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# A Natural Form of Learning Can Increase and Decrease the Survival of New Neurons in the Dentate Gyrus

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**ABSTRACT: Granule cells born in the adult dentate gyrus undergo a 4-week developmental period characterized by high susceptibility to cell death. Two forms of hippocampus-dependent learning have been shown to rescue many of the new neurons during this critical period. Here, we show that a natural form of associative learning, social transmission of food preference (STFP), can either increase or decrease the survival of young granule cells in adult rats. Increased numbers of pyknotic as well as phospho-Akt-expressing BrdU-labeled cells were seen 1 day after STFP training, indicating that training rapidly induces both cell death and active suppression of cell death in different subsets. A single day of training for STFP increased the survival of 8-day-old BrdU-labeled cells when examined 1 week later. In contrast, 2 days of training decreased the survival of BrdU-labeled cells and the density of immature neurons, identified with crmp-4. This change from increased to decreased survival could not be accounted for by the ages of the cells. Instead, we propose that training may initially increase young granule cell survival, then, if continued, cause them to die. This complex regulation of cell death could potentially serve to maintain granule cells that are actively involved in memory consolidation, while rapidly using and discarding young granule cells whose training is complete to make space for new naïve neurons. Published 2005 Wiley-Liss, Inc.[†]**

**KEY WORDS: hippocampus; adult neurogenesis; granule cell; learning; memory; cell death**

## INTRODUCTION

Although many new neurons are generated in the adult rat dentate gyrus each day, approximately half die within 4 weeks under normal laboratory conditions (Cameron and McKay, 2001; Dayer et al., 2003). During this period of vulnerability to cell death, the new granule cells appear to be important for some forms of hippocampus-dependent learning (Shors et al., 2001, 2002). In a reciprocal fashion, their own survival is altered by hippocampus-dependent learning tasks; both spatial Morris water maze learning and trace eyeblink classical conditioning have been shown to increase the survival rate of young granule cells (Gould et al., 1999; Ambrogini et al., 2000; Leuner et al., 2004). Paradoxically, other studies have found decreases or no change in BrdU-labeled cells with water maze training (Dobrossy et al., 2003; Ambrogini et al., 2004; Snyder et al., 2005).

Human and animal studies have shown that, in addition to spatial memory, the hippocampal region is critical for flexible expression of memories independent of spatial context (Alvarez et al., 1995, 2002; Bunsey and Eichenbaum, 1995; Dusek and Eichenbaum, 1997, 1998; Squire, 2004). One such form of memory is episodic/event memory, which in humans is generally accepted as a primary hippocampal function. Although it is unclear whether animals express episodic memory per se (Tulving, 2002; Clayton et al., 2003), a natural form of rodent learning, social transmission of food preference (STFP), shows many features of episodic memory in humans, including rapid one-trial learning, flexible expression, and hippocampal requirement for delayed but not immediate recall (Eichenbaum, 1998). The STFP learning task takes advantage of an association rodents automatically make between novel food odors and a specific component of rodent breath, carbon disulfide, within a single 10-min interaction (Galef, 1989; Eichenbaum, 1998; Clark et al., 2002). The resulting preference for the cued flavor persists for at least 3 months and can be expressed in a very different context from which it was learned, i.e., in the absence of social interaction and in different spatial contexts (Alvarez et al., 2002; Clark et al., 2002). Lesion or inactivation of the hippocampus causes a deficit in rats' preference behavior 1–2 days after training, but not less than 1 day or greater than 5 days after training (Alvarez et al., 2001; but see Burton et al., 2000; Winocur et al., 2001; Roberts and Shapiro, 2002). This timing of behavioral deficits suggests that the hippocampus is required for consolidation of information learned during STFP, but not for acquisition of the preference itself or for long-term storage or retrieval—similar to what is found in human studies of hippocampus-dependent memory (Cave and Squire, 1992; Milner et al., 1997). These episodic memory-like features, as well as the recently shown STFP-induced activation of CREB within the dentate gyrus specifically (Countryman et al., 2005), make STFP a valuable tool for elucidating the relationship between hippocampus-dependent learning and the survival of new granule cells.

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## MATERIALS AND METHODS

### Animals

All animal treatments conformed to NIH guidelines and were approved by the NIMH Animal Care and Use Committee. Adult (9–10 week old) male Long-Evans rats (Taconic) were singly housed under a 12:12 h light/dark cycle and provided with free access to food and water except as described later. After habituating to the routine conditions in the animal facility for 4–7 days, rats in Experiments 1–4 were each given a single injection of BrdU (300 mg/kg, i.p, 10 mg/ml in 0.9% NaCl/0.007 N NaOH, Roche, Indianapolis, IN). The day of BrdU injection was designated as Day 0 in all four of these experiments (Fig. 1) and rats were killed on Day 9, Day 14, or Day 16, i.e., when BrdU-labeled cells were 9, 14, or 16 days old.

In all experiments, additional rats of the same age and strain were used as demonstrators for STFP training (see later). Because of the large numbers of demonstrator rats needed, each experiment was split into two separate cohorts treated 1 day apart, and the same demonstrators were used for both cohorts. Each cohort consisted of 3 control rats and 3 trained rats, resulting in a total *N* of 6 rats undergoing training or testing in each group; a few rats were later excluded from analyses because of complete absence of BrdU labeling in the entire brain or problems with sectioning. Since no differences were observed across cohorts in any experiment, data from both cohorts were pooled.

### Experimental Design

#### *Preliminary experiments: analysis of STFP behavioral task*

Social interactions during STFP training (see later) were videotaped using a digital video camera (Sony night shot DRV120) to compare the behavior of control and trained rats. Two pairs of rats (one control rat with demonstrator and one trained rat with demonstrator) were videotaped during each of the three 20-min interactions on the training day for each cohort, so that all 6 control and 6 trained rats were taped once. The total time involved in social investigation, defined as sniffing of the demonstrator's nose and face by the subject, was scored from the videotapes for the first and second halves of the interaction period. Additionally, the latency and total number of aggressive postures adopted by trained or control rat toward the demonstrators were scored.

To measure the induction of corticosterone by the social interaction involved in STFP training, 6 control and 6 trained rats were trained for 1 day (three interactions) as described later and were decapitated within 1 min of the end of the last social interaction (between 3:00 and 4:00 PM). Home cage controls remained in the animal facility for the entire day and were decapitated within 1 min of removal from their home cage. Trunk blood was collected and centrifuged at 3,000 rpm for 10 min. Serum was pipetted off and stored at −20°C. Serum corticosterone level was measured with a Coat-A-Count rat corticosterone radioimmunoassay kit (TKRC1 from Diagnostic Products Corporation, Los Angeles, CA).

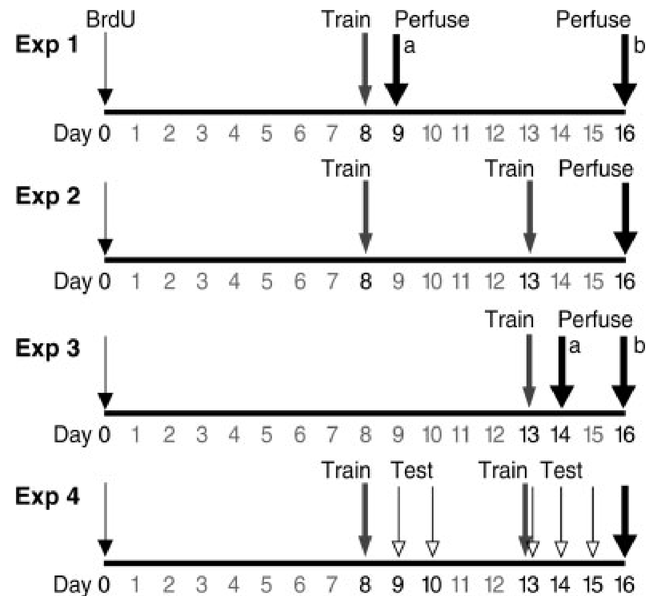



**FIGURE 1. Design of experiments 1–4. A single injection of BrdU was given on Day 0, and so each day, e.g., Day 16, corresponds approximately to the age of BrdU-labeled cells. Training for STFP and perfusion of rats were done on different days after BrdU injection. In Experiments 1 and 3, separate groups of rats were perfused 1 day after training (Experiments "1a" and "3a") or on Day 16 (Experiments "1b" and "3b"). Testing for STFP learning was done only in Experiment 4.**

#### *Experiment 1: STFP training on Day 8*

To examine the effect of STFP learning, 12 rats were given STFP training and 12 were given control training, as described later, on Day 8 after BrdU injection. Half of the rats in each group were perfused 1 day after training (Day 9, Experiment 1a) and the other half were perfused 8 days after training (Day 16, Experiment 1b) to examine short-term and longer-term effects of training on young granule cells (Fig. 1). These time points were chosen based on reports that learning alters survival between 1 and 2 weeks after granule cells are born (Gould et al., 1999; Ambrogini et al., 2004).

#### *Experiment 2: additional STFP training*

To investigate the effects of additional training, 6 rats were given STFP training on both Day 8 and Day 13 after BrdU injection. Six control rats received control training on both days. All rats were perfused 3 days after the second training day, on Day 16 (Fig. 1).

#### *Experiment 3: STFP training on Day 13*

To examine the effects of a training day on Day 13 alone, 24 rats were given STFP or control training 13 days after BrdU injection (Fig. 1). Half of each group was perfused 1 day later

(Day 14, Experiment 3a), and the remaining rats were perfused on Day 16 (Experiment 3b).

### Experiment 4: STFP training and testing

To examine the effects of both training and testing for STFP, 12 rats were given control or STFP training on Days 8 and 13 after BrdU injection as in Experiment 2. All rats were tested for food preference on Days 9–10 and 13–15 and perfused on Day 16 (Fig. 1).

## Behavioral Procedures

### Shaping

Rats were shaped to eat plain powdered chow (the NIH-07 formula used in the animal facility, Zeigler Bros., Gardner, PA) from 4-ounce straight-side polycarbonate jars (Nalgene 2116–0125, Nalge Nunc International, Rochester NY) for 2 days before training. Jars of powdered chow were attached with Velcro to transparent Plexiglas platforms (10 cm × 17 cm × 0.3 cm) and placed in the home cage instead of normal pelleted chow.

### Training

Control, trained, and demonstrator rats were all moderately food restricted by being given only 14–16 g of (pelleted) food the night before training (beginning at 4–5 PM). On the day of training, demonstrator rats were given ground chow mixed with a novel flavor and allowed to eat for 30 min. Pairs of flavors previously shown to be equally palatable to naive rats (Berger-Sweeney et al., 2000; Galef and Whiskin, 2000) were used: 2% cocoa was paired with 1% cinnamon (for all Day 8 training), and 0.7% basil was paired with 2% thyme (for all Day 13 training). The first cohort in each experiment was trained with cocoa (or basil) and the second cohort was trained with cinnamon (or thyme) to counterbalance flavors within each tested pair. Food cups were weighed before and after eating to ensure that all demonstrators ate at least 1 g of food. Immediately after eating, each demonstrator rat was placed into a subject's cage. Demonstrators and subjects were allowed to interact for 20 min, after which the demonstrators were returned to their home cages. Training was repeated two additional times using the same flavor and different demonstrators with a 60-min interval between interactions, according to the protocol of Alvarez et al. (2001). Control rats were treated identically to trained rats, except that their demonstrators ate plain powdered rat chow. Demonstrators for the trained group were used once each day to assure that they ate enough flavored food just before the interaction, but demonstrators for controls were rotated through all three control rats (requiring 12 demonstrators for six subjects).

### Testing

Control and trained rats were modestly food restricted by being given only 14–16 g of (pelleted) food the night before testing (beginning at 4–5 PM). During each testing session, all rats received one cup containing ground chow mixed with the trained flavor and one containing ground chow mixed with a novel flavor (the other flavor in the pair), and were allowed to eat for 45 min. Rats in the two cohorts were tested with the same two flavors, each of which was novel for a different cohort. The location of the two cups (right or left) was counterbalanced across rats. The food eaten was calculated by weighing the food cups before and after testing, and a preference score for each rat for each testing day was determined as follows: 100 × weight of trained food eaten/weight of all food eaten. Mean scores for each group were compared with chance performance (50%) using a one-group $t$-test (Prism, GraphPad).

## Histological Procedures

Rats were transcardially perfused with 4% paraformaldehyde (pH 7.4) under deep isoflurane anesthesia. Brains were removed from the skull, postfixed in 4% paraformaldehyde (pH 7.4) overnight, and then cryoprotected in 20% sucrose. Coronal 50 $\mu$m sections through the entire dentate gyrus were cut on a sliding microtome.

Series of every 12th section were mounted on slides and processed for BrdU immunohistochemistry using monoclonal anti-mouse anti-BrdU antibody (1:100, Becton-Dickinson, BD Biosciences, San Jose, CA), biotinylated goat anti-mouse IgG (1:200, Sigma, St. Louis, MO), avidin-biotin-horseradish peroxidase complex (Vector Labs, Vector Laboratories, Inc., Burlingame, CA), and cobalt-enhanced DAB (Sigma Fast Tabs), according to a previously published protocol (Dayer et al., 2003). Sections were then counterstained using cresyl violet and coverslipped under Permount (Fisher Scientific, Pittsburgh, PA).

Series of every 24th section from each brain were stained for the immature neuronal marker, crmp-4 (Minturn et al., 1995; Cameron and McKay, 2001), using polyclonal ab25 (1:20,000, generously provided by S. Hockfield), biotinylated anti-rabbit antisera (1:200, Sigma), avidin-biotin-horseradish peroxidase complex (Vector Laboratories), and cobalt-enhanced DAB (Sigma Fast Tabs) on free-floating sections. Sections were mounted on slides and coverslipped under Permount.

Series of every 12th section from each brain were double-labeled for BrdU and the mature neuronal marker NeuN simultaneously, using monoclonal mouse anti-NeuN (1:250, Polysciences, Warrington, PA) and monoclonal rat anti-BrdU (1:100, Accurate, Westbury, NY) followed by Alexa568-conjugated anti-mouse IgG (1:250) and Alexa488-conjugated anti-rat IgG (1:250, Molecular Probes, Eugene, OR). Sections were counterstained in bisbenzimide (Hoechst 33258), mounted on slides, and coverslipped under Immu-mount (Shandon, Waltham, MA).

Series of every 24th section from each brain were double stained simultaneously for BrdU and phospho-Akt (p-Akt), a promoter of cell survival and suppressor of cell death (Noshita et al., 2002), using a monoclonal anti-mouse anti-BrdU antibody (1:100, Becton-Dickinson) and a rabbit polyclonal anti-p-Akt (serine-473) antibody (Cell Signaling; 1:100), followed by Alexa 568-conjugated anti-mouse IgG (1:200) and Alexa 488-conjugated anti-rabbit IgG (1:200). Sections were mounted on slides and coverslipped under Immu-mount (Shandon).

Series of every 12th section from each brain were mounted on slides, counterstained with cresyl violet, and coverslipped under Permount for pyknotic cell counting.

## Histological Analysis

BrdU-labeled, crmp-4-labeled, and pyknotic cells were counted throughout each series at 400×, 1,000×, and 400× magnification, respectively, on slides that were coded to conceal treatment group. Crmp-4-labeled cells were counted only if a labeled cell body with proximal process was observed. Pyknotic cells were identified by the presence of rounded darkly stained condensed chromatin, lack of a nuclear membrane, and pale or absent cytoplasm (Sengelaub and Finlay, 1982; Sloviter et al., 1993b). Pyknotic cells are reliable indicators of apoptosis in the dentate gyrus (Sloviter et al., 1993a), and are found in numbers very similar to those seen with TUNEL-labeling (Gould et al., 1997).

Because STFP learning may use only specific subregions of the hippocampus (Winocur et al., 2001) and because region-specific changes in learning-induce cell survival have previously been reported (Ambrogini et al., 2000), cell counts were split according to their anatomical location. Sections not containing any ventral hippocampus (approximately Bregma −1.6 to −4.16 mm, according to Paxinos and Watson, 1998) were classified as rostral, while remaining sections (approximately Bregma −4.16 to −6.8 mm) were classified as caudal. These subdivisions are similar to previously described dorsal-ventral and septal-temporal divisions of the hippocampus (Dolorfo and Amaral, 1998; Moser and Moser, 1998). Cells on each section were classified as being in the suprapyramidal blade or infrapyramidal blade of the granule cell layer (gcl) or in the hilus. The location of pyknotic cells in the gcl was further subdivided into subgranular zone (sgz, defined as the inner 25% of the gcl) or outer gcl (i.e., the remaining 75% of the gcl).

Stereological counts and densities of each cell type were determined (see Discussion). Densities were calculated by dividing the cell count in each subregion, e.g., rostral suprapyramidal blade, by the total subregion volume, which was measured using a computerized analysis system (Neurolucida, Microbrightfield). Cell densities and counts from the gcl were analyzed using 3-way analysis of variance (ANOVA) with treatment (control vs. trained), region (rostral vs. caudal), and blade of the gcl (suprapyramidal vs. infrapyramidal) as factors. Data from the hilus were analyzed using 2-way ANOVA with treatment and region as factors. BrdU-labeled pyknotic cell counts were analyzed using Student's *t*-test because they were too low to split by region.

Fluorescently immunolabeled sections were analyzed at 400× on an Olympus BX60 epifluorescent microscope with FITC, TRITC, DAPI, and combination filters. Because NeuN, p-Akt, and BrdU all label nuclei, colabeling within the nucleus was the standard used for double-labeled cells. The percentages of NeuN-labeled and p-Akt-labeled BrdU-labeled cells were analyzed using Student's *t*-test, because these data were not split by region. Colabeling was confirmed and images for figures were acquired using an Olympus Fluoview 300 confocal microscope equipped with four lasers (405 Diode laser, Argon 488, HeNe 543, and HeNe 633). Each channel was separately scanned using a sequential configuration to avoid cross-talk between fluorescent labels. Confocal Z sectioning was performed at 0.5–1.0 $\mu$m intervals using UPlanFl 60× oil-immersion (NA = 1.25), or UPlanApo 100× oil-immersion (NA = 1.35) objectives. Images were acquired and three-dimensionally reconstructed using the Olympus Fluoview software, cropped and optimized in Photoshop 7.0 by making minor adjustments to contrast, and arranged with graphs in Canvas 8.0.

# RESULTS

## Preliminary Experiments: Analysis of STFP Behavioral Task

STFP training involves a social interaction between rats that have some superficial similarities to the resident-intruder stress paradigm (Takahashi et al., 1983; Heinrichs and Koob, 2001). Since stress could potentially alter hippocampus-dependent learning and its effects on young neuron survival (Prickaerts et al., 2004; Wong and Herbert, 2004), we examined the behavior of rats during the social interaction phase of STFP and their stress hormone levels immediately after.

During the first 10 min of the social interaction, trained rats spent significantly more time than controls investigating the faces of the demonstrators ($P = 0.0178$; 317 ± 35 s vs. 214 ± 18 s, respectively), suggesting that trained rats recognized the novelty of the food odor smelled on the demonstrator's breath. In the last 10 min, social investigation declined significantly in both groups, and all rats displayed primarily self-grooming or resting behavior. Occasional threatening or attack postures were shown by control and trained rats toward demonstrators and vice versa. No significant differences between control and trained rats in either the number of threats (2.0 ± 0.7 in both groups) or latency to threaten demonstrators (170 ± 65 s in controls and 172 ± 47 s in trained rats) were found.

Corticosterone levels immediately following STFP training were not significantly different ($P = 0.2017$) between trained rats (195.2 ± 25.2 ng/ml) and those that received control training (145.6 ± 25.3 ng/ml). Home cage rats had corticosterone levels (87.4 ± 29.3 ng/ml) that were not significantly different from control rats ($P = 0.1754$), but were significantly lower than those of trained rats ($P = 0.0234$).

The lack of difference between control and trained rats in corticosterone levels or aggressive behaviors suggests that histological differences between control and trained rats are unlikely to be caused by stress. The corticosterone level observed in trained rats, though significantly increased compared with home cage controls, was lower than the 300 ng/ml previously found after a Morris water maze swimming trial (Beiko et al., 2004). This modest increase in corticosterone might result from the novelty of the training experience, the mild food deprivation, the social interaction, or some combination of these factors.

## Experiment 1: STFP Training on Day 8

To examine the effect of STFP learning, rats were trained for STFP on Day 8 after BrdU injection and killed either 1 day or 8 days later (Fig. 1). BrdU-labeled cells were observed primarily along the hilar border of the gcl at both time points (Fig. 2A). No effect of STFP training on Day 8 was observed in BrdU-labeled cell density 1 day later (Fig. 2B, Table 1). However, on Day 16, i.e., 8 days after training, BrdU-labeled cell density was significantly higher in trained rats than in control rats (Fig. 2B, Table 1). Stereological cell counts showed similar results, although the increase on Day 16 showed only a trend toward significance (Table 1), likely because of higher variability in stereological count data (see Discussion). BrdU-labeled cell density was significantly higher in the rostral region than in the caudal region on both the days ($P = 0.0006$ on Day 1 and $P = 0.0019$ on Day 8). However, there were no significant interactions between treatment and either location parameter, indicating that training had the same effect in the rostral dentate gyrus, which corresponds approximately to the dorsal/septal dentate gyrus, as in the caudal (temporal/ventral) dentate gyrus. This is in contrast to the change limited to very rostral portion of the dentate gyrus after spatial water maze training reported by Ambrogini et al. (2000). Since no interactions were found between treatment and location in this or any of the following experiments, only main effects of treatment are shown throughout.

The hilus, which contains no BrdU-labeled granule neurons at any of the time points examined, was analyzed as a control region. No significant differences were found in counts or densities of BrdU-labeled cells in the hilus (Table 2), indicating that the effect of STFP on new granule cell survival is specific.

To look for active suppression of cell death following training, we immunostained for the phosphorylated form of Akt, a protein kinase that, when activated by phosphorylation, promotes cell survival (Noshita et al., 2002). Strong p-Akt staining was observed in more than 70% of BrdU-labeled cells 1 day after training (Fig. 3). The proportion of BrdU-labeled cells that stained for p-Akt was significantly higher in trained rats than in controls ($P = 0.0143$ by Student's unpaired $t$-test, Fig. 3D), suggesting that more of the young granule cells were actively stabilized, or protected from cell death, in the training group than in the control group. By 8 days after training, this difference had disappeared, and fewer than half of all BrdU-labeled cells stained for p-Akt ($P = 0.8216$ by Student's unpaired $t$-test, Fig. 3D).

Comparison of BrdU-labeled cell counts on Day 9 and Day 16 indicates a loss of BrdU-labeled cells in both treatment groups (Fig. 2B), consistent with constitutive death of young granule cells throughout this period (Dayer et al., 2003). We looked for additional evidence of cell death by counting BrdU-labeled and unlabeled pyknotic cells in the sgz and outer gcl. Small numbers of BrdU-labeled pyknotic cells were observed in the sgz in both treatment groups 1 day after training (Fig. 4A). Surprisingly, the density of these cells, though small, showed a significant *increase* in trained rats compared with controls ($P = 0.0262$, Fig. 4B) despite the decreased cumulative cell death found 7 days later. Stereological counts were too low and had too little variability to be analyzed statistically ($12 \pm 12$ in control vs. $24 \pm 4$ in trained rats). No BrdU-labeled pyknotic cells were observed 8 days after training. No significant treatment effects were observed in unlabeled pyknotic cell density in any portion of the gcl at either survival time ($P > 0.1$).

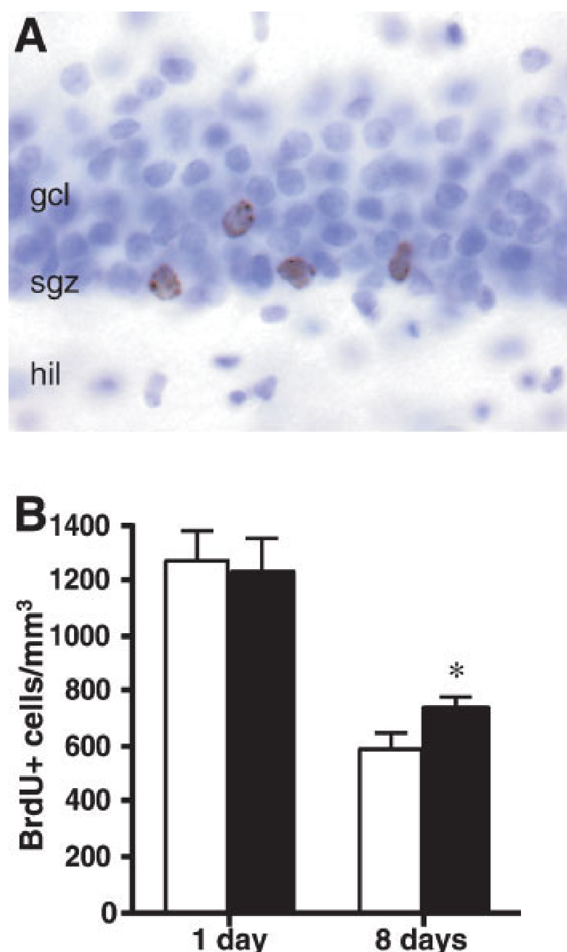



**FIGURE 2. STFP training 8 days after BrdU injection increased survival of BrdU-labeled granule cells. (A) Sixteen days after injection, BrdU-labeled cells were located primarily in the sgz, the portion of the gcl at the border of the hilus (hil). (B) One day after training (Day 9), rats trained for STFP (black bars, $N$ = 6) showed no difference in BrdU-labeled cell density from control rats (white bars, $N$ = 4). However, eight days after training (Day 16), trained rats ($N$ = 6) had significantly more BrdU-labeled cells than control rats ($N$ = 4). Bars represent mean + standard error of mean (SEM). * indicates significant difference from control ($P$ < 0.05) by 3-way ANOVA.**

## Experiment 2: Additional STFP Training

To investigate the effects of additional training, rats were trained on Days 8 and 13 after BrdU injection and were killed on Day 16 (Fig. 1). BrdU-labeled cell density was significantly lower in trained rats than in controls (Fig. 5, Table 1). This statistically significant difference was also seen in stereological

*TABLE 1.*

***BrdU-Labeled Cells in the GCL Following Training at Different Times After BrdU Injection***

| Experiment | Group | BrdU+ cell density | BrdU+ cell count |
|---|---|---|---|
| 1a: trn 8, kill 9 | Control ($N = 4$) | 1,280 ± 108 | 4,515 ± 301 |
| | Trained ($N = 6$) | 1,240 ± 118 ($P = 0.8089$) | 4,254 ± 355 ($P = 0.5880$) |
| 1b: trn 8, kill 16 | Control ($N = 4$) | 602 ± 62 | 2,271 ± 227 |
| | Trained ($N = 6$) | 746 ± 46 (**$P$ = 0.0296**) | 2,792 ± 223 (***P = 0.0593***) |
| 2: trn 8/13, kill 16 | Control ($N = 6$) | 980 ± 60 | 3,750 ± 222 |
| | Trained ($N = 6$) | 800 ± 70 (**$P$ = 0.0146**) | 2,696 ± 187 (**$P$ = 0.0001**) |
| 3a: trn 13, kill 14 | Control ($N = 6$) | 1,028 ± 56 | 3,744 ± 228 |
| | Trained ($N = 6$) | 1,000 ± 52 ($P = 0.5506$) | 3,482 ± 239 ($P = 0.2955$) |
| 3b: trn 13, kill 16 | Control ($N = 3$) | 1,012 ± 82 | 4,180 ± 342 |
| | Trained ($N = 6$) | 986 ± 60 ($P = 0.7634$) | 3,807 ± 241 ($P = 0.2584$) |
| 4: trn 8/13, test, kill 16 | Control ($N = 4$) | 1,072 ± 106 | 3,741 ± 240 |
| | Trained ($N = 4$) | 828 ± 84 (**$P$ = 0.0034**) | 2,880 ± 193 (**$P$ = 0.0052**) |

Densities (cells/mm$^3$) and stereological counts of BrdU-labeled (+) cells. $P$ values from 3-way ANOVA (BrdU+ cells) indicate statistically significant differences (**bold** face) or statistical trend (***bold italics***). trn, training; kill, killed.

cell counts (Table 1). No significant treatment effects were found on BrdU-labeled cells within the hilus (Table 2). No BrdU-labeled pyknotic cells were found in either treatment group, and the densities of unlabeled pyknotic cells in the sgz and outer gcl were not significantly different in trained rats compared with controls ($P > 0.1$). Staining for the mature neuronal marker NeuN revealed no significant difference in the proportion of BrdU-labeled cells double-labeled with NeuN between control (48 ± 2)% and trained rats (45 ± 5)%.

## Experiment 3: STFP Training on Day 13

To examine the effects of a training day on Day 13 alone, rats were trained for STFP on Day 13 after BrdU injection and were killed either 1 day later (on Day 14) or on Day 16 (Fig. 1). No significant treatment effects were found in BrdU-labeled cell density or count in the gcl on Day 14 or on Day 16 (Fig. 6A, Table 1). No significant treatment effects were found on BrdU-labeled cells in the hilus at either survival time point (Table 2). BrdU-labeled pyknotic cells were observed in some brains 1 day after STFP training, as in Experiment 1a. The density of these profiles was significantly increased in trained rats compared with controls (Fig. 6B,C); the stereological count was too low for analyzing statistically (2 ± 2 in control, 18 ± 6 in trained). No BrdU-labeled pyknotic cells were found in either treatment group 3 days after training, and no differences in sgz or outer gcl pyknotic cells were observed at either survival time point ($P > 0.1$).

*TABLE 2.*

***BrdU-Labeled Cells in the Hilus Following Training at Different Times After BrdU Injection***

| Experiment | Group | BrdU+ cell density | BrdU+ cell count |
|---|---|---|---|
| 1a: trn 8, kill 9 | Control ($N = 4$) | 244 ± 14 | 1,917 ± 284 |
| | Trained ($N = 6$) | 230 ± 14 ($P = 0.5196$) | 1,780 ± 203 ($P = 0.4706$) |
| 1b: trn 8, kill 16 | Control ($N = 4$) | 157 ± 20 | 1,368 ± 276 |
| | Trained ($N = 6$) | 157 ± 15 ($P = 0.9944$) | 1,410 ± 254 ($P = 0.8785$) |
| 2: trn 8/13, kill 16 | Control ($N = 6$) | 211 ± 16 | 1,752 ± 236 |
| | Trained ($N = 6$) | 243 ± 26 ($P = 0.3148$) | 1,700 ± 204 ($P = 0.7862$) |
| 3a: trn 13, kill 14 | Control ($N = 6$) | 209 ± 15 | 1,698 ± 206 |
| | Trained ($N = 6$) | 251 ± 23 ($P = 0.1393$) | 1,764 ± 205 ($P = 0.7141$) |
| 3b: trn 13, kill 16 | Control ($N = 3$) | 222 ± 22 | 2,016 ± 365 |
| | Trained ($N = 6$) | 270 ± 12 ($P = 0.0623$) | 2,268 ± 278 ($P = 0.2718$) |
| 4: trn 8/13, test, kill 16 | Control ($N = 4$) | 299 ± 25 | 2,055 ± 288 |
| | Trained ($N = 4$) | 276 ± 22 ($P = 0.4974$) | 2,244 ± 439 ($P = 0.5059$) |

Densities (cells/mm$^3$) and stereological counts of BrdU-labeled (+) cells. $P$ values from 2-way ANOVA show no statistically significant differences. trn, training; kill, killed.

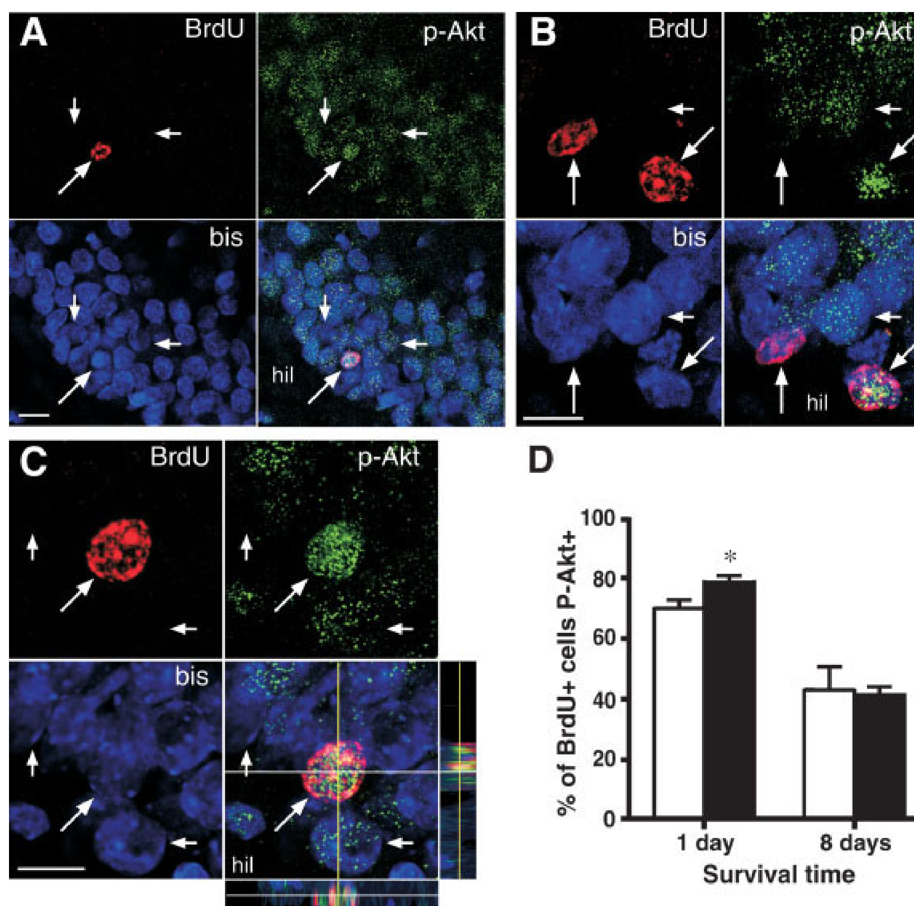



**FIGURE 3. The survival-promoting protein Akt was activated in new granule cells within 24 h of STFP training on Day 8. (A) A low magnification single confocal plane shows strong p-Akt immunostaining in a BrdU-labeled cell (long diagonal arrow) near the hilus (hil), while other granule cells, seen with bisbenzimide counterstain (bis), show moderate (horizontal arrow) or minimal p-Akt staining (vertical arrow). (B) A higher magnification image shows neighboring BrdU-labeled cells (long arrows), one with strong p-Akt staining (diagonal arrow), and the other with no detectable p-Akt staining (vertical arrow). A BrdU-negative granule cell with moderate p-Akt staining (horizontal arrow) is shown for comparison. *Z*-axis projection of 11 × 0.5 μm. (C) A BrdU-labeled granule cell with strong p-Akt staining (diagonal long arrow) is shown in color separation and orthogonal reconstruction. Moderately p-Akt stained (horizontal arrow) and p-Akt negative (vertical arrow) granule cells are shown for comparison. *Z*-axis projection of 9 × 0.5 μm. Calibration bars in A–C = 10 μm. (D) A significantly higher proportion of BrdU-labeled cells were also stained for p-Akt in trained rats (black bars) than in control rats (white bars) 1 day, but not 8 days, after training on Day 8. Bars represent mean + SEM. * indicates significant difference from control ($P = 0.0484$) by unpaired Student's *t*-test.**

## Experiment 4: STFP Training and Testing

To examine the effects of both training and testing for STFP, rats were trained on Days 8 and 13 after BrdU injection and killed on Day 16, as in Experiment 2, but were also tested for STFP preference after training (Fig. 1). Trained rats learned the STFP task, as indicated by their consumption of significantly more trained food than predicted by chance 24 h after each training session ($P < 0.05$; $P = 0.0291$ for cocoa/cinnamon and $P = 0.0087$ for basil/thyme by one-group *t*-test, Fig. 7A). Control rats' performance was at the level of chance at this time point ($P = 0.9078$ for cocoa/cinnamon and $P = 0.5881$ for basil/thyme by one-group *t*-test, Fig. 7A). Two days after training, trained rats no longer ate significantly more cued food than chance (Fig. 7A). Since previous studies have found that memory for STFP lasts for at least 3 months (Clark et al., 2002) and that rats develop aversions to foods they have eaten recently (Galef and Whiskin, 2003a), this loss of preference expression is likely to reflect the repeated testing protocol used in this experiment as opposed to loss of the STFP memory. The total amount of food consumed did not differ significantly between the control and trained groups at any time point ($P = 0.5773$; mean for all sessions, 3.8 ± 0.4 g and 4.2 ± 0.5 g, respectively).

BrdU-labeled cell density was significantly decreased in trained rats compared with controls (Fig. 7B, Table 1). The density of immature neurons labeled with crmp-4 was also significantly decreased in trained rats compared with controls ($P = 0.0237$ by 3-way ANOVA, Fig. 7B). The decreases in crmp-4-labeled cell density and BrdU-labeled cell density were similar in magnitude to each other and to the decrease in BrdU-labeled cell density seen in Experiment 2 (compare Figs. 5 and 7B). Stereological counts of BrdU-labeled cells also showed significant decreases

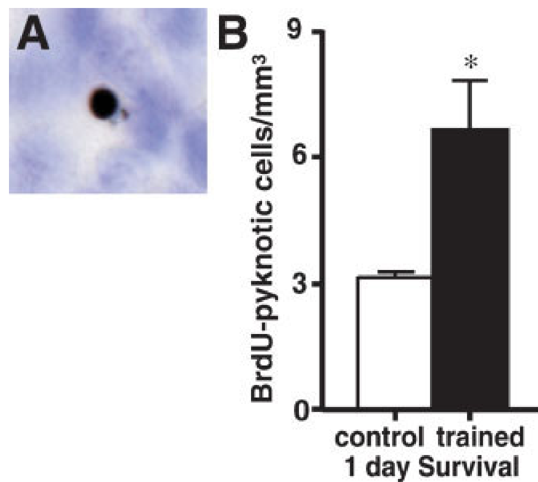



FIGURE 4. STFP training 8 days after BrdU injection increased death of new granule cells within 24 h. (A) An example of a 9-day-old BrdU-labeled pyknotic cell. These cells provide direct evidence for death of young granule cells 1 day after training; none were observed 8 days after training. (B) Rats trained for STFP ($N$ = 6) had more BrdU-labeled pyknotic cells than control rats ($N$ = 4) 1 day after training ($P$ = 0.0262 by unpaired Student's $t$-test, indicated by *). Bars represent mean + SEM.

with training (Table 1). Crmp-4-labeled cell counts in trained rats were not significantly lower than those in control rats, likely because of poorly-stained half-sections from one rat in each group, which affect stereological counts more then densities. No significant treatment effects were found on BrdU-labeled cells in the hilus (Table 2). The proportion of BrdU-labeled cells that were double-labeled with NeuN was not significantly different between control and trained rats ($P = 0.7089$; $(53 \pm 6)$% and $(51 \pm 3)$%, respectively).

No BrdU-labeled pyknotic cells were observed in either group, and no changes across treatment group were found in the number of unlabeled pyknotic cells in the sgz or outer gcl ($P > 0.1$). However, more unlabeled sgz pyknotic cells were observed in this experiment and the two experiments with 1-day survival after training (1a and 3a) than in the other experiments, in which rats were not disturbed the day before perfusion ($P = 0.0001$ by 2-way ANOVA, Fig. 7C). Additionally, more outer gcl pyknotic cells were found in this experiment (both treatment groups) than in any of the previous experiments ($P = 0.0012$ by 2-way ANOVA, Fig. 7D). These across-experiment pyknotic cell results should be considered preliminary, because experiments were performed at different times with different groups of rats.

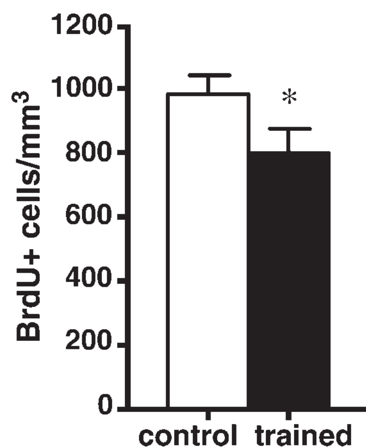
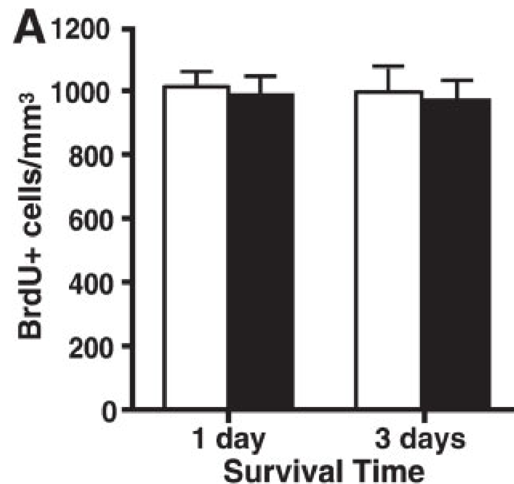



FIGURE 5. STFP training on 2 days (Days 8 and 13 after BrdU injection) decreases the number of young granule neurons surviving on Day 16. Rats trained for STFP ($N$ = 6) have a significantly lower density of BrdU-labeled cells than control rats ($N$ = 6). Bars represent mean + SEM. * indicates significant difference from control ($P$ < 0.05) by 3-way ANOVA.



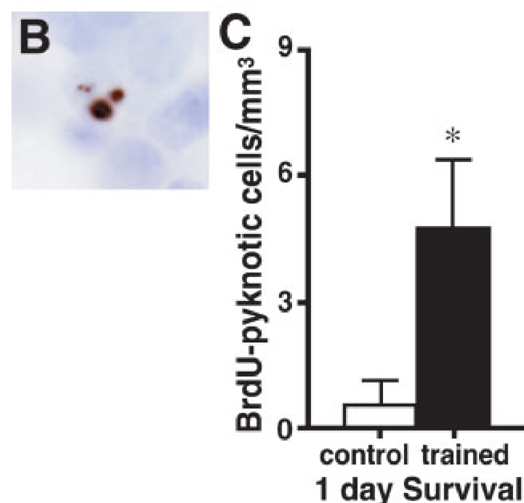




FIGURE 6. STFP training 13 days after BrdU injection increased cell death 1 day later, but had no significant effect on cumulative granule cell death by Day 16. (A) Rats trained for STFP (black bars) showed no significant difference in BrdU-labeled cell density from control rats (white bars) either 1 day ($P$ = 0.5506) or 3 days ($P$ = 0.7634) after training ($N$ = 6, 6, 3, 6 from left to right). (B) BrdU-labeled pyknotic cells such as this were seen 1 day after training on Day 13. (C) Rats trained for STFP on Day 13 after BrdU injection had significantly more BrdU-labeled pyknotic cells than control rats 1 day later ($P$ = 0.0299 by unpaired Student's $t$-test, indicated by *). Bars represent mean + SEM.

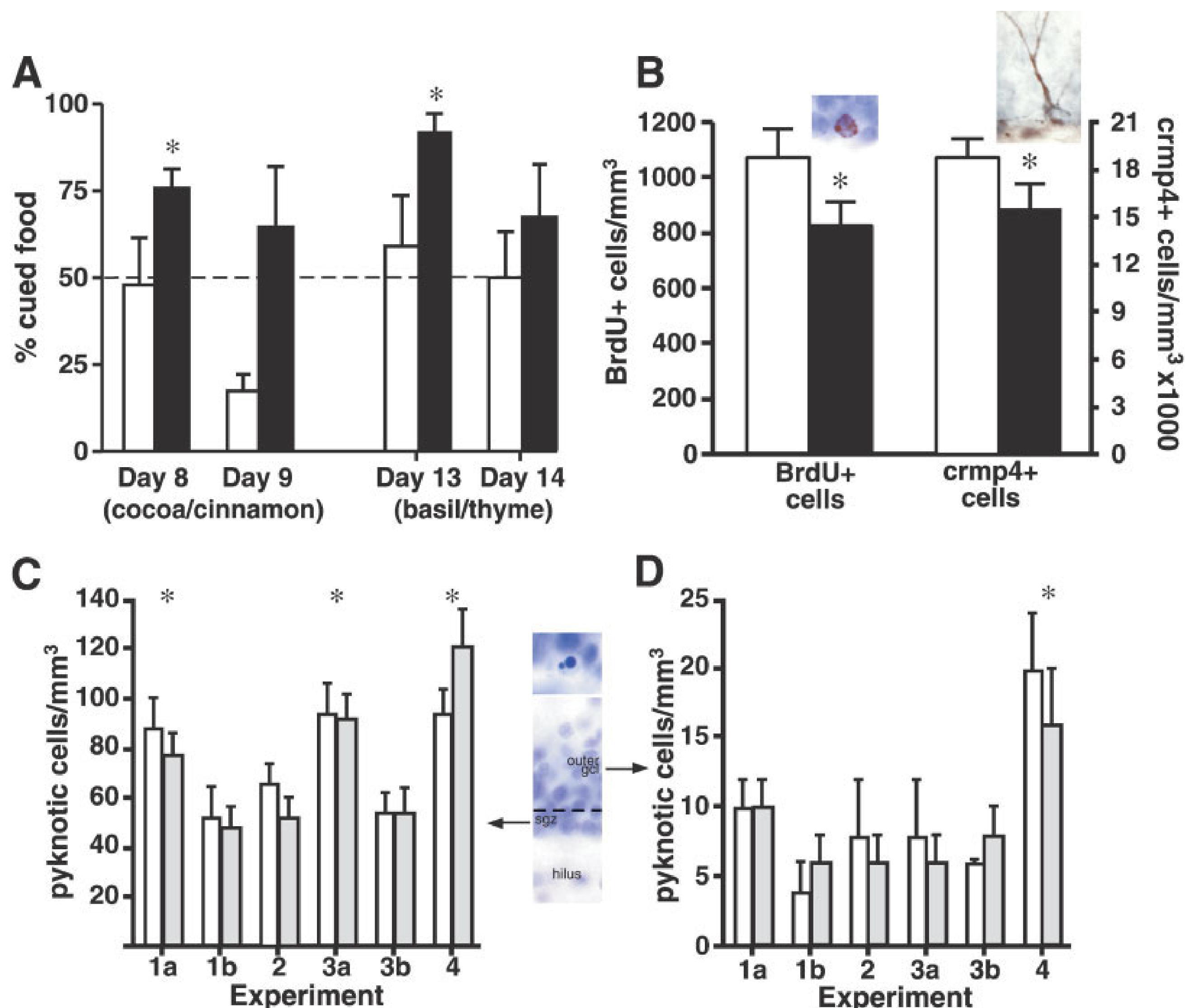



**FIGURE 7.** Two days of STFP training, each followed by testing, decreases survival of young granule neurons. (A) Trained rats learned the STFP task, as indicated by greater than chance performance the day after each training session ($N$ = 4); control rats had chance performance at this time point ($N$ = 4). Preference was no longer evident by the second day of testing, probably because of a competing preference for novel food rather than loss of memory (see text). (B) Trained/tested rats ($N$ = 4) had fewer BrdU+ cells and fewer total immature granule cells, stained with crmp-4, than control rats ($N$ = 4). Insets show typical BrdU-labeled and crmp-4-labeled cells. (C) In the sgz, no significant differences in pyknotic cell density were found between control and trained rats, but more pyknotic cells were seen in experiments that involved treatments (training or testing) 1 day before perfusion (Experiments 1a, 3a, and 4). Insets show a typical non-BrdU-labeled sgz pyknotic cell (top) and the portions of the gcl where pyknotic cells were counted. (D) In the outer gcl, where granule cells born during development reside, no differences in pyknotic cell density were found between control and trained rats. However, more outer gcl pyknotic cells were found in Experiment 4, which involved testing, than in other experiments. Bars represent mean + SEM. * indicates significant difference ($P$ < 0.05) from chance performance (in A, by one-group $t$-test), from control group (in B, by 3-way ANOVA), and from other experiments (in C and D, by 2-way ANOVA using treatment and experiment as factors).

## DISCUSSION

### Technical Considerations

The current study shows that a single day of STFP training on Day 8 after BrdU injection increases the survival of BrdU-labeled granule cells in the dentate gyrus, while STFP training on Days 8 and 13 decreases the survival of the young granule cells (Table 3). In both cases, the effect of STFP on BrdU-labeled cells is almost certainly due to cell death, rather than dilution of the BrdU label below the limits of detection, because control and trained groups were treated identically until Day 8, and disappearance of labeled cells through dilution is complete by Day 4 (Dayer et al., 2003).

*TABLE 3.*

***Summary of BrdU-Labeled Cell Density Results***

| | Experiment | BrdU+ cells | BrdU+ pyknotic cells |
|---|---|---|---|
| 1 day training, | 1a: trn 8, kill 9 | – | ↑ |
| kill on next day | 3a: trn 13, kill 14 | – | ↑ |
| 1 day training, | 1b: trn 8, kill 16 | ↑ | 0 |
| kill on day 16 | 3b: trn 13, kill 16 | – | 0 |
| 2 days training, | 2: trn 8/13, kill 16 | ↓ | 0 |
| kill on day 16 | 4: trn, test, kill 16 | ↓ | 0 |

+, labeled; ↑, increase ($P < 0.05$); ↓, decrease ($P < 0.05$); –, no significant change; 0, none found; trn, training; kill, killed.

The identity of the BrdU-labeled cells lost because of STFP learning cannot be directly determined, but because nearly all BrdU-labeled cells in the gcl express immature neuronal markers within 7 days of BrdU injection (Cameron and McKay, 2001; Brown et al., 2003), the lost cells are most likely to be young granule neurons. The very similar decrease seen in cells labeled with crmp-4, a marker specific for young neurons within the dentate gyrus (Minturn et al., 1995; Cameron and McKay, 2001), supports the neuronal identity of the affected cells.

Because the BrdU-labeled cell counts and densities observed in control rats in Experiment 1b were lower than those seen in Experiments 2–4, it appears that the effect seen in this experiment could result from unusually low numbers of BrdU-labeled cells in control rats rather than an increase due to training. We do not believe that this is the case, because across-experiment comparisons are much less reliable than within-experiment comparisons due to several factors that are not controlled for across experiments. These include variability in the housing environment, such as noise or other stressors either during development or just prior to BrdU injections (Tanapat et al., 2001; Mirescu et al., 2004); changes in the supplier colony such as genetic drift or undetected viruses; and differences in the lot or preparation of BrdU. Within each experiment, these factors are controlled for by purchasing, injecting, and analyzing rats assigned to control and trained groups at the same time.

BrdU-labeled and crmp-4-labeled cells were analyzed both as stereological counts and as cell densities for several reasons. Because the borders between the rostral and caudal dentate gyrus are not clearly defined by the anatomy or histology of the gcl, splitting into these subregions introduces variability and technically invalidates stereological counting methods (West, 1993). Densities are not affected by splitting into subregions and are more robust in the face of small technical problems such as unstained or misordered sections. Additionally, cell densities in the rostral vs. caudal dentate gyrus can be meaningfully compared, while comparisons of counts simply reflect where the dividing line is placed. In all the experiments, stereological counts and densities of BrdU-labeled cells agreed, i.e., both showed statistically significant changes or statistical trends in the same direction. In Experiment 1b, the stereological counts of BrdU-labeled cells reached only the level of a statistical trend ($P < 0.0593$), but the magnitude of the increase was nearly identical in both cases (23% for counts vs. 24% for density), suggesting that the lack of significance for count data simply reflects higher variability. In Experiment 4, the density of Crmp-4-labeled cells decreased significantly with training, while there was no significant change in cell count, but this lack of significance is most likely because of a technical problem (see Results).

## Magnitude of Change

The approximately 20% increase in BrdU-labeled cells observed in Experiment 1, though statistically significant, is modest compared with the near doubling shown following spatial navigation training and trace conditioning (Gould et al., 1999). These two learning protocols used many more trials than were used in our STFP protocol, but simply having more training does not produce a larger effect—a second day of STFP training reversed the effect rather than increasing the magnitude. Instead, the number of cells rescued by training may be related to the complexity of the task, with tasks like STFP that can be learned in a single trial affecting only a small proportion of young granule cells. This might allow rats living in a natural environment to record more of the presumably large number of memorable events they experience each day.

## Increased or Decreased Survival

A recent paper (Ambrogini et al., 2004) has suggested, based on comparison with an older study (Ambrogini et al., 2000), that the age of the young granule cells determines whether their survival is increased or decreased by spatial water maze learning. Our data suggesting BrdU-labeled, sgz, and outer gcl pyknotic cells subpopulation-specific responses to STFP are consistent with the idea that young, mature, and older granule cells do show different susceptibilities to learning- or experience-induced cell death. However, the specific age of the young granule cells was not the critical difference between increased and decreased survival in the current study: neither the result of STFP training 8-day-old cells nor the result of training 13-day-old cells explains the effect of training on both the days. The results of the current experiments also rule out differing effects of training and testing and differences in the length of the postlearning survival period as explanations for this change.

The most likely cause of the change from increased to decreased survival of young granule cells appears to be additional training; 1 day of STFP training either increased or had no effect on cell survival as of Day 16, while training on 2 days decreased cell survival (Table 3). Since rats were trained on different flavors on Days 8 and 13, decreased survival could be caused by the change in the cued flavor (from cocoa or cinnamon to basil or thyme). Loss of "trained cells" following a change in the trained odor would be an attractive mechanism for replacing outdated memories with more current information. However, a previous study found that rats given STFP

training on two odors at different times retained both preferences (Galef and Whiskin, 2003b), suggesting that training on a second odor should not replace the memory for the first odor. Therefore, it seems more likely that the decreased survival is produced by the continued repetition of similar training events. This idea appears consistent with the increased survival seen after 16 trials (Gould et al., 1999) and decreased survival after 50 trials (Ambrogini et al., 2004) previously found in the water maze. Another study (Dobrossy et al., 2003) found fewer BrdU-labeled cells after 8 days of spatial water maze training (32 trials) than after 4 days (16 trials), but it is not clear whether this change reflects cell survival because differential treatment occurred during the period when BrdU is visibly diluted by proliferation (Dayer et al., 2003).

Interestingly, an across-training reversal of synaptic efficacy across training sessions, from long-term potentiation (LTP) to long-term depression (LTD), has been shown in the dentate gyrus using another olfactory associative learning task (Truchet et al., 2002). This parallel switch in new granule cell survival and synaptic responses suggests that maintenance or loss of young granule cells may be analogous to, or possibly result from, the LTP-induced formation and LTD-induced retraction of dendritic spines that have been observed in dentate gyrus granule cells (Trommald et al., 1996; Luscher et al., 2000).

## Cell Death and Cell Survival

Although we have focused so far on contrasting the conditions that lead to increased vs. decreased granule cell survival, we found evidence that cell death and cell stabilization (active cell survival) are both induced soon after training. The simultaneous increases in these two opposing processes, as seen through p-Akt expression and pyknosis of BrdU-labeled cells, suggest that the immediate effect of STFP training may be to push immature granule cells toward a fate decision, i.e., a choice between maturing and dying. How individual cells might be chosen for death or stabilization is not clear. But since cells born on the same day respond differently, this choice seems likely to be based on a cell's unique circuitry rather than a global instruction. This in turn suggests that 1–2 week-old-granule cells may be linked into active circuits and in at least some respects already functioning as neurons.

The STFP-induced changes in pyknotic cells did not obviously parallel the cell death observed as loss of BrdU-labeled cells, most likely because of the timing differences inherent in the two methods. Pyknotic cells, histologically identified apoptotic cells (Sloviter et al., 1993a), are cleared within 2–3 h (Gould et al., 1997; Thomaidou et al., 1997) and therefore reflect cell death at the time the animal is perfused, while BrdU-labeled cell counts reflect cumulative cell loss over the entire experiment. BrdU-labeled pyknotic cell data, total sgz pyknotic cell data, and outer gcl pyknotic cell data all indicate that experience-related cell death begins within 24 h. Interestingly, only BrdU-labeled pyknotic cells showed changes in trained rats compared with controls, suggesting that the age range of granule cells affected specifically by training/learning might be relatively narrow. In contrast, the density of unlabeled pyknotic cells in the sgz, reflecting death of adult-born granule cells with a wide range of ages and maturity levels, was increased in all groups of rats that were removed from the housing facility the day before testing, while pyknotic cells in the outer gcl, where the oldest granule cells are located, were increased in all rats that were tested. Interestingly, the loss of these cells in the outer gcl, which are likely to have the largest axonal projections (Gaarskjaer, 1981; Hastings et al., 2002), is predicted by modeling to improve future learning by the network (Chambers et al., 2004).

## Why Would More Learning Lead to More Death?

Understanding why continued training leads to cell death will require additional information about the cells that die. It is possible that the dying cells are those that have not been incorporated into circuits and will no longer be needed once learning is complete—consistent with a "use it or lose it" rule (Swaab, 1991). Alternatively, the granule cells dying after extensive training may have been involved in early learning and initially saved before being killed. If repetition of events may trigger system consolidation and transfer of a durable memory trace or fact (e.g., cocoa is a safe food) into the cortex, the granule cells holding these event-based memories might become obsolete and could be discarded to make room for brand new, more plastic, granule cells. This second possibility, perhaps best described as "use it *then* lose it" provides a possible mechanism for the apparent loss of the STFP memory trace from the hippocampus after it is transferred to cortex (Winocur et al., 2001). However, it requires that granule cells function as memory storage devices long before they express mature neuronal markers, i.e., that maturity and functionality are not synonymous. It would also suggest that the granule cells that do survive for many months (Dayer et al., 2003; Kempermann et al., 2003; Leuner et al., 2004) are not all of the new granule neurons that captured records of episodic memories tagged as important (Morris and Frey, 1997). Instead, they may be those granule cells storing memories whose meanings have yet to be deciphered.

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