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More hippocampal neurons in adult mice living in an enriched environment

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Neurogenesis occurs in the dentate gyrus of the hippocampus throughout the life of a rodent[1–4], but the function of these new neurons and the mechanisms that regulate their birth are unknown. Here we show that significantly more new neurons exist in the dentate gyrus of mice exposed to an enriched environment compared with littermates housed in standard cages. We also show, using unbiased stereology, that the enriched mice have a larger hippocampal granule cell layer and 15 per cent more granule cell neurons in the dentate gyrus.

It has long been known that experience-dependent neuroanatomical plasticity occurs in the rat brain[5–9]. Environmental enrichment or the 'combination of complex inanimate and social stimulation'[10] has been used to induce experience-dependent neuroplasticity. Morphological changes in the hippocampus include increases in hippocampal thickness[11,12], dendritic arborization[13] and the number of glial cells[12]. Enrichment is normally compared with standard laboratory conditions, although the type of enrichment offered in this study still represents a deprived condition compared to conditions in the wild[10,14]. To test whether exposure to an enriched environment could lead to higher numbers of neurons in the dentate gyrus, we performed the following experiment.

Twenty-one-day-old mice were randomly distributed in two experimental groups and grew up under either standard or enriched conditions for 40 days (Fig. 1). During the last 12 days of this period they received one daily intraperitoneal injection (50 mg per kg body weight) of bromodeoxyuridine (BrdU; Sigma). The thymidine analogue BrdU is incorporated into the DNA of dividing cells and can be detected immunohistochemically in their progeny[4,15]. One day after the last injection, five mice from each group were perfused with 4% paraformaldehyde. The remaining animals were tested in a Morris water maze for five consecutive days; during these five days and for an additional 23 days they lived in their respective environments.

Four weeks after the last BrdU injection, the remaining mice were perfused with 4% paraformaldehyde. Immunohistochemistry for BrdU was done and the number of labelled cells was determined using unbiased counting techniques. Results revealed no statistically significant difference in the number of BrdU-positive cells in the dentate gyrus between the two groups one day after the last injection (post-injection, p.i.). This implies that enriched conditions have little or no influence on the proliferative activity of progenitor cells in the dentate gyrus. At 4 weeks p.i., however, a highly significant difference in the number of BrdU-positive cells was found (Figs 2a and 3a, b). Enriched mice had 57% more labelled cells per dentate gyrus than controls. This suggests that environmental enrichment has a survival-promoting effect on proliferating neuronal precursor cells in the dentate gyrus.

Triple labelling for BrdU, calbindin-D_{28K} (a granule cell marker[16]) and glial fibrillary acidic protein (GFAP, an astrocytic marker) was performed. We used confocal microscopy to determine the phenotype of the BrdU-positive cells at 4 weeks p.i. (Fig. 3c). The distribution of phenotype did not differ between the groups: 57 and 61% in controls and in enriched animals, respectively, of the BrdU-positive cells were also calbindin-positive, indicating a neuronal differentiation to a granule-cell phenotype. Multiplying the number of BrdU-labelled cells and the respective neuronal ratio leads to the conclusion that on average at least 2,490 of the new neurons per dentate gyrus that were born over 12 days (the period of BrdU injections) survived in enriched animals compared with ~1,330 new neurons in controls.

In the dentate gyrus of control animals, 16% of the BrdU-positive cells were also GFAP-positive, whereas 27% were neither calbindin- nor GFAP-positive. The respective numbers for the enriched group are 11 and 29%. Thus enriched mice showed on average ~450 surviving new astrocytes in the dentate gyrus versus ~380 in controls, consistent with earlier reports that enriched housing causes an increase in glial cells[6,12].

Early reports have suggested that enriched housing produces an increased 'hippocampal depth'[11]. We determined stereologically the absolute volume of the granule cell layer and found a statistically significant increase of ~15% in the enriched group (Fig. 2b). This change of hippocampal morphology has been attributed to a wider arborization of dendrites[13], varying sizes of neuronal nuclei[17] or more glial cells[12]. A higher number of neurons as a result of enriched living could contribute to the volumetric change. When we determined stereologically the absolute number of granule cells in the dentate gyrus, we found that enriched animals contained on average more than 310,000 granule cells, as opposed to 270,000 in controls (Fig. 2c). This is an increase of more than 40,000 neurons, or at least

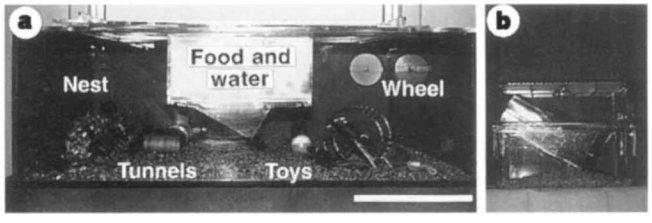

Figure 1 a, Special cage for the enriched housing of 12 mice. **b**, For comparison, a standard cage for 4 control mice is shown at the same scale. Scale bar, 25 cm.

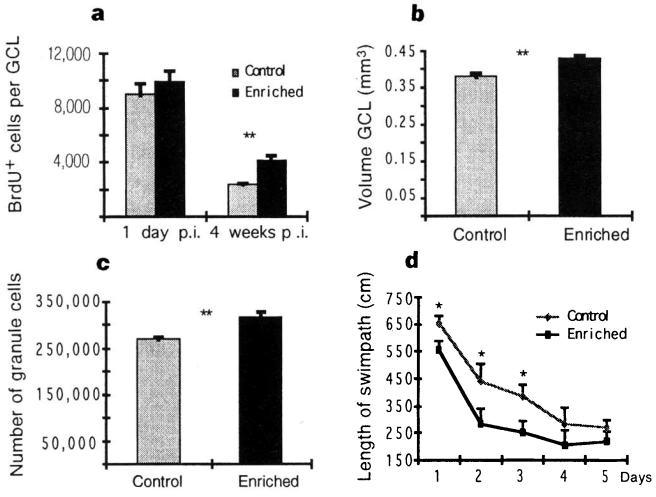

Figure 2 a–d, Four parameters in which enriched mice differ from their control littermates. **a**, Number of BrdU-positive cells per hippocampal granule cell layer (GCL). The density of BrdU-positive cells was determined stereologically (cells per mm^3) and multiplied by the absolute volume of the dentate gyrus. Significance level is $P < 0.005$ (*t*-test). **b**, Volume of the dentate gyrus. At 4 weeks p.i., the volume of the hippocampal dentate gyrus was significantly ($P < 0.005$, *t*-test) increased in the enriched group. **c**, Absolute granule cell number in the dentate gyrus. At 4 weeks p.i., enriched mice had significantly more granule cell neurons ($P < 0.01$). The underlying neuronal density was 6.47 ± 0.25 per sample volume in controls and 6.56 ± 0.14 in enriched mice and thus not influenced by environmental enrichment. All data are given as mean ± standard error. *N* was 5 per group at 1 day p.i., and 7 per group at 4 weeks p.i. **d**, In the spatial learning task (Morris water maze), enriched mice (lower curve) have a significantly shorter swim path ($P < 0.05$, *t*-test) on days 1 to 3 of testing. On day 1 there was no significant difference between the groups in the first two trials, indicating equal baselines. The time to reach the platform (latency) paralleled this curve and reached the significance level on day 2. At all time points, no difference in the average swim speed could be found, and on a transfer test without a platform on day 6 no differences between groups were detected (data not shown).

15%. Although no matching stereological data for C57B16 mice are available, the baseline neuronal cell counts in the dentate gyrus falls well into the range determined for other strains[18].

We conclude that environmental enrichment has a survival-promoting effect on the progeny of neuronal precursor cells in the hippocampus of mice and that these neurons add to an increased granule cell number and hippocampal volume in these animals.

The subgranular zone of the dentate gyrus is the only region of the hippocampus in which adult neurogenesis occurs. As environmental enrichment theoretically might have a stimulating effect on quiescent stem cells in other subregions, we evaluated the CA fields, but did not find BrdU-positive cells in numbers exceeding the very low and scattered ubiquitous background of dividing cells that can be found throughout the hippocampus (Fig. 3d–g). The hilar area (CA4) is an exception, as a certain rate of cell genesis, although no neurogenesis, is known to occur here[3,4]. At 4 weeks p.i., we counted 824 ± 105 (mean ± standard error) BrdU-positive hilar cells in controls compared with 886 ± 119 in enriched animals ($P > 0.7$; *t*-test). The hilar volume did not change either and was $0.43 \pm 0.02\,mm^3$ in controls and $0.47 \pm 0.03\,mm^3$ in enriched animals ($P > 0.69$; *t*-test). These data are indicative of a rather specific effect of enriched environment on the population of newborn neurons and astrocytes in the subgranular zone.

The regulatory mechanisms underlying neurogenesis are not known and so it is unclear how an enriched environment can influence this regulation. Studies on both experience-dependent neuroplasticity[9] and neurogenesis in the adult hippocampus[19] support a role for steroid hormones in this regulation, possibly mediated through pathways involving the activation of glutamatergic receptors[19,20].

From *in vitro* and *in vivo* studies, it is known that trophic factors, including epidermal growth factor and fibroblast growth factor 2 (refs 21–23) can influence the fate of neuronal progenitor cells, although data on experience-dependent regulation of these factors are still lacking. It is likely that a precise temporal and spatial regulation of several factors is required for survival and differentiation of endogenous neuronal precursor cells.

To demonstrate that the environmental enrichment employed in our study was sufficient to induce the behavioural improvements in

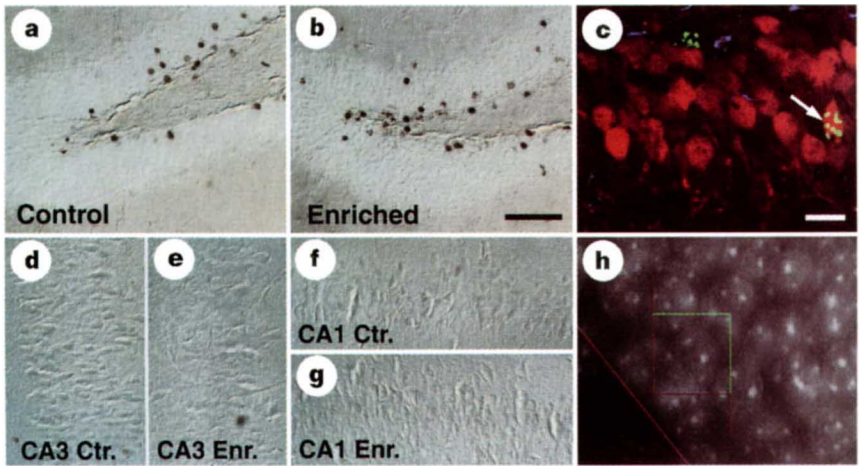

Figure 3 BrdU immunohistochemistry and phenotyping. **a** and **b**, Comparison of BrdU labelling in controls (Ctr.) and enriched (Enr.) mice at 4 weeks p.i. (for quantification, see Fig. 2a). Scale bar, 80 μm. **c**, Confocal microscopic image; arrow indicates double labelling of a BrdU-positive cell (yellow-green) with calbindin D_{28K} (red). GFAP is blue. The hilar region is at the top of the image. On the border between hilus and granule cell layer is a BrdU-positive, calbindin-negative cell. Scale bar, 12 μm. **d–g**, Representative areas in the CA3 (**d**, **e**) and CA1 (**f**, **g**) fields mice from both groups with very few BrdU-positive cells (Scale bar in **b**, is 100 μm for **d–g**). **h**, Hoechst 33342-stained granule cells and superimposed $15\,\mu m \times 15\,\mu m^2$ counting frame for the stereological determination of the absolute granule cell number (see Methods).

a spatial learning task reported previously[24,25], we tested our animals in a Morris water maze and found a moderate, though significant improvement in mice exposed to the enriched environment (Fig. 2d). However, we cannot conclude that a greater number of neurons in the dentate gyrus leads to enhanced behavioural performance. But it is likely that a combination of increased neurons, synapses and dendrites, as well as factors still unknown, contribute to the enhanced performance induced by exposure to an enriched environment. □

Methods

Housing conditions. 24 female C57B16 mice (Harlan Sprague Dawley) were obtained at the age of weaning (21 days) and randomly distributed into two groups. Twelve mice were placed in standard conditions (4 per cage); another 12 were put in a specially designed cage of 1 m^2 ground area. This cage was equipped with paper tubes, nesting material, a rearrangeable set of plastic tubes, a tunnel made from sisal with various openings and a running wheel. All animals received food and water *ad libitum*. In addition to the standard food, however, animals in the 'enriched' cage received extra treats, including cheese, crackers, apples, popcorn and whole-grain nibble bars.

Behavioural testing. Mice in the 2-month survival group (4 weeks p.i.) were tested in a Morris water maze for 5 consecutive days immediately following the final BrdU injection. Mice were tested in a water maze with a computerized tracking and analysis system (San Diego Instruments) which measured the time to reach the platform (latency) and the length of the swim path. The escape platform was hidden 1 cm below the surface of the water, which had been made opaque by adding non-toxic white paint, and kept at a constant position. Each animal was tested for 4 trials per day, each lasting 40 s and beginning from 4 different start points that were randomly varied each day. If an animal did not find the platform it was set on it at the end of the trial. Animals were allowed to rest on the platform for 15 s.

Immunohistochemistry. Immunohistochemistry for the detection of BrdU requires a pretreatment of tissue sections to denaturate DNA[4]. All staining was done on free-floating 40-μm sections. A monoclonal mouse-anti-BrdU antibody (Boehringer Mannheim; 1:400) was used in combination with avidin–biotin complex (Vector Laboratories) and a horse-anti-mouse-IgG-antibody conjugated with biotin (Vector; 1:167).

Immunofluorescent triple-labelling was done as previously described[4]. Calbindin was detected by a polyclonal rabbit-anti-calbinding antibody (Swant; 1:1,000) and a Texas-red-conjugated secondary antibody (Jackson; 1:167). GFAP was detected by a polyclonal guinea-pig anti-GFAP antibody (Advanced Immuno; 1:250) and a CY-5-conjugated secondary antibody (Jackson, 1:167). All primary and secondary antibodies were applied in cocktails with 3% horse or donkey serum and 0.1% Triton X-100 in Tris-buffered saline. To determine the phenotypes of BrdU-positive cells, 25 cells in each of 2 randomly chosen sections out of 4 were examined for each animal on a confocal microscope (Biorad/Zeiss).

Stereology. The total number of BrdU-positive cells in the subgranular layer and the corresponding sample volumes were determined in 7–9 coronal sections, 240 μm apart, containing dentate gyrus. For numbers from the hilus (CA4), we proceeded accordingly and defined as hilus that volume that is enclosed by the dentate gyrus and a virtual straight line that closes its two blades. For cell counts, the optical disector method was used to avoid oversampling errors (for reviews, see 26–28). The absolute volumes of the dentate gyrus and the hilus (CA4) were determined in a parallel haematoxylin-and-eosin-stained series of sections. For all volumetric measurements, the Cavalieri estimation was used[26]. The absolute granule cell number was estimated using a semiautomatic stereology system, Stereoinvestigator 1.0 (MicroBrightfield). For this estimation, a series of sections, 480 μm apart, was stained with Hoechst Dye 33342 (Sigma; 0.5 mg ml^{-1} tris-buffered saline for 15 min). A 100-μm grid was projected over each section and granule cells in fields within the granule cell layer were counted in a 15 μm × 15 μm × 40 μm sample volume, disregarding the cells that were in sharp focus in the uppermost focal plane. A 60 × S-Plan-Apo oil objective with a numerical aperture of 1.40 was used on an Olympus BH-2 microscope equipped with a Panasonic CCTV video camera, and all counts were done on the video image (Fig. 3h). The resulting density was multiplied by the total volume to yield the absolute cell number.

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Deactivation and reactivation of somatosensory cortex after dorsal spinal cord injury

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Sensory stimuli to the body are conveyed by the spinal cord to the primary somatosensory cortex. It has long been thought that dorsal column afferents of the spinal cord represent the main pathway for these signals[1–3], but the physiological and behavioural consequences of cutting the dorsal column have been reported to range from mild and transitory[4–8] to marked[9–13]. We have re-examined this issue by sectioning the