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Neurogenesis and Learning: Acquisition and Asymptotic Performance Predict How Many New Cells Survive in the Hippocampus

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Abstract

Previous research has shown that some associative learning tasks prevent the death of new neurons in the adult hippocampus. However, it is unclear whether it is mere exposure to the training stimuli that rescues neurons or whether successful learning of the task is required for enhanced neuronal survival. If learning is the important variable, then animals that learn better given the same amount of training should retain more of the new cells after learning than animals that do not learn as well. Here we examined the effects of training versus learning on cell survival in the adult hippocampus. Animals were injected with BrdU to label a population of cells and trained one week later on one of two trace conditioning tasks, one of which depends on the hippocampus and one that does not. Increases in cell number occurred only in animals that acquired the learned response, irrespective of the task. There were significant correlations between acquisition and cell number, as well as between asymptotic performance and cell number. These data support the idea that learning and not simply training increases the survival of the new cells in the hippocampus.

Keywords

neurogenesis; BrdU; dentate gyrus; hippocampus; associative learning; trace conditioning; time

The hippocampus produces and integrates new cells into the granule cell layer throughout adult life, most of which become neurons (Cameron, Woolley, McEwen, & Gould, 1993; Markakis & Gage, 1999; Lledo, Alonso & Grubb, 2006). Thousands of new cells are produced in the adult hippocampus each day (Christie & Cameron, 2006), but a large percentage of them die within a few weeks (Cameron, et al., 1993; Dayer, Ford, Cleaver, Yassaee, & Cameron, 2003; McDonald & Wojtowicz, 2005). The death of some cells can be prevented, however, by experiences that involve certain types of learning tasks (Gould, Tanapat, Hastings, & Shors, 1999a; Hairston, Little, Scanlon, Barakat, Palmer, Sapolsky, & Heller, 2005; Leuner, Mendolia-Loffredo, Kozorovitskiy, Samburg, Gould, & Shors, 2004; Leuner, Waddell, Gould, & Shors, 2006a; Shors, Miesegaes, Beylin, Zhao, Rydel and Gould, 2001; Leuner, Gould, & Shors, 2006b). For example, cells generated one week before training on these tasks are more likely to survive than cells that are generated in the hippocampus of naïve animals (Leuner, et

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al., 2006a; Gould, Beylin, Tanapat, Reeves, & Shors, 1999b). After training, the cells remain in the dentate gyrus (DG) for months where they presumably become incorporated into the adult hippocampus (Leuner, et al., 2004; van Praag, Schinder, Christie, Toni, Palmer, & Gage, 2002; Ramirez-Amaya, Marrone, Gage, Worley, & Barnes, 2006).

The tasks that reportedly enhance cell survival are those that depend on the hippocampus for learning, such as trace conditioning using an eyeblink response to assess performance and spatial learning using the Morris water maze (Leuner, et al., 2006a; Gould, et al., 1999b; Shors, 2004). Tasks that are similar in procedure, but do not depend on the hippocampus do not enhance cell survival. These tasks include delay eyeblink conditioning and the visible platform task (Shors, 2004). Also, new cells in the DG appear to be involved in aspects of hippocampal dependent learning, because depletion of the new cells is associated with deficits in some types of hippocampal-dependent learning (Shors, et al., 2001; Saxe, Battaglia, Wang, Malleret, David, Monckton, Garcia, Sofroniew, Kandel, Santarelli, Hen, & Drew, 2006; Winocur, Wojtowicz, Sekeres, Snyder, & Wang, 2006; Madsen, Kristjansen, Bolwig, & Wortwein, 2003). Thus, the evidence to date suggests that new cells in the hippocampus are sensitive to training on tasks that depend on the hippocampus and may also be used in performing the learned response.

Despite these data, a number of questions remain about the effects of training on cell survival. One question is whether learning is important or whether exposure to the training procedure is sufficient. In the initial studies, the number of cells from animals that reached a predetermined criterion during training on trace eyeblink conditioning was compared to the number in animals that either learned a task that did not depend on the hippocampus or were exposed to unpaired stimuli (Leuner, et al., 2004; Gould, et al., 1999b). Thus, the effects of training in animals that did not learn the response were not evaluated. The other question is whether tasks that do not necessarily depend on the hippocampus could rescue new cells from death. For example, there is a training task that is very similar to trace conditioning – it possesses a 500 ms trace interval between the conditioned stimulus (CS) and the unconditioned stimulus (US) – but instead of the US alone, the US is presented simultaneously with another CS. Importantly, learning this task does not depend on the hippocampus (Bangasser, Waxler, Santollo, & Shors, 2006). These two questions were addressed in the following experiment. First, a population of new cells was labeled with bromodeoxyuridine (BrdU) one week before training. Groups of rats were then trained either on trace conditioning, which is hippocampal-dependent (Beylin, Gandhi, Wood, Talk, Matzel, & Shors, 2001; Solomon, Vander Schaaf, Thompson, & Weisz, 1986) and increases cell survival (Gould, et al., 1999b), or on a trace conditioning task which is hippocampal-independent, referred to here as contiguous trace conditioning (CTC) (Bangasser, et al., 2006) (Figure 1a). The number of new cells that remained in the hippocampus after training was determined for all animals, irrespective of how well they learned.

General procedures and eyeblink conditioning

Male Sprague-Dawley rats (n=37), 350–450 g, 65 days old, were individually housed with *ad libitum* food and water and were maintained on a 12h light/dark cycle. For the assessment of hippocampal cell survival, rats were injected i.p. once with BrdU (200mg/kg), which incorporates DNA of dividing cells during the S-phase of the cell cycle. Naive rats (n = 13) were kept undisturbed in their home cages, whereas one to two days after injection, the remaining animals (n = 24) were implanted with headstages and electrodes for eyeblink conditioning. During surgery, rats were first anesthetized with pentobarbital (15 mg/kg) and maintained on isoflurane and oxygen. Two pairs of electrodes (insulated stainless steel wire 0.005") were attached to a head stage and implanted through the upper eyelid. Following recovery (7 days after BrdU injection), rats were given 45 min to acclimate (no stimuli

presented) to the conditioning environment and baseline blinking was assessed by recording responses during 100 random intervals of 500 ms.

Twenty-four hours after acclimation and eight days after the BrdU injection, a group (n=12) was trained with the trace procedure and another group (n=12) with CTC (200 trials/day for four days and an intertrial interval 25 ± 5 s). A white noise generator attached to a speaker administered a white noise (83 db) CS and a shock generator delivered an eyelid shock (0.7 mA) as the US. Trace conditioning consisted of a 250 ms CS presentation, followed by a 500 ms trace interval, and a 100 ms US presentation. The procedure of the CTC was similar to the trace procedure except that the CS was presented again simultaneously with the US (Figure 1a). Eyeblinks that occurred during the trace interval were considered conditioned responses (CRs) and were detected by changes in eyelid electromyographic (EMG) activity with electrodes connected to a differential amplifier with a 300–500 Hz band pass filter (amplified 10K and digitized at 1 kHz). Changes in EMG activity during the trace interval were compared to baseline recordings 250ms before CS onset. If the activity exceeded a minimum of 0.5 mV and a maximum amplitude of the baseline by >4 standard deviations and persisted for >7 ms, it was considered a conditioned response.

Twenty-four hours after the last day of training (13 days after the BrdU injection), rats were deeply anaesthetized with sodium pentobarbital (100 mg/kg) and intracardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were extracted and post-fixed in 4% paraformaldehyde for up to 48 hrs, and were later transferred to 0.1M phosphate buffer.

Immunohistochemistry for BrdU

Coronal sections (40 μ m) were cut through the entire DG of one hemisphere (randomly chosen) of the brain with an oscillating tissue slicer. For BrdU peroxidase staining, a 1:12 series of sections were mounted onto glass slides and pretreated by heating in 0.1 M citric acid (pH 6.0). Tissue was then incubated in trypsin, followed by 2N HCl and overnight in primary mouse anti-BrdU (1:200) and 0.5% Tween 20. The next day, tissue was incubated for 1 hr in biotinylated anti-mouse antibody (1:200), then in avidin-biotin-horseradish peroxidase (1:100), and lastly in diaminobenzidine. After rinsing in phosphate buffer, slides were counterstained with cresyl violet and cover-slipped with Permount. For quantitative analysis, estimates of total numbers of BrdU-labeled cells were determined using a modified unbiased stereology protocol (Gould, et al., 1999b; West, Slomianka, & Gundersen, 1991). BrdU-labeled cells in the subgranular zone (SGZ), granule cell layer (GCL) and hilus on every twelfth unilateral section throughout the entire rostrocaudal extent of the DG were counted blindly at 1,000X on a Nikon Eclipse E400 light microscope, avoiding cells in the outermost focal plane. The number of cells was multiplied by 24 to obtain an estimate of the total number of BrdU-labeled cells in the hippocampus.

Conditioning was evaluated using repeated measures ANOVA. Blocks of training trials were used as the repeated measures (blocks of 20 trials for the first 100 and blocks of 100 for the remaining 700 trials) and the type of training procedure (Trace versus CTC) as the independent measure. The type of training procedure did not alter the % of CRs that were emitted [$F(1, 22) = 0.01$; $p > 0.05$], nor was there an interaction between blocks of training trials and type of training ($p > 0.05$). Thus, responding during training with trace and CTC was similar (Figure 1b). As expected, there was a main effect of trials [$F(11, 242) = 13.39$; $p < 0.001$], as the percentage of CRs increased across blocks.

To evaluate the potential effect of overall performance during training on cell survival, animals were categorized into those that reached a criterion of 60% CRs during training (good learners) or those that did not (poor learners), as used in previous studies (Leuner, et al., 2004). Of the good learners, 7 had been trained with the standard trace procedure and 6 had been trained with

CTC. Of the poor learners, 5 had been trained with trace and 6 with CTC. As expected, those classified as good learners emitted a greater % of CRs across blocks [$F(11,66)=11.65$; $p<0.001$; $F(11,55)=9.49$; $p<0.001$ separate repeated measures ANOVA for good learners trained for 800 trials on Trace or CTC, respectively], whereas the poor learners did not ($p>0.05$) (Figure 1b). Within-subjects comparisons indicated that the %CRs in animals that learned well (reached 60% CRs) did not further increase during the last 200 trials of training ($p>0.05$), indicating that they had reached asymptotic performance. There was no difference in spontaneous eyeblink rates between good learners and poor learners before any training occurred [$F(1,22)=0.10$; $p>0.05$] (data not shown).

Overall, learning rather than training increased the number of cells that remained in the hippocampus one day after training had ceased [$F(1,34)=0.76$; $p>0.05$] (Figure 2a). The animals that reached a criterion of 60% CRs in either task (Trace or CTC) possessed more labeled cells than did naïve animals that were kept in their home cages during the training procedure [Trace: $F(1,18)=9.53$; $p<0.01$; CTC: $F(1,17)=4.78$; $p<0.05$] (Figure 2a). This effect of learning was evident in the combined counts from subgranular zone and granule cell layer [Trace: $F(1,18)=5.75$; $p<0.05$; CTC: $F(1,17)=5.24$; $p<0.05$] (Figure 2a) and did not occur in the hilus ($p>0.05$; data not shown). Since the majority of cells in the subgranular zone and granule cell layer mature into neurons (Christie & Cameron, 2006), these data suggest that learning rescues cells that will become neurons. Moreover, the animals that learned well possessed more cells after training than did the animals that learned poorly [Trace: $F(1,10)=15.9$; $p<0.005$; CTC: $F(1,10)=5.16$; $p<0.05$] (Figures 2a and 3). Again, the effect of learning was evident in the subgranular zone and granular cell layer [Trace: $F(1,10)=5.08$; $p<0.05$; CTC: $F(1,10)=6.72$; $p<0.05$] (Figure 2b), but not in the hilus ($p>0.05$; data not shown). The number of BrdU labeled cells in animals that reached criterion (good learners) did not differ between animals that were trained on trace or CTC ($p>0.05$).

The number of BrdU labeled cells in subgranular and granule cell layer of individual animals correlated with the % of CRs during training on the third session (trials 400–600) [$r=0.54$; $p<0.01$] (Figure 2b) and the last session (trials 600–800) [$r=0.49$; $p<0.05$] (Figure 2c). The number of cells also correlated with the total number of CRs that were emitted across all 800 trials of training [$r=0.42$; $p<0.05$]. The number of BrdU labeled cells (SGZ and GCL) did not correlate with the %CRs during training on the first (trials 1–200) or second (trials 200–400) session. The number of cells in the hilus did not correlate with the %CRs during any session of training ($p>0.05$).

In this experiment, cells that were born one week before trace conditioning were more likely to survive provided that learning occurred. The type of training task was inconsequential: that is, learning during training with the standard trace procedure in which the stimuli are discontinuous was effective, as was training with a trace procedure in which contiguity is established by simultaneous presentation of the CS and the US together after the trace interval. These results confirm previous findings showing that learning a trace conditioning procedure enhances the survival of newly generated cells in the adult hippocampus (Gould, et al., 1999b) and that discontinuity between the CS and the US is not a necessary feature for this effect to occur (Leuner, et al., 2006a). In a recent study, we found that animals with hippocampal lesions could associate the CS with a US across a trace interval, provided that the CS was presented again in combination with the US (Bangasser, et al., 2006). Here we find that learning under these training conditions increased the number of new cells that survived, indicating that the newly generated cells are not responding exclusively to tasks that depend on the hippocampus for learning. There is one potential caveat to this conclusion. In the lesion study (Bangasser, et al., 2006), conditioning was assessed with fear conditioning (freezing) rather than an eyeblink response, as used here. It seems unlikely that the choice of behavioral response would matter and therefore, we tentatively conclude that the increase in cell survival

is not limited exclusively to learning that depends on the hippocampus. Additionally, just because the CTC task does not require the hippocampus for learning, it does not mean that the hippocampus is not used when it is present.

Irrespective of the training regimen, animals that learned better by the end of training retained more new cells in their hippocampus than those that did not learn as well. The cells were located in the subgranular zone and granular cell layer, where post-mitotic daughter cells reside as they differentiate into neurons (Christie & Cameron, 2006). There was no effect of learning on the number of cells that remained in the hilus, where fewer new neurons reside. In previous studies, the vast majority of the cells (~80%) that remained in the hippocampus after learning possessed neuron-specific markers (Leuner, et al., 2004; Leuner, et al., 2006a; Gould, et al., 1999b). It is therefore assumed that the cells here would become neurons, if they were not already. The increase in BrdU cell number after learning was significant, although proportionately less here than in some previous studies (Leuner, et al., 2004; Gould, et al., 1999b). The reasons for the differences probably reflect, at least in part, the fact that more cells were present in the naïve controls. This could be due to the age of the animals when they were injected with BrdU. Even in adulthood, the number of new cells decreases significantly between about 2 and 9 months of age (McDonald & Wojtowicz, 2005). In the initial study (Gould, et al., 1999b), we used adult animals, but did not confine our measurements to young adults (~65 days of age) used here and more recently (Leuner, et al., 2006a). Also, the overall level of conditioning achieved after 800 trials even in those that reached criterion (i.e. the good learners) was not as high as in other studies, which regulates the number of cells that survive.

The results from these studies indicate that learning and not training increases the survival of new cells in the dentate gyrus. The effects are therefore not attributable to “enriched environment” or movements associated with the training procedure. This supports previous results indicating no effect of unpaired stimuli on cell survival (Leuner, et al., 2004; Gould, et al., 1999b). Exactly what determines whether a given task will increase cell survival is unclear at this time, but most likely involves differences in the electrophysiological responses during training. Trace conditioning is known to enhance cell excitability in the hippocampus (Moyer, Thompson, & Disterhoft, 1996), but so does delay conditioning (Berger, Rinaldi, Weisz, & Thompson, 1983), which does not rescue the new cells from death (Leuner, et al., 2006a; Gould, et al., 1999b). More subtle differences in how hippocampal neurons respond to trace conditioning (Gilmartin & McEchron, 2005) likely account for the differential effects of the various training procedures on neurogenesis.

Here we report several correlations between the amount of learning and the number of cells that remained in the dentate gyrus. Other investigators have also reported correlations (Drapeau, Mayo, Aurousseau, Le Moal, Piazza and Abrous, 2003, Kempermann, & Gage, 2002), although typically between cells generated and performance on hippocampal-dependent tasks. For example, the number of cells born in the hippocampus of aged animals, weeks after training, correlated with their performance on a spatial maze task (Drapeau, et al., 2003). The effect reported here is different in that the correlation emerges as a function of learning itself and thus reflects the fate of cells that were already present at the time of the learning experience. In a previous study, we did find that the degree of responding early in training (200 trials) correlated with the number of new cells that survived (Leuner, et al., 2004). However, the animals were not trained to asymptote, and therefore we do not know which ones would have learned, given the opportunity. Here we show that animals that learned after training for 800 trials retained more cells than animals that did not learn, but were trained for as many trials. Therefore, it can be concluded that the effect of trace conditioning and perhaps other training tasks on neurogenesis is related to learning and not simply to training. Moreover, the correlation between the number of learned responses and cell number that occurs early in training is maintained until the end of training when most animals have reached asymptote. These data

suggest that acquisition rescues the cells from death and the number of cells at the end of training relates to the level of performance that was achieved.

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Figure 1.

A. Schematic diagram of the trace and CTC conditioning procedures. During trace conditioning, an 83-dB, 250 ms burst of white noise conditioned stimulus (CS) was separated from a 100 ms, 0.7 mA periorbital shock unconditioned stimulus (US) by a 500 ms trace interval. During contiguous trace conditioning (CTC) the CS was presented again after the 500 ms trace interval, simultaneously with the US for 100 ms.

B. *Training in CTC versus trace conditioning did not differentially affect learning:* Behavioral data from animals that learned well (reached 60% CRs) during training on CTC or trace and animals that learned poorly (poor learners) during training on CTC or trace. Data are

represented as a mean percentage of CRs $\pm$ SEM with the first 100 trials divided into 20 trial blocks and the remaining 700 trials divided into blocks of 100.

Figure 2. Learning during trace conditioning or CTC enhanced the survival of new born cells in the dentate gyrus

A. Bars represent mean number of BrdU-labeled cells in the SGZ and GCL of the dentate gyrus $\pm$ SEM of naïve animals good learners and poor learners trained either on trace conditioning or CTC. * $p < 0.05$ difference between good learners and naïve animals, as well as difference between good learners and poor learners.

C. There was a positive correlation between the performance of individual animals during the third day of training (400–600 trials, trained either on CTC or trace) and the number of BrdU labeled cells in the SGZ and GCL of the dentate gyrus.

D. There was also a positive correlation between the performance of individual animals during the last 200 trials of training (600–800 trials, trained either on CTC or trace) and the number of BrdU labeled cells in the SGZ and GCL of the dentate gyrus.

Figure 3. BrdU labeled cells (shown with black arrows) in the dentate gyrus of hippocampus from similar sections of an animal that learned well during training (reached 60% CRs) and an animal that learned poorly. Images were magnified 1000x.