Environmental stimulation of 129/SvJ mice causes increased cell proliferation and neurogenesis in the adult dentate gyrus

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New neurons are continuously born in the dentate gyrus of the adult mouse hippocampus, and regulation of adult neurogenesis is influenced by both genetic and environmental determinants. Mice of the 129/SvJ strain have significantly less hippocampal neurogenesis than other inbred mouse strains [1] and do not perform well in learning tasks. Here, the impact of environmental stimuli on brain plasticity during adulthood of 129/SvJ mice was studied using 'enriched environments' where mice receive complex inanimate and social stimulation [2,3]. In contrast to our earlier reports on mice of the C57BL/6 strain – which are competent in learning tasks and in which environmental stimulation did not influence cell proliferation [4,5] – environmentally stimulated 129/SvJ mice were found to have twice as many proliferating cells in the dentate gyrus compared with mice in standard housing. Environmental stimulation fostered the survival of newborn cells in 129/SvJ mice; this effect had also been seen in C57BL/6 mice. Phenotypic analysis of the surviving cells revealed that environmental stimulation resulted in 67% more new neurons. In combination with our earlier results, these data indicate a differential impact of inheritable traits on the environmental regulation of adult hippocampal neurogenesis. In addition, we observed behavioral changes in environmentally stimulated 129/SvJ mice.

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Results and discussion

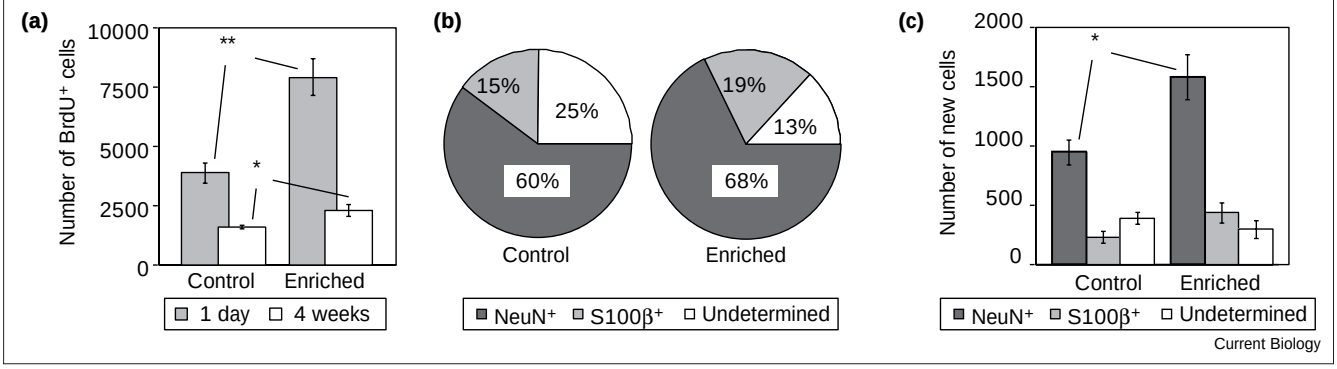
Mice of the 129/SvJ strain spent 40 days in an enriched environment and were given daily injections of the proliferation marker bromodeoxyuridine (BrdU; 50 µg per g body weight) during the last 12 days of this period. The brains of five animals from this experimental group (Enr-129), and five animals from a control group (Ctr-129) that had been kept in standard housing, were then examined immunohistochemically. In contrast to C57BL/6 mice, which do not show any change in proliferation in response to environmental stimulation [4,5], Enr-129 mice had a significantly greater number of BrdU-labeled cells in the subgranular zone of the dentate gyrus than Ctr-129 mice (Figures 1a,2; $p = 0.0017$). This finding of increased 'proliferative activity' can be interpreted as, firstly, an increased number of dividing cells, secondly, a higher rate of cell division or, thirdly, a combination of both.

At 4 weeks after the last injection of BrdU, when the brains of the remaining mice were examined, significantly more BrdU-positive cells were found in Enr-129 than in Ctr-129 mice (Figures 1a,2; $p = 0.0186$). This implies that net cell survival was increased in Enr-129 mice. However, in relative terms, survival of newly generated cells was actually lower in Enr-129 (29%) than in Ctr-129 (40%) mice. This is in contrast to our earlier findings with C57BL/6 mice, where the experience-related net effect on adult hippocampal neurogenesis was due to a survival-promoting effect on newborn cells in the subgranular zone [5]. Data from the stereological analysis of the volume of the granule cell layer and the total granule cell number is in the Supplementary material published with this paper on the internet.

When BrdU-labeled cells were examined by triple immunohistochemistry, the percentage of cells that co-labeled for the neuronal marker protein NeuN or the astrocytic marker S100β did not differ between Enr-129 and Ctr-129 mice, but the percentage of $BrdU^+$ cells that did not express NeuN or S100β was significantly lower in Enr-129 than in Ctr-129 mice (Figure 1b; $p = 0.0247$). To estimate the absolute number of newly generated neurons ($BrdU^+ NeuN^+$) and astrocytes ($BrdU^+ S100\beta^+$), the phenotypic percentages (Figure 1b) were multiplied by the absolute number of BrdU-labeled cells at 4 weeks after injection. The results were 1573 ± 191 (mean ± standard error of the mean) new neurons in Enr-129 versus 943 ± 104 in Ctr-129 mice (Figure 1c; $p = 0.0158$), indicating a 67% net increase in hippocampal neurogenesis in Enr-129 mice.

These results indicate that although environmental stimulation of 129/SvJ mice elicited as strong a neurogenic response in the dentate gyrus as reported for C57BL/6 mice [5], detailed analysis revealed that in the 129/SvJ mice this similar net effect involved a stimulation of cell proliferation which had been absent in C57BL/6 mice. Thus, strain differences not only influence the baseline rate of adult hippocampal neurogenesis [1], but also

Figure 1



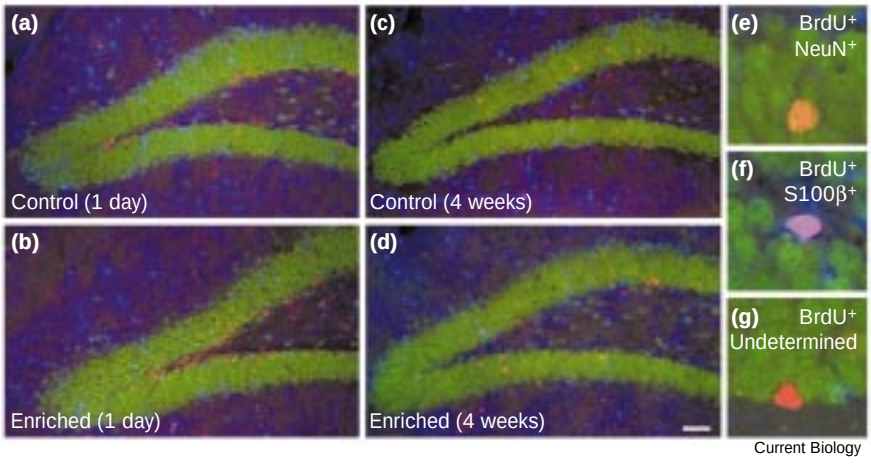
Quantification of BrdU$^+$ cells and phenotype distribution in Ctr-129 (control) and Enr-129 (enriched) mice. (a) The number of BrdU$^+$ cells in the granule cell layer. The number of cells labeled with BrdU 1 day after the last injection is an indicator of proliferative activity. The number of cells labeled 4 weeks after the last injection reflects survival of newborn cells. (b) Phenotypic distribution of surviving BrdU$^+$ cells at 4 weeks after BrdU injection. The percentage of neurons was determined by NeuN labeling and the percentage of astrocytes by S100β labeling. Cells that did not label positively for either NeuN or S100β were denoted of undetermined phenotype. (c) The absolute number of new cells by phenotype. The total number of surviving BrdU$^+$ cells of the different phenotypes as in (b) per dentate gyrus was calculated, based on stereological analysis. (a,c) The error bars indicate standard error of the mean. Two asterisks denote $p < 0.01$; one asterisk, $p < 0.05$.

influence how adult hippocampal neurogenesis is regulated in response to environmental stimuli. Proliferation, survival and differentiation of progenitor cells and their progeny are each separately influenced by inheritable traits and are not uniformly upregulated in response to environmental stimulation [1,5].

The degree to which adult hippocampal neurogenesis in 129/SvJ mice could be stimulated was not necessarily predictable from the low baseline of neurogenesis [1]. Because the relative increase in proliferating cells 1 day after injection was greater than the increase in the number of surviving BrdU-labeled cells 4 weeks later, the neurogenic potential in these mice was apparently not exhausted. It remains to be determined, however, whether increased proliferation in the subgranular zone indeed generates a larger pool of cells from which even more new neurons could be generated upon different or more complex environmental stimulation than under the conditions of this experiment.

Ex vivo experiments have shown that multipotent neural stem cells can be isolated from the hippocampus of adult rats [6]. *In vivo*, multipotentiality has not yet been demonstrated, but cell proliferation, neurogenesis, and astrogenesis in the dentate gyrus of adult mammals have been

Figure 2



Confocal microscopic analysis of neurogenesis in the dentate gyrus of Ctr-129 (control) and Enr-129 (enriched) mice. BrdU-labeled cells are visualized with Texas red (red). Neurons are identified with antibodies against NeuN conjugated with FITC (green), and astrocytes identified with antibodies against S100β conjugated with CY5 (blue). (a) Ctr-129 and (b) Enr-129 mice at 1 day after the last injection of BrdU. Environmental stimulation resulted in an approximately twofold greater number of BrdU-labeled cells. (c,d) After 4 weeks, surviving BrdU-labeled cells migrated into the granule cell layer and differentiated into either (e) neuronal, (f) astrocytic, or (g) undetermined phenotypes. The scale bar in (d) represents 50 μm for (a–d) and 7.5 μm for (e–g).

recognized since the early 1960s [7,8]. Proliferation in the subgranular zone can be experimentally influenced by a variety of factors, including glucocorticoid hormone levels [9], glutamatergic deafferentation [10], excitotoxicity [11] and — possibly via a combination of these factors — stress [12]. The effects of these factors on steps of neuronal development beyond cell proliferation remain to be determined.

Hypotheses have emerged that consider the functional relevance of adult hippocampal neurogenesis [4,5,11–13]. Because the hippocampus is established as a critical structure in several types of memory [14], the production of neurons in the adult hippocampus might have cognitive implications. Correlations between neurogenesis and behaviorally measurable hippocampal function may provide insight into both the regulation and functional relevance of adult hippocampal neurogenesis.

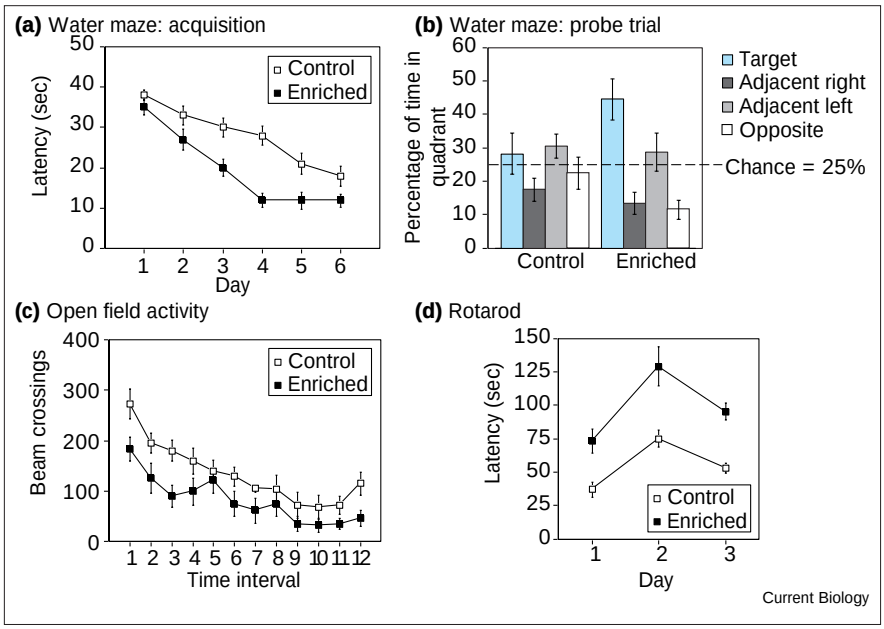
In contrast to C57BL/6, mice of the 129 strain perform poorly in various behavioral tests (see references in [15]) including tasks that examine spatial learning [16,17]. Compounding the problems with strain 129 mice is the existence of many substrains, sometimes ill-defined, that differ considerably from each other and may or may not show the characteristics 'typical' of the strain [18]. Despite these problems, strain 129 mice are widely used in gene targeting ('knockout') experiments [15,19] to understand the molecular basis of cognitive behavior and and how it correlates with morphology and physiology [20–22].

As in our earlier studies [4,5], we used the Morris water maze task to test the effects of environmental stimulation on cognitive function mediated by the hippocampus. The water maze task is widely used as a test of spatial memory in rodents and requires normal hippocampal function [23]. Overall analysis of water maze performance showed that Enr-129 mice navigated to the hidden platform in a significantly shorter time than Ctr-129 mice (Figure 3a; $p = 0.0026$), took a significantly shorter swim path to do so (528 ± 41 cm versus 640 ± 40 cm; $p = 0.0294$) and also swam significantly faster than Ctr-129 mice (26.9 ± 0.5 cm/sec versus 22.9 ± 0.7 cm/sec; $p = 0.0172$). In a subsequent probe trial in which the platform was removed, Enr-129 mice spent on average 28.1 ± 3.6 sec out of 60 sec (~47%) in the target quadrant compared with the 17.1 ± 3.6 sec (~29%) spent there by Ctr-129 mice (Figure 3b; $p = 0.0482$). Ctr-129 mice swam 465.9 ± 98.0 cm in the target quadrant, whereas Enr-129 mice swam 748.5 ± 93.4 cm in this quadrant ($p = 0.0574$). The mean distance to the target point during the probe trial was 38.4 ± 3.1 cm in Enr-129 and 49.1 ± 5.0 cm in Ctr-129 mice ($p = 0.0813$). Two-way analysis for quadrants and groups revealed an interaction term with $p = 0.0410$. Although non-cognitive factors are likely to contribute to these results, the results are consistent with an improvement in cognitive function [24]. The parallel experience-induced increase in hippocampal neurogenesis and general improvements in performing the learning task suggests that some relationship exists between the two, but at present the link remains correlational.

In the activity chamber, Enr-129 mice showed significantly less motor activity over the 1 h test period ($p = 0.0219$) than Ctr-129 mice (Figure 3c). Enr-129 mice

Figure 3

Behavioral analysis of Ctr-129 (control) and Enr-129 (enriched) mice. (a) In the water maze acquisition phase, the time the mice took (latency) to find the hidden platform on each of 6 days was determined. (b) In the water maze probe trial where the platform was removed on the seventh day, there was a significant temporal preference of Enr-129 mice for the target quadrant. Percentage trial times in the four pool quadrants are plotted; the 25% chance that mice are found in a particular quadrant is indicated. (c) In the open field activity chamber, the number of beam breaks per 5 min interval is shown, indicating movements of the mice. (d) Performance of mice on the rotarod. The time taken for the mice to fall is shown. All error bars indicate standard error of the mean.



were able to stay on the rotarod (accelerating at 20 rpm^2) for almost twice as long as Ctr-129 mice (Figure 3d; $p = 0.0013$). These additional experience-related effects suggest that the effects of environmental stimulation on 129/SvJ mice are broad and not restricted to the hippocampus. This implies that the genetic constraints that seemingly hamper the performance of 129/SvJ mice are in part dependent on housing conditions.

Consequently, it is problematic when strains are categorized as 'poor learners' or as having impaired performance on a learning task, or when certain anatomical measures are thought of as 'genetically fixed'. To a certain degree, what is considered a genetically determined baseline already reflects a specific response to the environment. In strain difference studies and knockout experiments 'normalized' baselines are obtained under identical conditions for all strains, but for any given strain these conditions may not be necessarily the most appropriate to investigate complex physiological processes. Our results imply that inheritable traits influence these 'normalized' patterns of morphology and function as well as the dynamic changes that occur in these systems.

Materials and methods

A total of 25 female 129Sv/J mice were obtained from Jackson Laboratory at the age of weaning (P21). Upon arrival they were randomly assigned to the two experimental groups: 13 mice lived in an enriched environment, consisting of one large cage with a rearrangeable set of tunnels, toys and running wheels as described [4,5], and 12 control mice were held 3 per cage in standard laboratory cages. For water maze testing, the hidden platform variant of the test was used with a protocol consisting of four trials per day over 6 days and a probe trial where the platform was removed on the seventh day. BrdU injections, the preparation of tissue, immunohistochemistry, immunofluorescence and unbiased counting techniques were performed as described [4] and are detailed in the Supplementary material. Morphological data were quantified using stereological procedures to achieve reliable intergroup comparisons. All numbers were obtained from the left dentate gyrus, except for phenotypical analysis which included samples from both hemispheres.

Supplementary material

Additional methodological and analytical details are published with this paper on the internet.

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Supplementary material

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Materials and methods

BrdU injections

After 28 days in their respective environments all mice received one daily intraperitoneal injection of 10 mg/ml BrdU (5-bromo-2-deoxyuridine; Sigma) in sterile 0.9% NaCl solution. The daily dose was 50 μg/g body weight and was given for 12 consecutive days; 1 day after the last injection of BrdU, five animals from each group were perfused as described below. The remaining animals lived for 4 weeks more in their respective experimental conditions. During this period they were tested behaviorally.

Behavioral testing

Activity chamber: overall activity and habituation to a new environment were examined using an automated activity recording chamber (San Diego Instruments). The mice were placed individually in the empty dark box for 1 h. Interruptions in an array of infrared beams caused by movements of the mouse were recorded and analyzed in 5 min intervals.

Rotarod: to test motor coordination and aspects of procedural learning, the mice were tested on a rotarod task (Columbus Instruments) for three consecutive days with three trials per day. The animals were placed on the rotating rod at a starting speed of 5 rpm. When the animal had found balance, the trial was started and the rod accelerated with 20 rpm^2 to a maximum of 65 rpm (equivalent to a maximum 3 min trial). The time the mouse could stay on the accelerating rod was recorded as 'latency to fall'.

Water maze: spatial learning was examined in the hidden platform variant of the water maze task. The escape platform was hidden 1 cm below the surface of the water, which had been made opaque with non-toxic white paint; the platform remained at a fixed position. The pool was round, with a diameter of 120 cm and the water temperature was 20°C. The test was carried out on six consecutive days with four trials per day and an inter-trial interval of 10 sec on the platform. The four starting points were rotated daily. Time (latency) and swim path to find the platform were recorded by an automated video tracking system (San Diego Instruments). If the animal had not found the platform after a maximum of 40 sec, it was placed on the platform.

Tissue preparation

The mice were killed with an overdose of anesthetics and perfused transcardially with 4% paraformaldehyde in cold phosphate buffer. The brains were removed. They were stored in the fixative overnight and then transferred into 30% sucrose. After an additional 24 h they were cut coronally at 40 μm on a sliding microtome (AO Scientific Instruments) from a dry ice-cooled block. The sections were stored at –20°C in cryoprotectant containing 25% ethylene glycol, 25% glycerin, and 0.05 M phosphate buffer.

Antibodies

The following primary antibodies were used: monoclonal mouse anti-BrdU (Boehringer Mannheim) 1:400 for conventional light microscopy, or monoclonal rat anti-BrdU (Accurate) 1:100 for immunofluorescence; monoclonal mouse anti-NeuN, clone A60 (kindly provided by R.J. Mullen, University of Utah, Salt Lake City, USA) 1:20, and polyclonal rabbit-anti S100ß (S. Want, Bellinzona, Switzerland) 1:2000.

For immunohistochemistry with the peroxidase technique, biotinylated horse anti-mouse IgG (Jackson), 6 μl/ml, was used as secondary antibody and detected with avidin-biotin-peroxidase complex (ABC, Vectastain Elite, Vector Laboratories) at 9 μl/ml. For indirect immunofluorescence the following secondary antibodies were used (all 6 μl/ml): donkey anti-rabbit IgG (Jackson), conjugated with CY5; donkey anti-mouse IgM and IgG (Jackson), conjugated with FITC; and goat anti-rat IgG (Jackson), conjugated with Texas red.

All primary and secondary antibodies were diluted in TBS containing 0.1% Triton X-100 and 3% horse or donkey serum (TBS-plus).

Pretreatment for BrdU immunohistochemistry

For BrdU immunohistochemistry on tissue sections the DNA had to be denatured. After blocking endogenous peroxidases with H_2O_2 (see below), the sections were incubated in 50% formamide in 2 × SSC-buffer (0.3 M NaCl, 0.03 M sodium citrate) at 65°C for 2 h, washed in 2 × SSC, incubated in 2 N HCl for 30 min at 37°C, and washed in 0.1 M borate buffer (pH 8.5) for 10 min.

Immunohistochemistry

For quantitation of BrdU-labeled cells, every sixth 40 μm section was used. Endogenous tissue peroxidases were blocked by treating free-floating sections with 0.6% H_2O_2 in TBS for 30 min. After BrdU pretreatment (see above) and washing in TBS, sections were blocked in TBS-plus containing horse serum for 1 h and then incubated in primary antibody in TBS-plus overnight at 4°C. After rinses in TBS, the sections were incubated in the secondary antibody in TBS-plus for 4 h at room temperature. ABC Elite reagent (Vector Laboratories) was applied for 1 h. Diaminobenzedine (DAB; Sigma) was applied as a substrate for the peroxidase reaction for 5 min at a concentration of 0.25 mg/ml in TBS with 0.01% hydrogen peroxide and 0.04% nickel chloride. Sections were thoroughly washed, and mounted with coverslips.

Immunofluorescence

For the triple-labeling for BrdU, NeuN and S100ß, every twelfth section throughout the dentate gyrus was used. After pretreatment (see above) and blocking with TBS-plus with donkey serum, sections were incubated in an antibody cocktail with antibodies against BrdU, NeuN and S100β for 36 h at 4°C. After washing in TBS and TBS-plus, a cocktail of secondary antibodies (Texas red to detect BrdU, FITC for NeuN, and CY5 for S100β) was applied for 4 h at room temperature. Sections were washed again, and mounted with coverslips in polyvinyl alcohol with diazabicyclo-octane (DABCO) as an anti-fading agent.

Fluorescent signals were detected using a confocal laser scanning head (Biorad MRC 1000) on a Zeiss inverted microscope. Fifty cells per animal were analyzed. Counts were performed blind to the groups. Images were processed with Adobe Photoshop 4.0 (Adobe Systems). Only general contrast enhancements, color level adjustments and channel mergings were carried out and images were not otherwise digitally manipulated.

Stereology

In principal, the experimental set-up for stereology was the same as in our previous studies [1,4]. In contrast to our earlier study with young adult C57BL/6 mice [5], however, a new video system was used and volumetric measurements were done on Hoechst 33342 rather than H&E stained sections. The total number of granule cells was determined in every sixth section in a series of 40 μm coronal sections using a three-dimensional counting volume [S1] in a variant of the optical disector principle [S2]. Systematic sampling of unbiased counting frames of 15 μm on a side with a 200 μm matrix spacing was achieved

using a semi-automatic stereology system (StereoInvestigator, MicroBrightfield Inc) and a 60 × SPlanApo oil objective (1.4 NA). Nuclei of granule cells were stained with Hoechst 33342 (Molecular Probe; 0.5 mg/ml tris-buffered saline for 15 min). Nuclei intersecting the uppermost focal plane (exclusion plane) and those that intersected the exclusion boundaries of the counting frame were excluded from the count. All counts were done blind to groups and treatment conditions. Cells were counted according to the optical disector principle through a 40 μm axial distance. The reference volume of the granule cell layer was determined by summing the traced areas of the granule cell layer in each section and multiplying the result by the distance between sections sampled (240 μm). The average (mean) granule cell number per disector volume was multiplied by the reference volume to estimate the absolute granule cell count. Because BrdU-positive cells were relatively rarely encountered, sampling of these cells was done exhaustively throughout the extent of the granule cell layer in its rostro-caudal extension, modifying the optical disector procedure to exclude the top focal plane only. The resulting number of BrdU-positive cells was then related to the granule cell layer volume per section and multiplied by the reference volume to give an estimate of the total number of BrdU-positive cells per dentate gyrus.

Stereological analysis of the dentate gyrus at 4 weeks after the last injection of BrdU revealed that Enr-129 had a more densely packed granule cell layer. The average number of granule cells per 9000 μm^3 sample volume was 9.2 ± 0.2 in Ctr-129 and 10.0 ± 0.2 in Enr-129 mice ($p = 0.0210$). No change in the absolute volume of the granule cell layer was detectable ($0.34 \pm 0.01\ mm^3$ in Ctr-129 and $0.32 \pm 0.02\ mm^3$ in Enr-129 mice; $p = 0.5051$), resulting in an average estimated total granule cell count of $3.44 \pm 0.11 \times 10^5$ in Ctr-129 and $3.56 \pm 0.24 \times 10^5$ in Enr-129 mice ($p = 0.6827$), per hippocampus. In environmentally stimulated C57BL/6 mice we had found a statistically significant greater volume of the granule cell layer and a 15% increase in the total granule cell number [4,5]. Comparing the volumetric measures obtained at 1 day after the last injection of BrdU to calculate a second estimate of the total granule cell layer volume in 129/SvJ mice, we found a volume of $0.29 \pm 0.02\ mm^3$ in Ctr-129 and of $0.37 \pm 0.03\ mm^3$ in Enr-129 mice ($p = 0.0497$). A cautious interpretation would be that the volumetric data are consistent with measurable effects on total granule cell numbers in 129/SvJ mice, but may be confounded here by biological variance.

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