

Learning enhances adult neurogenesis in the hippocampal formation

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Thousands of hippocampal neurons are born in adulthood, suggesting that new cells could be important for hippocampal function. To determine whether hippocampus-dependent learning affects adult-generated neurons, we examined the fate of new cells labeled with the thymidine analog bromodeoxyuridine following specific behavioral tasks. Here we report that the number of adult-generated neurons doubles in the rat dentate gyrus in response to training on associative learning tasks that require the hippocampus. In contrast, training on associative learning tasks that do not require the hippocampus did not alter the number of new cells. These findings indicate that adult-generated hippocampal neurons are specifically affected by, and potentially involved in, associative memory formation.

The involvement of the hippocampal formation in learning has been recognized for decades^{1,2}, but the cellular mechanisms underlying this association remain enigmatic. The hippocampal formation produces new neurons throughout adulthood in many vertebrates, from birds to primates^{3–8}; however, although the number of neurons produced is high, the function of these cells is unknown. One possibility is that these new cells are important in hippocampus-dependent learning, a view consistent with reports that generalized environmental complexity enhances the number of new hippocampal neurons in adult birds and mice^{3,6}. However, previous studies have not explored the possibility that hippocampus-dependent learning specifically alters the fate of adult-generated hippocampal neurons.

To determine whether associative learning affects adult-generated hippocampal neurons, we examined the fate of new cells labeled with the thymidine analog bromodeoxyuridine (BrdU) in adult rats after a number of different behavioral manipulations. We used two different hippocampus-dependent tasks (classical conditioning of the eyeblink response using a trace protocol^{9–12} and spatial navigation learning in a Morris water maze¹³) and two hippocampus-independent learning tasks (classical eyeblink conditioning using a delay protocol⁹ and cue training in a Morris water maze¹³).

RESULTS

In the classical conditioning protocol, all of the animals that received paired training reached the criteria of 60% conditioned responses (CRs) to the conditioned stimulus (CS) by the end of training. None of the unpaired animals reached criteria. Rats exposed to paired training increased their CRs over 800 trials, whereas rats exposed to unpaired training did not ($F_{7,133} = 12.61$, $p < 0.0001$). Of rats exposed to paired training, there was no overall significant difference in percent CRs between the trace and delay protocols ($F_{1,19} = 4.24$, $p = 0.05$). By the last day of training,

levels of performance were indistinguishable between groups exposed to trace and delay conditioning. In the maze task, there was no difference in latency between animals trained with a visible platform versus a submerged platform over the course of training ($F_{1,10} = 2.88$, $p = 0.12$). As expected, the rats showed a decrease in latency over trials ($p < 0.01$).

Our previous [³H]thymidine-autoradiographic study demonstrated that the number of new cells in the dentate gyrus of adult control rats increases between two hours and one week after DNA synthesis, and then declines dramatically by two weeks after labeling⁵. Similar results were obtained for the number of BrdU labeled cells in the dentate gyrus of control rats at different survival times following BrdU injection (2096.0 ± 61.2 at 2 hours after BrdU injection, 3874.5 ± 121.0 at 24 hours after BrdU injection and 2130.0 ± 175.1 at two weeks after BrdU injection; $F_{3,11} = 146.35$, $p = 0.0001$). It is likely that the decrease in labeled cells observed between one week and two weeks following DNA synthesis is due to cell death as opposed to label dilution, because the number of silver grains per labeled cell does not decrease between these time points after [³H]thymidine injection⁵, and BrdU-labeled pyknotic or degenerating cells are observed in the dentate gyrus between these time points after BrdU injection (E.G., unpublished data).

To determine whether learning alters the survival of new cells in the dentate gyrus, we engaged animals in behavioral tasks during the time when the numbers of new cells normally diminish. In animals that received BrdU one week before training, learning either of the hippocampus-dependent tasks dramatically increased the number of BrdU-labeled cells in the dentate gyrus compared to naive controls ($F_{2,15} = 137.40$, $p = 0.0001$ for trace conditioning; $F_{3,20} = 266.40$, $p = 0.0001$ for maze training) when the brains were examined 24 hours after the last day of training (Fig. 1a, b, e and f). This difference seems to be specific to the dentate gyrus because learning did not affect the number of

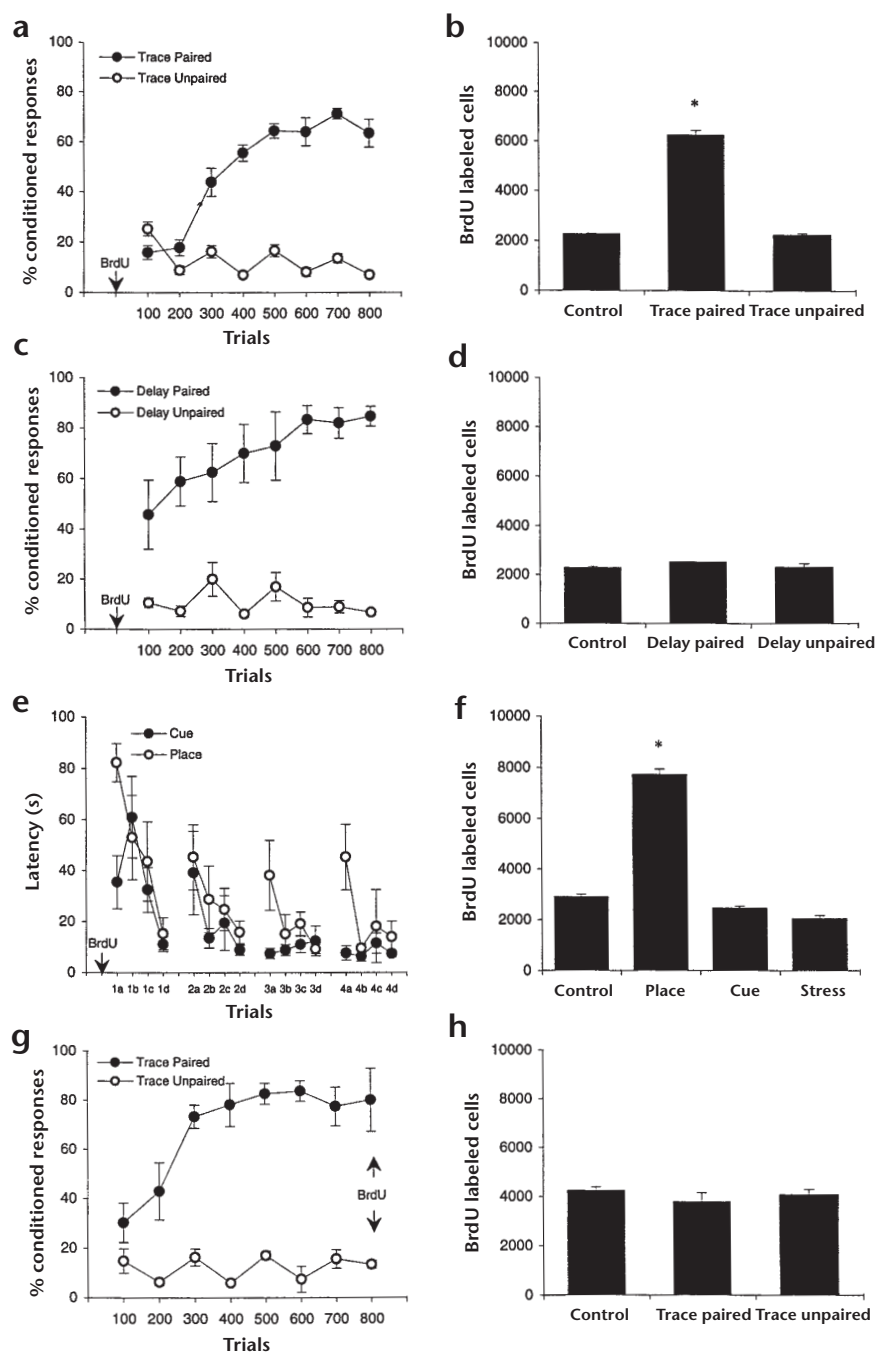


Fig. 1. Learning that requires the hippocampus, but not other types of learning or a similar experience in the absence of overt learning, increased the numbers of adult-generated hippocampal granule neurons. **(a)** Acquisition of the trace eyeblink conditioned response (trace paired) and the unpaired condition (trace unpaired). **(b)** Total numbers of BrdU-labeled cells in the dentate gyrus of these animals following trace conditioning (trace paired). These animals received BrdU injections one week before training and were perfused 24 hours after the last day of training. Bars represent mean \pm standard error ($n = 6$). *significant difference ($p < 0.01$) from other groups. **(c)** Acquisition of the delay eyeblink conditioned response (delay paired) and the unpaired condition (delay unpaired). **(d)** Total numbers of BrdU-labeled cells in the dentate gyrus of these animals following delay conditioning. These animals received BrdU injections one week before training and were perfused 24 hours after the last day of training. ($n = 5-6$). **(e)** Acquisition of place and cue learning in the Morris water maze. **(f)** Total numbers of BrdU-labeled cells in the dentate gyrus of these animals following spatial (place) or cue (cue) training. These animals received BrdU injections one week before training and were perfused 24 hours after the last day of training. ($n = 6$). **(g)** Acquisition of the trace eyeblink conditioned response (trace paired) and the unpaired condition (trace unpaired) from animals injected with BrdU on the last day of training after all animals had reached learning criterion. These animals were perfused 24 hours after the BrdU injection. **(h)** Total numbers of BrdU-labeled cells in the dentate gyrus of these animals following trace conditioning (trace paired). ($n = 5-6$).

BrdU-labeled cells in the subventricular zone, a region lining the wall of the lateral ventricles that produces new cells in adulthood¹⁴ (in the trace-conditioning experiment, 4820.6 ± 271.5 for naive controls, 4993.8 ± 214.7 for trace-paired stimuli, 5323.7 ± 212.7 for trace-unpaired stimuli, $F_{2,15} = 0.09$, $p = 0.3598$; in the water-maze experiment, 4668.2 ± 382.2 for naive controls, 4949.2 ± 412.8 for place-trained rats; 5359.0 ± 756.2 for cue-trained rats; 5066.2 ± 539.5 for swim-stress rats, $F_{3,20} = 0.279$, $p = 0.8538$). After hippocampus-dependent learning, the majority of new hippocampal cells were located in the granule cell layer and expressed a marker of immature granule neurons, Turned-on-after-division, a 64-kD protein (TOAD-64) or a marker of mature granule neurons, the calcium-binding protein calbindin,

but not an astroglial marker, glial fibrillary acidic protein (GFAP; Fig. 2a-d). When examined one week after the end of training (three weeks after BrdU injection), the number of BrdU labeled cells in the dentate gyrus remained elevated in the hippocampus-dependent learning groups, but the percentage of BrdU labeled cells that expressed cell-specific markers did not differ between any of the groups. When examined 1 week after the end of training, that is, approximately 3 weeks after BrdU injection, $\sim 57\%$ of BrdU labeled cells expressed TOAD-64 ($F_{3,20} = 0.311$, $p = 0.8169$), $\sim 11\%$ of BrdU-labeled cells expressed GFAP ($F_{3,20} = 0.572$, $p = 0.6402$), and $\sim 71\%$ of BrdU-labeled cells expressed calbindin ($F_{3,20} = 0.572$, $p = 0.6402$). No differences were observed in the volume of the granule cell layer with any

training (in the trace-conditioning experiment, $3.08 \pm 0.37 \text{ mm}^3$ for naive controls, $3.25 \pm 0.51 \text{ mm}^3$ for trace-paired animals, $3.21 \pm 0.38 \text{ mm}^3$ for trace-unpaired animals, $F_{2,15} = 0.043$, $p = 0.958$; in the water-maze experiment, $3.20 \pm 0.19 \text{ mm}^3$ for naive controls, $3.19 \pm 0.23 \text{ mm}^3$ for place-trained rats, $3.11 \pm 0.23 \text{ mm}^3$ for cue-trained rats, $2.95 \pm 0.24 \text{ mm}^3$ for swim-stress rats, $F_{3,20} = 0.302$; $p = 0.8236$).

Exposure to similar environmental conditions and production of the same number and types of motor responses in the absence of overt learned responses had no effect on the number of BrdU-labeled cells in the dentate gyrus. Specifically, exposure to the same number of eyeblink-conditioning stimuli presented in an explicitly unpaired manner using either trace (trace unpaired) or delay (delay unpaired) stimulus parameters did not affect the number of BrdU-labeled cells compared to naive controls (Fig. 1a–d). Similarly, rats exposed to the water maze for the same amount of time as the place-trained group but without a platform exhibited no difference in the number of BrdU-labeled cells compared to naive controls (Fig. 1e and f).

Training on associative learning tasks that do not require the hippocampus was similarly ineffective at altering the number of BrdU-labeled cells. Rats trained on a Cue Test in the water maze did not exhibit more neurons after training than naive controls (Fig. 1e and f). Similarly, acquisition of the conditioned response during delay eyeblink conditioning did not result in more new neurons after training (Fig. 1c and d; $F_{2,12} = 1.889$, $p = 0.1936$).

It is likely that the increased number of BrdU-labeled cells in the dentate gyrus following hippocampus-dependent learning is, at least in part, the result of enhanced cell survival, because significant differences among groups in the number of pyknotic, or degenerating cells were observed in the subgranular zone. The presence of degenerating cells in the subgranular zone, the region of cell proliferation in the dentate gyrus, in control rats, has been reported previously¹⁵. Animals exposed to either of the hippocampus-dependent tasks (trace-eyeblink conditioning or place learning in a water maze) showed fewer pyknotic cells than all other groups (in the trace-conditioning experiment, 220.0 ± 41.9 for naive control, 81.8 ± 24.3 for trace-paired animals, 214.8 ± 46.5 for trace-unpaired animals, $F_{3,15} = 12.339$, $p = 0.0007$; in the water-maze experiment, 208.7 ± 30.9 for naive controls, 50.0 ± 8.4 for the place-trained group, 222.0 ± 48.1 for the cue-trained group, 236.0 ± 34.2 for the swim-stress group, $F_{3,20} = 16.527$, $p = 0.0001$). BrdU-labeled pyknotic cells were observed occasionally in the subgranular zone, but these profiles were relatively rare (0–36 per dentate gyrus), and labeled cells were never observed in the dentate gyrus after hippocampus-dependent learning.

To assess whether training alters the rate of cell proliferation, additional groups of animals received BrdU during, rather than before, training on the trace eyeblink protocol. In this experiment, the number of new cells produced in the dentate gyrus did not differ between conditioned animals and naive controls (Fig. 1g and h; $F_{2,13} = 0.011$, $p = 0.9892$). No BrdU-labeled pyknotic cells were observed in the dentate gyrus of animals in any group injected with BrdU 24 hours before perfusion.

DISCUSSION

These results demonstrate a direct association between hippocampus-dependent learning and neurons generated in the adult hippocampal formation. Types of learning that depend on the hippocampus, including trace eyeblink conditioning and spatial water-maze training, increased the number of newly generated neurons in the dentate gyrus of the hippocampal region.

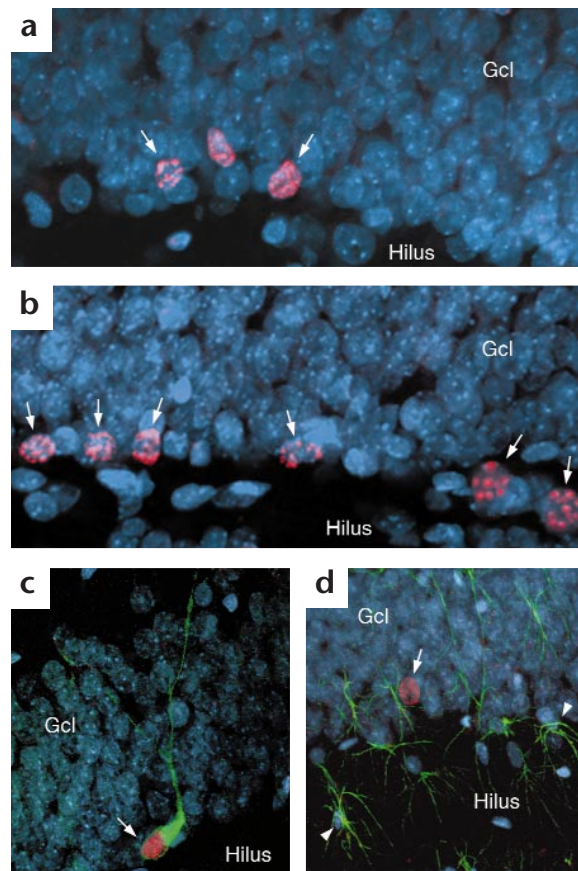


Fig. 2. The number of new neurons in the granule cell layer (Gcl) of adult rats increases following spatial learning in the Morris water maze. Confocal laser scanning microscopic images of BrdU labeled cells (arrows) reveal a difference in number between control (a) and spatial learning (b) adult rats. The vast majority of BrdU labeled cells (arrows) had the morphology of granule neurons and were immunoreactive for the marker of immature neurons TOAD-64 (c) but not the astroglial marker GFAP (d). GFAP-positive astrocytes that are not BrdU labeled are indicated by arrowheads.

Conversely, learning that does not require the hippocampus, including delay-eyeblink conditioning and cue-maze training, did not alter the numbers of new granule neurons compared to naive controls. It should be noted that animals that learned tasks not requiring the hippocampus and those in the various control groups, including naive controls and those exposed to the same stimuli but without explicit learning, did maintain a significant number of new neurons. Because all of these conditions are likely to involve some form of learning, the possibility that a basal number of new neurons, that is, the control number, is maintained by learning of an unspecified nature can not be ruled out. However, because exposure to both delay-eyeblink conditioning and cue-maze training are known to activate, but not require, the hippocampal formation^{16,17}, these results suggest that for learning to further enhance the number of new hippocampal neurons, the animal must be engaged in a task for which this brain region is essential.

It is likely that the changes we observed in the number of BrdU-labeled cells are primarily the result of hippocampus-dependent learning enhancing the survival of new granule cells, as opposed to the proliferation of granule-cell precursors, for the

following reasons. First, hippocampus-dependent learning increased the number of cells produced before, but not during, training. Second, hippocampus-dependent training enhanced the number of BrdU-labeled cells in the dentate gyrus during the time when the number of new cells is known to diminish in laboratory controls. The number of new cells in the dentate gyrus increases between 2 hours and 1 week after DNA synthesis and then declines dramatically by the two week time point (our results and ref. 5). This decrease in labeled cells is most likely the result of cell death, and not label dilution from continual cell proliferation, because no decrease in the number of silver grains per labeled cell was observed between one and two weeks after [³H]thymidine injection in our previous study⁵. Third, hippocampus-dependent learning decreased the number of pyknotic cells in the subgranular zone of the dentate gyrus compared to controls. Although we cannot rule out the possibility that hippocampus-dependent learning had some effect on cell proliferation, available evidence strongly suggests that the increased number of BrdU-labeled cells we observed is the result of enhanced survival. Consistent with our results, previous studies have reported that environmental complexity increases the number of adult-generated hippocampal neurons in birds and mice, presumably by enhancing cell survival^{3,6}. Studies³ of black-capped chickadees demonstrated a naturally occurring seasonal fluctuation in hippocampal neurogenesis that correlates positively with engagement in spatial learning behaviors, that is, seed storage and retrieval. However, several variables, including stress, social interaction, nutrition and learning opportunities, differ between captivity versus wild living in the case of birds, and between standard laboratory cage housing versus 'enriched environment' conditions in the case of mice. Our results demonstrate that it is hippocampus-dependent learning, but not experience in the absence of explicit learning or learning that does not require the hippocampus, that enhances the number of adult-generated neurons.

Another study in this issue reports that water-maze training in mice does not alter the number of new neurons in the dentate gyrus³⁴. In that study, animals were injected with BrdU during place training. These results are essentially consistent with ours in that we also did not observe an enhanced number of labeled cells in the dentate gyrus of animals injected with BrdU during training. Rather, we observed an enhanced survival of those cells that were generated before training. Rescue of adult-generated cells by certain types of learning may occur only during a specific 'sensitive period' following the production of a new cell. The results of our study suggest some new cells require this type of input for survival between 1 and 2 weeks after mitosis, a time when adult-generated granule cells appear to be forming connections with the CA3 region (N.B. Hastings and E. Gould, unpublished data). Hippocampus-dependent learning may facilitate the integration of adult-generated cells into existing circuitry and insure their survival.

Our previous studies have identified factors that alter the production of new granule neurons by affecting the proliferation of granule cell precursors. We have shown that adrenal steroids suppress the proliferation of granule cell progenitors by activating an NMDA-receptor-dependent excitatory pathway^{18,19}. Additionally, stressful experiences, known to increase levels of adrenal steroids and hippocampal glutamate release²⁰, also diminish the proliferation of granule cell precursors in the dentate gyrus of adult tree shrews and marmoset monkeys^{7,8}. Although the experiments in the present report were not designed to assess the effects of stress on cell proliferation, the effects of stress on the survival of adult-generated cells were considered in the spatial

learning study. In this case, animals exposed to swim stress, that is, those that were in the water for the same duration as the place-learning animals, showed no change in the number of BrdU-labeled cells in the dentate gyrus. These results suggest that stress does not alter the survival of recently produced neurons, a finding consistent with our previous data showing that adrenal steroids are only necessary for the maintenance of mature, and not immature, granule neurons in the adult dentate gyrus²¹. Because time is required for new cells to become integrated into functional circuitry, it is unlikely that acute stress, which affects cell proliferation and not survival, would have an immediate impact on hippocampal function via this mechanism. However, continually diminished production of new cells resulting from chronic stress or corticosterone treatment may contribute to performance decrements in hippocampus-dependent tasks under some conditions^{22,23}.

There are many theories of hippocampal function, including the assertion that the hippocampus is necessary for acquiring associations between discontinuous events, either temporal or spatial²⁴, or for the acquisition of declarative as opposed to procedural memories²⁵. In addition to acquisition, studies have provided evidence that the hippocampus has a transient role in memory²⁵. Our results suggest that learning about space (spatial navigation learning) and time (trace classical conditioning) under relatively specific conditions has a trophic effect on adult-generated hippocampal neurons. The direct relationship between hippocampus-dependent learning and the survival of adult-generated hippocampal neurons suggests a function for these new neurons in certain types of learning. The immature status of adult-generated hippocampal cells may make them uniquely qualified to form synaptic connections rapidly and to participate in the transient storage of information.

METHODS

Adult male Sprague Dawley rats (300–350 g) from the Princeton University animal colony were injected with BrdU (200mg/kg) and trained on either a classical-conditioning task or a maze-learning task one week later ($n = 5-6$ for each group). Previous studies have shown that under normal laboratory conditions, the number of new cells in the dentate gyrus declines significantly between one and two weeks after DNA synthesis⁵. To test the possibility that training would alter the survival of new cells, we trained animals between one and two weeks after BrdU injection. The rats were perfused transcardially with 4.0% paraformaldehyde in 0.1 M phosphate buffer 24 hours or 7 days after the last day of training. To determine whether learning affects cell proliferation, we injected rats with BrdU during training and after learning criterion had been reached, and perfused them 24 hours later.

Classical conditioning. For classical conditioning, the rats were anesthetized and implanted with four subcutaneous electrodes around the eyelid^{26,27}. For animals injected with BrdU before training, electrode implantation occurred three days after BrdU injection. For those injected with BrdU during training, electrode implantation occurred one week before BrdU injection. Two electrodes delivered the unconditioned stimulus (US) and two recorded eyelid electromyographic (EMG) responses. Rats were acclimated to the conditioning apparatus for one hour, and spontaneous blink rate was recorded. Twenty-four hours later, rats were exposed to paired or unpaired stimuli using a trace protocol, or paired or unpaired stimuli using a delay protocol (200 trials per day for 4 consecutive days). During trace conditioning, a 83-dB, 250-ms burst of white noise (CS) was separated from a 100-ms, 0.7-mA periorbital shock (US) by a 500-ms trace interval. The intertrial interval (ITI) was 20 ± 10 s. Trace eyeblink conditioning using these parameters is hippocampus dependent; that is, acquisition of this task is prevented by hippocampal lesions (A. Beylin, A. Talk, C. Ghandi, L. Matzel and T.J. Shors, unpublished data). During unpaired training, rats received the same number

of CS and US exposures, but in an explicitly unpaired manner. The maximum EMG response occurring during a 250-ms prestimulus baseline recording period was added to four times its standard deviation. Responses that exceeded that value and had a width of at least three ms were considered eyeblinks. Eyeblinks were considered conditioned responses (CRs) if they occurred at least 250 ms after CS onset. In the unpaired-trace protocol, eyeblinks were counted if they occurred 250 ms after CS onset. Three groups of animals were trained for trace-paired or trace-unpaired protocols or were naive controls. Two groups that received BrdU one week before the start of training were perfused at either 24 h ($n = 6$ per group) or 7 days after the end of training ($n = 6$ per group). One group that received BrdU during training, after learning criterion had been reached, was perfused 24 hours later ($n = 5-6$ per group).

During delay conditioning, a 320-ms, 83-dB CS coterminated with an 80-ms, 0.7-mA US. In this protocol, eyeblinks were considered CRs if they occurred at least 80 ms after CS onset. During unpaired training, rats received the same number of CS and US exposures except in an explicitly unpaired manner. In the unpaired-delay protocol, eyeblinks were counted if they occurred at least 80 ms after CS onset. These animals, in addition to a separate group of naive control animals, were perfused 24 hours after the last day of training ($n = 5-6$ per group). Delay eyeblink conditioning is not hippocampus dependent; hippocampal lesions do not impair this type of learning in rabbits or rats²⁸ (A. Beylin, A. Talk, C. Ghandi, L. Matzel and T.J. Shors, unpublished data).

Morris water-maze training. For maze training^{29,30}, groups of rats were trained on a cue test using a visible platform, on a place test using a submerged platform or in a swim-stress condition in which each rat was time-yoked to a place-test-trained rat. Acquisition of the place test, but not the cue test, is disrupted by hippocampal lesions¹³. The water maze (175 × 75 cm) was filled with room-temperature water and nontoxic white paint. Prominent posters and objects surrounded the maze. Rats were exposed to 4 trials per day for 4 days with an ITI of 60 s. During cue testing, the platform was visible and randomly moved to one of four quadrants on each trial. During place testing, the platform was submerged in the same quadrant on all trials. For both the cue and place tasks, the rat was randomly placed into a quadrant facing the maze wall. Latency to reach the platform was timed, and swim path was videotaped. The rat remained on the platform for 30 s. Additional animals were time-yoked to rats in the place test; these rats were placed in the maze for the same amount of time but without a platform. The ITI for these animals was 90 s (total time that rats trained with the cue and place tests were allowed to remain on the platform added to the 60 s ITI). This experiment was done twice—once with animals perfused 7 days after the last day of training ($n = 6$ per group) and once with animals perfused 24 h after the last day of training ($n = 6$ per group).

Histological procedures. Brains of all rats were processed immunohistochemically for combined BrdU and markers of several cell types, including TOAD-64, a marker of immature neurons³¹, GFAP, a marker of astroglia⁵, and calbindin, a marker of mature granule neurons⁶, using peroxidase or fluorescent methods. For stereological analysis, coronal sections (40 μm) were cut throughout the entire hippocampal function with an oscillating tissue slicer. The sections were mounted onto slides and incubated in H₂O₂, rinsed, permeabilized with trypsin, denatured in 2 N HCl, rinsed and incubated with normal horse serum and mouse monoclonal antibody against BrdU (Novocastra, Newcastle upon Tyne, UK, 1:250). The sections were rinsed in PBS and reacted immunohistochemically using a Vectastain ABC Elite kit with nickel-enhanced diaminobenzidine (DAB). The sections were then rinsed and incubated in a second primary antibody: anti-GFAP (Santa Cruz Biotechnology, Santa Cruz, California; 1:5000), anti-TOAD 64 (gift of Susan Hockfield 1:10,000) or anti-calbindin (Chemicon, Temecula, California 1:750). Following several rinses, the sections were reacted immunohistochemically, omitting nickel from the DAB solution, and then counterstained for Nissl with cresyl violet and coverslipped with Permount. For immunofluorescence, the sections were processed for BrdU labeling as described above but with anti-mouse CY-3 (Sigma, 1:300) followed by incubation in anti-TOAD-64, anti-GFAP or anti-calbindin. These latter antigens were visualized with either goat anti-rabbit-Alexa 488 (Molecular Probes, Eugene, Oregon, 1: 1000 for TOAD-64 and calbindin) or biotinylated rabbit anti-

goat (Vector, Burlingame, California, 1:200) followed by avidin-CY2 (Amersham Pharmacia, Piscataway, New Jersey, 1:1000). The sections were dried, counterstained with the DNA dye Hoechst 44323 and coverslipped under PBS and glycerol. Control sections were processed as described above with omission of the primary antisera.

Stereological analysis of the number of BrdU-labeled cells was done on peroxidase-stained tissue on coded slides using a modified version of the optical fractionator method³². BrdU-labeled cells on every twelfth section throughout the dentate gyrus were counted, omitting cells in the outermost plane of focus to avoid counting cell caps. The same stereological methods were used for counting the number of degenerating or pyknotic cells in the subgranular zone. Pyknotic cells were defined by darkly stained, condensed spherical chromatin, lack of a nuclear membrane and pale or absent cytoplasm¹⁵. BrdU-labeled pyknotic cells were also counted, although these profiles were rare. The volume of the granule cell layer was determined for each animal using Cavalieri's principle³³. For purposes of comparison, we also examined the number of BrdU-labeled cells in the subventricular zone (SVZ), a region lining the wall of the lateral ventricles and known to produce cells in adulthood¹⁴. For this analysis, we counted the number of BrdU-labeled cells in the SVZ present on coronal sections throughout the dentate gyrus (every twelfth section throughout the entire hippocampal region). This analysis includes a substantial part of the SVZ but excludes the anterior portion. For double labeling, the percentage of BrdU-labeled cells that expressed TOAD-64, calbindin or GFAP was determined by counts of labeled cells on a minimum of six sections throughout the dentate gyrus using an Olympus BX-60 fluorescent microscope. A minimum of 50 labeled cells were examined for each animal. Immunofluorescent double-labeled cells were verified using a Zeiss Axiovert confocal laser scanning microscope (510LSM). For statistical analysis, ANOVA was followed by Neuman Keuls *post hoc* comparisons.

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