cell*, **10**, 515–519.
Day, K., Shefer, G., Richardson, J.B., Enikolopov, G. & Yablonka-Reuveni, Z. (2007) Nestin-GFP reporter expression defines the quiescent state of skeletal muscle satellite cells. *Dev. Biol.*, **304**, 246–259.
Deng, W., Aimone, J.B. & Gage, F.H. (2010) New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? *Nat. Rev. Neurosci.*, **11**, 339–350.
Encinas, J.M. & Enikolopov, G. (2008) Identifying and quantitating neural stem and progenitor cells in the adult brain. *Method. Cell Biol.*, **85**, 243–272.
Encinas, J.M. & Sierra, A. (2012) Neural stem cell deforestation as the main force driving the age-related decline in adult hippocampal neurogenesis. *Behav. Brain Res.*, **227**, 433–439.
Encinas, J.M., Vaahtokari, A. & Enikolopov, G. (2006) Fluoxetine targets early progenitor cells in the adult brain. *Proc. Natl. Acad. Sci. USA*, **103**, 8233–8238.
Encinas, J.M., Hamani, C., Lozano, A.M. & Enikolopov, G. (2011a) Neurogenic hippocampal targets of deep brain stimulation. *J. Comp. Neurol.*, **519**, 6–20.
Encinas, J.M., Michurina, T.V., Peunova, N., Park, J.H., Tordo, J., Peterson, D.A., Fishell, G., Koulakov, A. & Enikolopov, G. (2011b) Division-coupled astrocytic differentiation and age-related depletion of neural stem cells in the adult hippocampus. *Cell Stem Cell*, **8**, 566–579.
Enikolopov, G. & Overstreet-Wadiche, L. (2008) Transgenic reporter lines for studying adult neurogenesis. In Gage, F.H., Kempermann, G. & Song, H. (Eds), *Adult Neurogenesis*. CSHL Press, pp. 81–100.
Fok, W., Zhang, Y., Salmon, A.B., Bhattacharya, A., Gunda, R., Jones, D., Ward, W.F., Richardson, A. & Perez, V. (2012) Short-term treatment with rapamycin and dietary restriction have overlapping and distinctive effects in young mice. *J. Gerontol. A-Biol.*, **68**, 108–116.
Galea, L.A. (2008) Gonadal hormone modulation of neurogenesis in the dentate gyrus of adult male and female rodents. *Brain Res. Rev.*, **57**, 332–341.
Gleiberman, A.S., Encinas, J.M., Mignone, J.L., Michurina, T., Rosenfeld, M.G. & Enikolopov, G. (2005) Expression of nestin–green fluorescent protein transgene marks oval cells in the adult liver. *Dev. Dynam.*, **234**, 413–421.
Gleiberman, A.S., Michurina, T., Encinas, J.M., Roig, J.L., Krasnov, P., Balordi, F., Fishell, G., Rosenfeld, M.G. & Enikolopov, G. (2008) Genetic approaches identify adult pituitary stem cells. *Proc. Natl. Acad. Sci. USA*, **105**, 6332–6337.
Harrison, D.E., Strong, R., Sharp, Z.D., Nelson, J.F., Astle, C.M., Flurkey, K., Nadon, N.L., Wilkinson, J.E., Frenkel, K., Carter, C.S., Pahor, M., Javors, M.A., Fernandez, E. & Miller, R.A. (2009) Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature*, **460**, 392–395.
Hattiangady, B. & Shetty, A.K. (2008) Aging does not alter the number or phenotype of putative stem/progenitor cells in the neurogenic region of the hippocampus. *Neurobiol. Aging*, **29**, 129–147.
Hayes, N.L. & Nowakowski, R.S. (2002) Dynamics of cell proliferation in the adult dentate gyrus of two inbred strains of mice. *Brain Res. Dev. Brain Res.*, **134**, 77–85.
Houtkooper, R.H., Williams, R.W. & Auwerx, J. (2010) Metabolic networks of longevity. *Cell*, **142**, 9–14.
Kuhn, H.G., Dickinson-Anson, H. & Gage, F.H. (1996) Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. *J. Neurosci.*, **16**, 2027–2033.
Lagace, D.C., Fischer, S.J. & Eisch, A.J. (2007) Gender and endogenous levels of estradiol do not influence adult hippocampal neurogenesis in mice. *Hippocampus*, **17**, 175–180.
Leuner, B., Kozorovitskiy, Y., Gross, C.G. & Gould, E. (2007) Diminished adult neurogenesis in the marmoset brain precedes old age. *Proc. Natl. Acad. Sci. USA*, **104**, 17169–17173.
Lugert, S., Basak, O., Knuckles, P., Haussler, U., Fabel, K., Gotz, M., Haas, C.A., Kempermann, G., Taylor, V. & Giachino, C. (2010) Quiescent and active hippocampal neural stem cells with distinct morphologies respond selectively to physiological and pathological stimuli and aging. *Cell Stem Cell*, **6**, 445–456.
Mendez-Ferrer, S., Michurina, T.V., Ferraro, F., Mazloom, A.R., Macarthur, B.D., Lira, S.A., Scadden, D.T., Ma'ayan, A., Enikolopov, G.N. & Frenette, P.S. (2010) Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature*, **466**, 829–834.
Mercken, E.M., Carboneau, B.A., Krzysik-Walker, S.M. & de Cabo, R. (2012) Of mice and men: the benefits of caloric restriction, exercise, and mimetics. *Ageing Res. Rev.*, **11**, 390–398.
Mignone, J.L., Kukekov, V., Chiang, A.S., Steindler, D. & Enikolopov, G. (2004) Neural stem and progenitor cells in nestin-GFP transgenic mice. *J. Comp. Neurol.*, **469**, 311–324.
Mignone, J.L., Roig-Lopez, J.L., Fedtsova, N., Schones, D.E., Manganas, L.N., Maletic-Savatic, M., Keyes, W.M., Mills, A.A., Gleiberman, A., Zhang, M.Q. & Enikolopov, G. (2007) Neural potential of a stem cell population in the hair follicle. *Cell Cycle*, **6**, 2161–2170.
Miller, R.A., Harrison, D.E., Astle, C.M., Baur, J.A., Boyd, A.R., de Cabo, R., Fernandez, E., Flurkey, K., Javors, M.A., Nelson, J.F., Orihuela, C.J., Pletcher, S., Sharp, Z.D., Sinclair, D., Starnes, J.W., Wilkinson, J.E., Nadon, N.L. & Strong, R. (2011) Rapamycin, but not resveratrol or simvastatin, extends life span of genetically heterogeneous mice. *J. Gerontol. A-Biol.*, **66**, 191–201.
Ming, G.L. & Song, H. (2011) Adult neurogenesis in the mammalian brain: significant answers and significant questions. *Neuron*, **70**, 687–702.
Olariu, A., Cleaver, K.M. & Cameron, H.A. (2007) Decreased neurogenesis in aged rats results from loss of granule cell precursors without lengthening of the cell cycle. *J. Comp. Neurol.*, **501**, 659–667.
Paliouras, G.N., Hamilton, L.K., Aumont, A., Joppe, S.E., Barnabe-Heider, F. & Fernandes, K.J. (2012) Mammalian target of rapamycin signaling is a key regulator of the transit-amplifying progenitor pool in the adult and aging forebrain. *J. Neurosci.*, **32**, 15012–15026.
Park, J.H. & Enikolopov, G. (2010) Transient elevation of adult hippocampal neurogenesis after dopamine depletion. *Exp. Neurol.*, **222**, 267–276.
Plumpe, T., Ehninger, D., Steiner, B., Klempin, F., Jessberger, S., Brandt, M., Romer, B., Rodriguez, G.R., Kronenberg, G. & Kempermann, G. (2006) Variability of doublecortin-associated dendrite maturation in adult hippocampal neurogenesis is independent of the regulation of precursor cell proliferation. *BMC Neurosci.*, **7**, 77.
Sahay, A. & Hen, R. (2007) Adult hippocampal neurogenesis in depression. *Nat. Neurosci.*, **10**, 1110–1115.
Sahay, A., Wilson, D.A. & Hen, R. (2011) Pattern separation: a common function for new neurons in hippocampus and olfactory bulb. *Neuron*, **70**, 582–588.
Samuels, B.A. & Hen, R. (2011) Neurogenesis and affective disorders. *Eur. J. Neurosci.*, **33**, 1152–1159.
Stangl, D. & Thuret, S. (2009) Impact of diet on adult hippocampal neurogenesis. *Genes Nutr.*, **4**, 271–282.
Tanapat, P., Hastings, N.B., Reeves, A.J. & Gould, E. (1999) Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. *J. Neurosci.*, **19**, 5792–5801.
Zhao, C., Deng, W. & Gage, F.H. (2008) Mechanisms and functional implications of adult neurogenesis. *Cell*, **132**, 645–660.