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Calorie Restriction Alleviates 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 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 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 show that CR increases the number of dividing cells in the dentate gyrus (DG) 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.

Keywords

adult neurogenesis; stem cells; aging; calorie restriction; rapamycin

Introduction

Adult hippocampal neurogenesis is linked to behavior (e.g., facilitating pattern separation in similar contexts), response to therapy (e.g., mediating 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 proliferative activity of specific subpopulations of stem cells (Lugert *et al.*, 2010). Radial glia-like hippocampal neural stem cells are largely quiescent in the adulthood. Our results suggest that upon activation they undergo a rapid series of divisions followed by astrocytic differentiation and, while physically still present in the 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 getting rise to new neurons may help to ameliorate the age-related cognitive deficits.

Restriction of calorie intake can increase the lifespan of a diverse range of species, including mammals (Anderson & Weindruch, 2010; Houtkooper *et al.*, 2010; Anderson & Weindruch, 2012; Mercken *et al.*, 2012). Lifespan can also be significantly extended by prolonged treatment with the 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 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 based on their expression of the 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.

Materials and Methods

Animals

Nestin-GFP reporter mouse line is described in (Mignone *et al.*, 2004). Nestin-GFP homozygous transgenic mice, previously backcrossed to C57BL/6 animals for over 10 generations, were bred and initially housed in the animal facility of Cold Spring Harbor Laboratory in a standard light- and temperature-controlled environment (12-hour light/dark cycle; light on at 7:00 AM; 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. Prior to six months of age, all mice were maintained with access to standard Purina Mills Test diet *ad libitum*. Starting at 6 months, mice were switched onto 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). Control group received standard diet *ad libitum*. CR was established by feeding mice 80% of *ad libitum* consumption for one week and then lowering it to 60% for the remainder of the study (Fok, 2012). Mice in the rapamycin group were fed *ad libitum* the Purina Mills chow containing 14 ppm of encapsulated rapamycin in the diet as described (Harrison *et al.*, 2009; Miller *et al.*, 2011; Fok, 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 sacrificed for analysis (control n=12: 5 females and 7 males; CR n=12: 5 females and 7 males; rapamycin n=12, 6 females and 6 males). Diet treatment on the remaining mice was continued for an additional 10 months, at which point all remaining mice were sacrificed for analysis.

All mice were monitored daily for the 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 which 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 euthanized humanely.

Mouse maintenance was following the guidelines for the use and treatment of laboratory animals from the National Institutes of Health and all procedures were approved by Animal

Care and Use Committees of Cold Spring Harbor Laboratory and University of Texas Health Science Center.

Double S phase pulse labeling of dividing cells and immunohistochemistry

To label proliferating cells, all mice received single injections of 5-chloro-2-deoxyuridine (CldU) (128 mg/kg, i.p.; Sigma) and 5-iodo-2-deoxyuridine (IdU) (173 mg/kg, i.p.; Sigma) 24 hr and 2 hr prior to euthanasia, respectively. After an overdose of anesthetic the mice were transcardially perfused with 30 ml of phosphate buffered saline (PBS) and 30 ml of 4% paraformaldehyde (PFA) in PBS, pH 7.4. The extracted brains were further post-fixed in 4% PFA 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 50 µm thickness by using a vibratome (Vibratome). The brain sections were sequentially collected and subsets of sections at 100 µm intervals were taken for immunohistochemistry and analysis. Brains of 4 mice (of the total 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 2N HCl at 37°C for 1 hr for detection of CldU- and/or IdU-incorporating stem and progenitor cells. The denatured sections were neutralized with 0.1 M borate, pH 8.0 twice for 20 min. The sections were rinsed with washing solution (PBS with 0.2% Triton X-100) and incubated for blocking and permeabilization in PBS with 2% Triton X-100 and 5% goat serum (GS) at room temperature (RT) for 2 hr. Particularly for the double labeling of CldU and IdU, the brain sections were pre-incubated with rat anti-CldU (Accurate Chemicals, OBT-0030; 1:500 dilution) for 12 hr at RT 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% GS) containing primary antibodies: chicken anti-GFP (Aves Labs, GFP-1020; 1:400 dilution), rat anti-CldU (Accurate Chemicals, OBT-0030; 1:500 dilution), mouse anti-IdU (BD Bioscience, 347580; 1:300 dilution), rabbit anti-GFAP (Dako, Z-0034; 1:500 dilution), and guinea pig anti-doublecortin (DCX) (Millipore, AB2253, 1:500 dilution). The primary antibody reaction was extended for 2 hr at RT and the sections were rinsed with washing solution. The sections were then incubated for 2 hr at RT with Alexa Fluor (AF) fluorescent dye-conjugated goat secondary antibodies AF-405, AF-488, AF-568, or AF-633 (Invitrogen; 1:400 dilution). 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 described (Encinas & Enikolopov, 2008; Park & Enikolopov, 2010; Encinas *et al.*, 2011a; Encinas *et al.*, 2011b). 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 x4 pixel binning, with 12 bits image depth, and the 40x objective air lens. Optical serial Z stack were sliced at 1.8 µm intervals, directed from the bottom to the top. To diminish the potent crosstalk which 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 analyzed for quantification by using Velocity 6.0.1 (PerkinElmer). Quantification of immunolabeled cells was achieved by several image view options, including extended focus, Z planes, and 3D opacity-elicited rotation. Representative images were collected using a laser scanning microscope LSM 710 (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 S.E.M. We used the 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 nonparametric (Kruskal-Wallis test) test, followed by post-hoc comparisons using t-tests or Mann-Whitney tests, was appropriate. Statistical analysis was performed using Prism6 software (GraphPad Software, Inc.).

Results

We exposed Nestin-GFP mice to standard diet *ad libitum* (control group), 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 three experimental groups survived by 18 months of age (12 months of treatment). We did not see any reliable difference in the lifespan of those mice that died between 18 and 28 months (i.e., between 12 and 22 months treatment time points). This is compatible with other lifespan studies where 30-40% mice survived at that age (Harrison *et al.*, 2009; Miller *et al.*, 2011) (i.e., the timespan and sample size of our experiment would prevent observing reliable differences in longevity between the treatment groups). However, we noticed that CR-exposed females had lower incidence of age-related signs and illnesses: when most of the animals (31 mice) showed loss of hair, cataracts, tumors, hunchback, dermatitis, or protruding xyphoid, only one of the CR-exposed females showed such signs.

We next examined whether long term (12 months) exposure of adult mice to CR or rapamycin can change the level of hippocampal neurogenesis as judged by the incorporation of nucleotide analogues CldU and IdU. We injected CldU and 24 hr later determined the total number of CldU-positive cells. The number of CldU⁺ cells in the subgranular zone (SGZ) of the DG was increased in CR animals compared to both control and Rapamycin ($KW_{2,29}=6.498$, $P=0.039$; CR 2.0 fold increase over Control $MW_2-P=0.042$; CR 2.5 fold increase over Rapamycin $MW_2-P=0.025$). There was no difference between the control and the rapamycin-fed mice (Fig. 1A). However, when we analyzed the results for males and females separately, we found that CR increased the number of CldU-positive 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, $MW_1-P=0.056$; CR 4.2 fold increase over rapamycin, $MW_2-P=0.016$). Rapamycin did not affect the CldU-positive cell count in females or males (Fig. 1B). Note that in this and following series of experiments the low number of labeled cells reflects the advanced age of the analyzed animals, with the number of dividing cells in old rodent brain over hundred times lower than in the young (e.g., 1 month old) 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 to get reliable estimates (not shown).

Incorporation of nucleotide analogues, such as BrdU, CldU, or IdU, marks all cells that were in S phase of the cell cycle at the time of the nucleotide injection. This includes 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). 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 based on their expression of GFP (Mignone

et al., 2004; Enikolopov & Overstreet-Wadiche, 2008). Therefore, we next analyzed the number of GFP-expressing cells which were labeled with CldU (i.e., dividing neural progenitors) in the SGZ. The number of CldU⁺GFP⁺ cells was increased CR-exposed mice compared to both the control and the rapamycin-treated 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-positive cells do not correspond to neural progenitors. Similar to 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-exposed females was increased 3.9 fold compared to females maintained on the normal diet and 5.6 fold compared to the 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 the control and the rapamycin-treated females (Fig. 1D). In contrast, there was no statistically significant difference between the counts from all three groups when only the 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 analyzed 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 hr after CldU, 2 hr before the analysis). While the numbers of IdU cells were less than those for CldU (because CldU-labeled cells had a chance to undergo mitoses and thus increase their number), the general trend for IdU labeled cells matched that of CldU cells, with an increase in the number of labeled cells in female, but not male, mice exposed to CR (*Female CR 4.0 fold increase over control*, $MW_2-P=0.032$) (Fig. 2A, B). Likewise, the trend towards an increase in response to CR in female mice was observed when only the GFP-expressing IdU-labeled progenitors were analyzed (*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,D).

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

The majority of GFP-labeled cells in the reporter line DG correspond to the amplifying neural progenitors, while a smaller fraction corresponds to radial glia-like stem cells. These cells are largely quiescent under normal conditions, with only a small fraction of these cells labeled by thymidine analogs (Encinas *et al.*, 2006; Encinas *et al.*, 2011b). These quiescent neural progenitors (QNP) can be distinguished from the rest of GFP-expressing 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 dentate gyrus o and of individual stem cells in the young brain for comparison (Fig. 3A-C). While the overall number of labeled QNPs was, as expected, very low in all groups, their number was higher

in CR-exposed females (*Females CR 3.7 fold increase over control t -P=0.06; CR 5.1 fold increase over rapamycin t -P=0.05*) (Fig. 4A,B). Since such increase may reflect a change in either the total number of QNPs or 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 the control group) (Fig. 4C,D). This suggests that prolonged exposure to CR does not change the overall number of hippocampal neural stem cells but 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 be later translated into an increased number of young neurons, we determined the number of doublecortin (DCX)-positive cells in the DG of the experimental groups. While the trend was similar to the results with dividing progenitors, there was no significant difference in the number of DCX-expressing cells (except for a decrease in the CR-exposed males) (Fig. 4E,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 age-dependent decrease in progenitor cell division is to a large degree due 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, while the number of dividing stem cells has 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 in 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 a 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 happens 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 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 will appear 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 where 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 which express DCX (although compatible with the general trend for 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 maturation of young neurons is controlled independently of progenitor cell proliferation (Plumpe *et al.*, 2006). Further studies, perhaps by using a more targeted immunohistochemical approaches, fate-mapping, or behavioral analysis, will help to clarify this issue.

CR 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 QNP cells in rapamycin-treated females; however, this increase is not translated in the increased number of dividing progenitors or DCX-positive progenitors). —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. While 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 a different degree. Our results on the gender difference in response to CR may reflect the fact that the gonadal hormones have profound effect of neurogenesis (Tanapat *et al.*, 1999; Galea, 2008) and that the reproductive function and sex hormones levels decrease earlier in females than in males. Since CR reduces the manifestation of physiological age-related changes (Harrison *et al.*, 2009; Anderson & Weindrich, 2010; 2012), it is possible that a slower impairment of the female hormone status acts to support a higher level of hippocampal neurogenesis even in older females. Interestingly, while the number of analyzed animals was not sufficient for statistically significant estimates, females exposed to CR not only had more robust neurogenesis, but showed less 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; Day *et al.*, 2007; Mignone *et al.*, 2007; Gleiberman *et al.*, 2008; 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 young neurons in individual mice after different periods of CR of and their performance in behavioral tasks, to determine whether life-long or even short-term CR may benefit the cognitive function of the aging animals.

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Abbreviations

CldU	5-chloro-2-deoxyuridine
CR	calorie restriction
DCX	doublecortin
DG	dentate gyrus
GS	goat serum
IdU	5-iodo-2-deoxyuridine
PFA	paraformaldehyde
PBS	phosphate buffered saline
QNP	quiescent neural progenitor
RT	room temperature
SGZ	subgranular zone
MW₂	two-tailed Mann-Whitney test

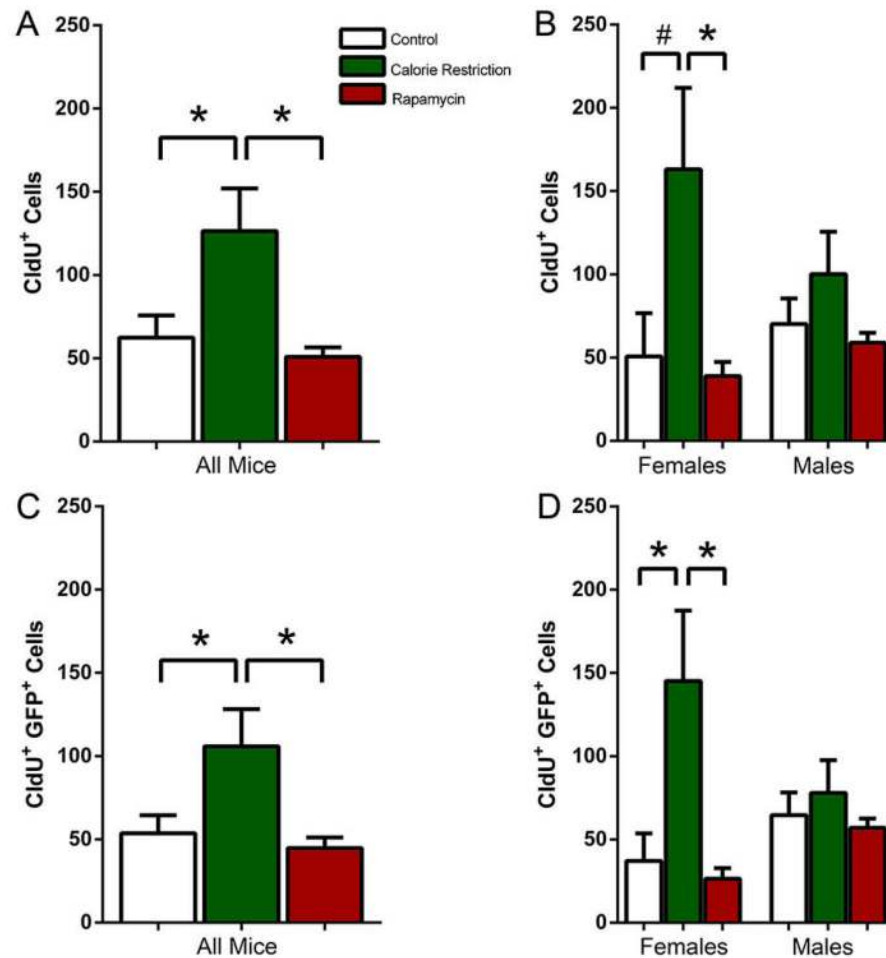


Figure 1.

(A, B) CldU-labeled cells in all mice (A) and separately in females and males (B) after 12 months exposure to CR or rapamycin. (C, D) CldU-labeled Nestin-GFP-positive cells in all mice (C) and separately in females and males (D) after 12 months exposure to CR or rapamycin. ANOVA and Kruskal Wallis tests indicate significance of the changes in all groups except males. # corresponds to $p < 0.1$ for difference between the control and the CR groups in B. * corresponds to $p \leq 0.05$.

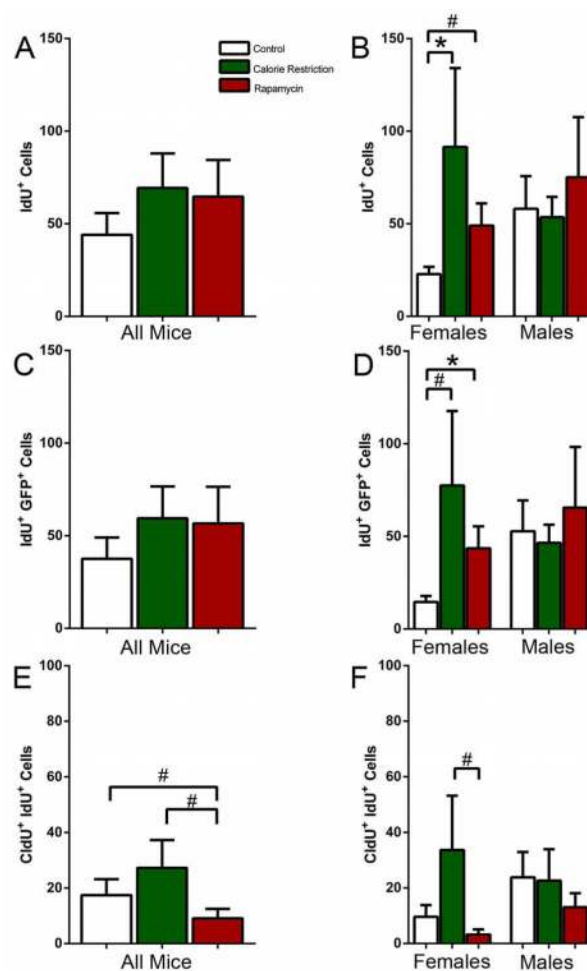


Figure 2.

(A, B) IdU-labeled cells in all mice (A) and separately in females and males (B) after 12 months exposure to CR or rapamycin. (C, D) IdU-labeled Nestin-GFP-positive cells in all mice (C) and separately in females and males (D) after 12 months exposure to CR or rapamycin. (E, F) Double labeled CldU⁺IdU⁺ cells in all mice (E) and in females and males (F) after 12 months exposure to CR or rapamycin. # corresponds to $p < 0.1$, * corresponds to $p \leq 0.05$.

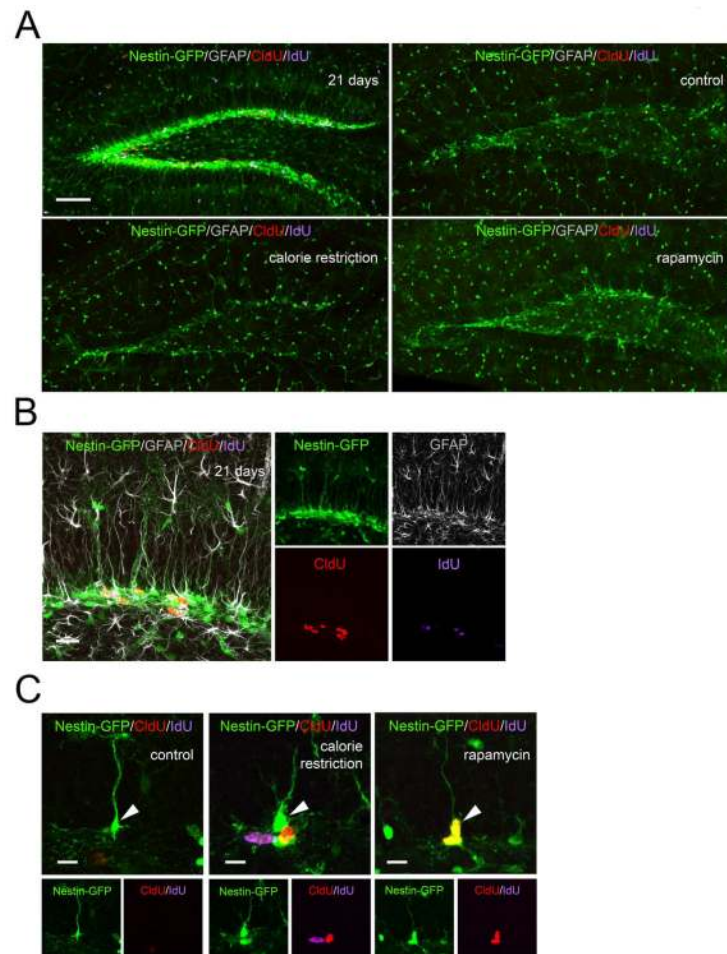
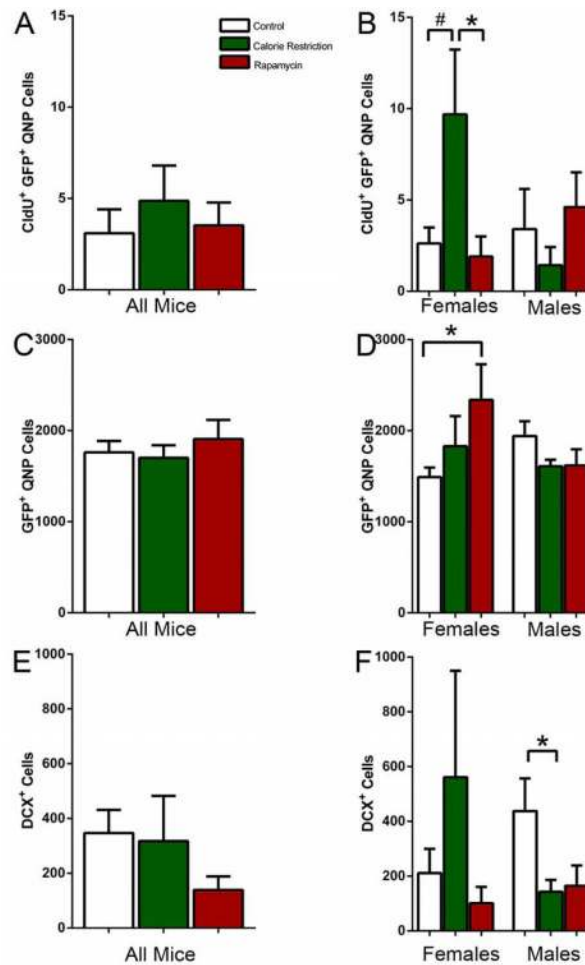


Figure 3.

(A) Dividing stem and progenitor cells after 12 months of CR or rapamycin treatment. Upper left panel shows the DG of young (21 days) Nestin-GFP mice for comparison. Note the large number of Nestin-GFP cells and of dividing CldU-positive, IdU-positive, and CldU/IdU-double-positive cells in the young, but not the old, brain. (B, C) QNPs change their morphology and division in the DG of old mice (C) compared to young mice (B; shown here for comparison purposes). Note the large number of Nestin-GFP cells and of dividing CldU-positive, IdU-positive, and CldU/IdU-double-positive cells in the young brain. Brain sections were also probed with anti-GFAP antibody to reveal astrocytes and the GFAP-positive radial processes in QNP cells; note the correspondence between the GFP-positive and GFAP-positive radial processes. In (C) are examples of dividing cells in the vicinity of Nestin-GFP QNPs cells; note the changed morphology of QNPs (arrows) in old mice (thicker processes with increased branching and ramifications). Also note double labeled CldU⁺/IdU⁺ stem and progenitor cells. Markers (Nestin-GFP, GFAP, CldU, and IdU) are indicated on the individual panels. Scale bar is 100 μm in (A), 20 μm in (B), and 10 μm in (C).

**Figure 4.**

(**A, B**) CldU-labeled Nestin-GFP positive stem cells (QNPs) in all mice (**A**) and separately in females and males (**B**) after 12 months exposure to CR or rapamycin. (**C, D**) Total number of Nestin-GFP-positive QNP cells in all mice (**C**) and separately in females and males (**D**) after 12 months exposure to CR or rapamycin. (**E, F**) DCX-labeled neuroblasts and newborn immature neurons in all mice (**A**) and separately in females and males (**B**) after 12 months exposure to CR or rapamycin. # corresponds to $p=0.06$ in **B**; * corresponds to $p \leq 0.05$.