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Neurogenesis May Relate to Some But Not All Types of Hippocampal-Dependent Learning

Tracey J. Shors,[1]* David A. Townsend,[1]
Mingrui Zhao,[1] Yevgenia Kozorovitskiy,[2] and
Elizabeth Gould[2]

[1]*Department of Psychology and Center for Collaborative Neuroscience, Rutgers University, Piscataway, New Jersey*

[2]*Department of Psychology, Princeton University, Princeton, New Jersey*

ABSTRACT: The hippocampal formation generates new neurons throughout adulthood. Recent studies indicate that these cells possess the morphology and physiological properties of more established neurons. However, the function of adult generated neurons is still a matter of debate. We previously demonstrated that certain forms of associative learning can enhance the survival of new neurons and a reduction in neurogenesis coincides with impaired learning of the hippocampal-dependent task of trace eyeblink conditioning. Using the toxin methylazoxymethanol acetate (MAM) for proliferating cells, we tested whether reduction of neurogenesis affected learning and performance associated with different hippocampal dependent tasks: spatial navigation learning in a Morris water maze, fear responses to context and an explicit cue after training with a trace fear paradigm. We also examined exploratory behavior in an elevated plus maze. Rats were injected with MAM (7 mg/kg) or saline for 14 days, concurrent with BrdU, to label new neurons on days 10, 12, and 14. After treatment, groups of rats were tested in the various tasks. A significant reduction in new neurons in the adult hippocampus was associated with impaired performance in some tasks, but not with others. Specifically, treatment with the antimitotic agent reduced the amount of fear acquired after exposure to a trace fear conditioning paradigm but did not affect contextual fear conditioning or spatial navigation learning in the Morris water maze. Nor did MAM treatment affect exploration in the elevated plus maze. These results combined with previous ones suggest that neurogenesis may be associated with the formation of some but not all types of hippocampal-dependent memories. ***Hippocampus 2002;12:578–584.*** **© 2002 Wiley-Liss, Inc.**

KEY WORDS: spatial maze; fear; trace memory; context; dentate gyrus

INTRODUCTION

Nearly 40 years ago, it was reported that the hippocampus continues to produce new neurons in the adult rat (Altman and Das, 1965). This prescient report was followed by several studies that collectively present a compelling case that the new cells produced in adulthood differentiate into granule neurons. We now know that new cells in the hippocampus become incorporated into the granule cell layer, attain the morphological and biochemical characteristics of neurons (Cameron et al., 1993; Okano et al., 1993; Gould et al., 2001), develop synapses on their cell bodies and dendrites (Kaplan and Bell, 1984; Kaplan and Hinds, 1977), extend axons into the CA3 region (Stanfield and Trice, 1988; Hastings and Gould, 1999), and generate action potentials (van Praag et al., 2002). In addition, this process appears to occur in virtually all mammalian species examined, including humans (Eriksson et al., 1998; reviewed in Hastings et al., 2000). Until recently, however, the number of new cells produced in adulthood was vastly underappreciated. Recently, Cameron and McKay (2001) demonstrated that approximately 9,000 new cells are produced every day in the adult rat hippocampus, most of which differentiate into neurons. This extrapolates to >250,000 new cells added to the dentate gyrus every month, a staggering figure, given that the structure only possesses 1–2 million granule cells (Boss et al., 1985; West et al., 1988).

The significant number of new cells generated in adulthood suggests that they may play an important role in learning processes, a possibility first outlined by Barnea and Nottebohm (1994). Indeed, there are several intriguing correlations between the number of new neurons and performance on hippocampal-dependent learning tasks (reviewed in Gould et al., 1999b). For example, learning of some hippocampal-dependent tasks, such as trace eyeblink conditioning and spatial navigation in the Morris water maze, increases the number of new granule cells by enhancing either cell survival or proliferation (Gould et al., 1999a; Lemaire et al., 2000). We recently proposed that a significant removal or depletion of new hippocampal neurons would affect hippocampal-dependent learning. To test this hypothesis, we reduced the generation of new neurons with an antimitotic agent known as methy-

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*Correspondence to: Tracey J. Shors, Department of Psychology, Center for Collaborative Neuroscience, 152 Frelinghuysen Road, Rutgers University, Piscataway, NJ 08854-8020. E-mail: shors@rci.rutgers.edu
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lazoxymethanol acetate (MAM). After 2 weeks of treatment, the number of new cells was dramatically reduced, and animals were impaired in their ability to learn the hippocampal-dependent task of trace eyeblink conditioning, whereas they were still able to learn the hippocampal-independent task of delay conditioning (Shors et al., 2001). There was no detrimental effect of MAM treatment on motor activity, pain sensitivity, or electrophysiological responses in the hippocampus, suggesting that impairment in trace conditioning was not attributable to indirect effects of the treatment on performance of the response or gross hippocampal function. The idea that newly generated neurons are involved in the formation of hippocampal-dependent memories was further supported since the ability to acquire them was restored once the population of new neurons was replenished.

These data, along with those from previous studies, suggest that newly generated neurons in the adult hippocampus are not only affected by, but participate in the learning process. However, it remains unknown whether depletion of new cells affects other types of hippocampal-dependent learning. To investigate this further, we tested animals treated with MAM on spatial learning in a Morris water maze, a task whose acquisition is dependent on the hippocampus (Riedel et al., 1999). In a second experiment, we addressed whether MAM treatment affects the formation of a different type of trace memory; that acquired during fear conditioning. Like that of the eyeblink response, acquisition of trace memories using a fear paradigm depends on an intact hippocampal formation (McEchron et al., 1998). If adult-generated neurons are involved in the formation of trace memories per se, then their depletion should similarly affect acquisition of this type of learning, regardless of the type of behavioral response that is measured.

As an animal associates a neutral stimulus with a fearful one, it also learns to fear the context in which the stimuli were presented. This type of learning, known as contextual fear conditioning, is reportedly dependent on the hippocampal formation (Kim and Fanselow, 1992; Phillips and LeDoux, 1992). For similar reasons, as noted above, we examined whether depletion of new neurons would affect acquisition of contextual fear about the training environment. Finally, there are reports that individual differences in response to novelty correlate with cell proliferation in the dentate gyrus, suggesting a relationship between anxiety-related behaviors and neurogenesis (Lemaire et al., 1999). Therefore, we also tested whether neuron depletion was associated with changes in anxiety and exploratory behavior in the elevated plus maze.

MATERIALS AND METHODS

Subjects

Male Sprague-Dawley rats (220–250 g) were housed individually in hanging wire cages and left undisturbed for 1 week after arrival at the animal facility. They had unlimited access to water and laboratory chow and were maintained on a 12:12 light/dark cycle with light onset at 7:00 AM. Rats were injected daily with MAM (7 mg/kg) or saline for 14 days. A higher dose of MAM was used in this study compared with our previous studies, in which 5 mg/kg MAM was used. The lower dose of MAM was variable in its reduction of BrdU-labeled cell number, whereas the higher does (7 mg/kg) produced a more reliable depletion. The animals were injected with BrdU (75 mg/kg) on days 10, 12, and 14 of MAM treatment at least 2 h after injection with MAM.

Spatial Navigation Test

Rats were treated with saline (n = 8) or MAM (n = 8) and 48 h later trained in the spatial navigation task of the Morris water maze. A maze (175 × 75 cm) was filled with room temperature water and nontoxic black paint. Prominent posters and objects surrounded the tank. Rats were exposed to four trials per day for 4 days with an intertrial interval (ITI) of 60 s. An escape platform was located 1.5 cm below the water's surface, which was invisible to the rat. For each trial, the rat was placed in a random quadrant of the tank, facing the tank wall, and provided 60 s to locate and mount the platform. Latency to reach the platform was recorded. If the rat failed to locate the platform, it was placed there for 30 s. Each rat was provided 30 s on the platform and was placed in a separate environment between trials. The day after training, a probe trial was conducted without a platform. The rat was placed in the tank for 60 s, and time spent in the quadrant where the platform had been was recorded.

Fear Conditioning

Rats were treated with saline (n = 8) or MAM (n = 9) and 24 h later acclimated to the training environment for 30 min. The conditioning environment consisted of four conditioning boxes each surrounded by a soundproof chamber. Within each chamber was a 26 × 22-cm freeze monitor box (SD Instruments, San Diego, CA) with a metallic grid floor connected to the shock generator and clear Plexiglas sides and removable lid. Vertical and horizontal movements of animals were recorded with a photo-beam activity system. On the walls of each enclosing soundproof chamber were detachable panels with 3-inch alternating black and white lines. The detachable panel lines were either configured in a horizontal pattern or a vertical pattern. Rats were not presented with any stimuli during the 30-min period. After 2 min, a baseline measurement of movement was recorded for 3 min, designated the acclimation baseline. Animals were returned to their home cage and boxes were cleaned with Brillianize plastic cleaner (Benicia, CA).

The following day, rats were returned to the same freeze monitor box in which they had been acclimated. After 2 min, they were exposed to 10 trials of paired stimuli using a trace paradigm with an intertrial interval of 210 s. For each trial, a pure-tone conditioning stimulus (CS) (82 dB, 15 s, 5 ms rise/fall time) was followed by a 30-s trace interval followed by a footshock unconditioned stimulus (US) (0.5 s, 1 mA) delivered through the grid floor of the freeze monitor box (see Fig. 2B). One day later, rats were placed in the conditioning chamber for 5 min and no conditioning stimuli were presented. After 2 min, movements over 3 min were recorded and used as a measure of fear associated with the training context. Rats were returned to their home cages for 30 min. They were then placed in a novel testing chamber. The context was changed by

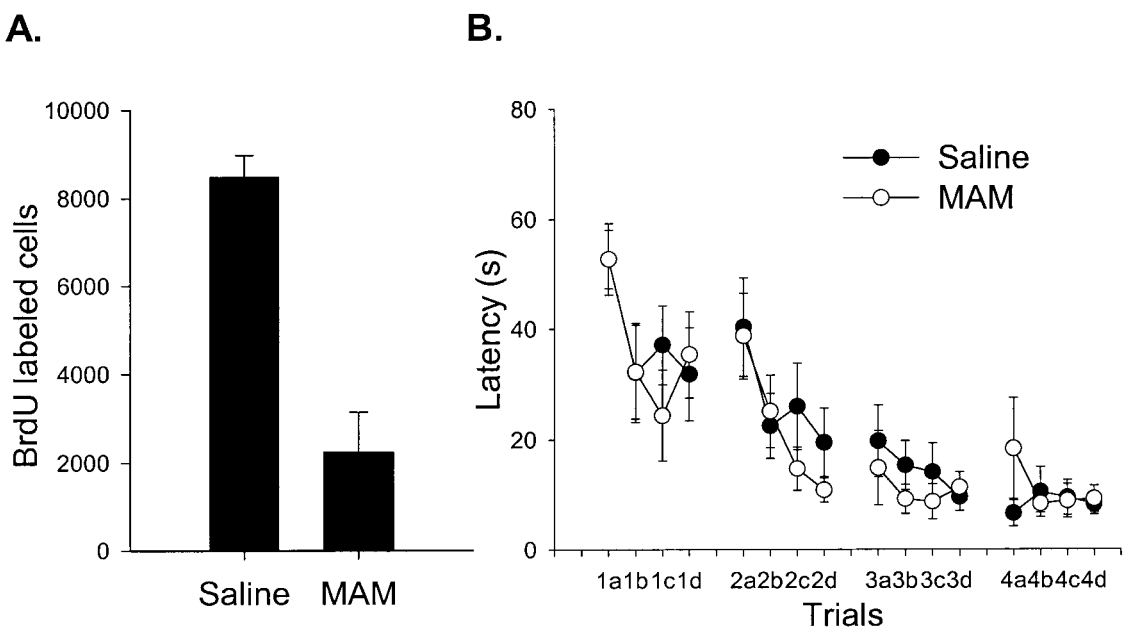

FIGURE 1. Depletion of adult-generated neurons does not affect spatial maze learning. A: Total number of BrdU-labeled cells in the dentate gyrus after 2 weeks of treatment with saline or methylazoxymethanol acetate (MAM). Bars represent mean ±SEM. B: Latency to locate the platform across 16 trials (4 per day) of maze training.

altering the stripes by 90-degrees, replacing grid floors with black Plexiglas floors, using differently scented cleaning solution, and changing the light source without altering the luminosity. After 2 min, movements over 3 min was recorded and used as the baseline for the novel context. Then 10 CS were delivered with an ITI of 240 s (no US). The amount of movement during the tone (15 s) and during the trace interval (30 s after CS offset) was measured. Fear was measured as a decrease in % movements during the tone and trace intervals. It is presented as a percentage of one of two baselines, either the movements during acclimation to the training context (before training) or in the novel context after training.

Plus Maze

At 24 h after testing with fear conditioning, rats were evaluated for performance in the elevated plus maze. The maze consisted of an elevated (53-cm) plus-shaped track. Two of the arms of the maze were enclosed by walls (40 cm in height), with the other two arms and the central intersection open. All arms were 50 cm in length, and the central intersection was 5 cm square. A single 40-watt bulb lit the maze. Rats were confined in the maze for 10 min. Each trial began when a rat was placed in the center facing a closed arm. The entire trial was videotaped from above. Entries into an arm were counted only if all four paws entered the arm. The number of entries into each type of arm (closed or open) and the total time spent in the arms were calculated for each rat. A generalized anxiety and fear score was calculated for each rat by creating a ratio of the total time spent in open arms divided by the total time spent in both open and closed arms (×100). In between rats, the maze was cleaned with alcohol to remove urine and other scent markings.

Histology

At 24 h after testing in behavioral studies, rats were overdosed with sodium pentobarbital and transcardially perfused with paraformaldehyde (4%). Brains were removed, dissected, and stored in phosphate-buffered saline (PBS). Half brains were cut on an oscillating tissue slicer (40-μm-thick sections) throughout the dentate gyrus. Tissue was stained for BrdU, using peroxidase and fluorescence techniques. For peroxidase, tissue was heated at 75°C for 1 h, followed by 2 N HCl for 1 h. After rinsing in PBS, sections were incubated overnight at 4°C in mouse monoclonal anti-BrdU (1:250; Novocastra). Sections were reacted using a standard Vector ABC kit. Slides were coded before quantitative analysis.

Stereological analyses of number of BrdU-labeled cells in one side of the dentate gyrus were conducted on every 12th section of peroxidase stained tissue throughout this brain region. For each section, the number of BrdU-labeled cells was determined in the dentate gyrus excluding those in the outermost focal plane to avoid counting cell caps. Resulting numbers were tallied and multiplied by the number of intervening sections and by both hemispheres.

RESULTS

Effects of MAM on Spatial Learning

BrdU labeling was conducted on a subset of animals in each group (n = 4). Treatment with MAM (7 mg/kg) for 14 days reduced the number of adult-generated cells in the dentate gyrus of the hippocampus [$F(1,6) = 37$; $P < 0.001$] (Fig. 1A). Despite the

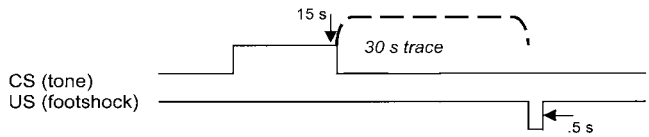

FIGURE 2. Schematic of fear conditioning paradigm for trace conditioning in which the conditioned stimulus (CS) and unconditioned stimulus (US) are separated in time.

reduction in neurogenesis, MAM had no effect on performance in the spatial cue version of the Morris water maze as measured by a change in latency to locate the hidden platform across the 16 trials of training [$F(15,210) = 0.65$; $P = 0.83$]. Nor was there an overall change in latency after MAM treatment ($P = 0.62$) (Fig. 1B). For both groups, latency to reach the platform decreased across trials [$F(15,210) = 638$; $P < 0.00001$]. There was also no effect of MAM treatment on performance during the probe trial [$F(1,14) = 0.005$; $P = 0.94$].

Effects of MAM on Trace Fear Conditioning

BrdU labeling was conducted on a subset of animals in each group (n = 4). Treatment with MAM (7 mg/kg) for 14 days reduced the number of adult-generated cells in the dentate gyrus [$F(1,6) = 72.56$; $P < 0.0005$] (see Fig. 3A). Using a baseline obtained during the acclimation period before training, analysis of variance (ANOVA) showed that treatment with MAM reduced the fear associated with the trace interval during the first three CS-alone trials on testing day [$F(1,15) = 6.06$; $P < 0.05$], as well as during the first trial ($P < 0.05$) (Figs. 2 and 3C). MAM treatment also reduced the fear response during the CS on those same trials [$F(1,15) = 4.40$; $P = 0.05$] (Fig. 3B). With respect to the response after the trace interval (when the US had occurred during training), there was also an effect of MAM [$F(1,15) = 6.20$; $P < 0.05$] (Fig. 3D). Animals treated with saline reduced their movements when the US would have occurred by more than 90%, whereas those treated with MAM only reduced their movements by ~50%.

These effects of MAM on trace fear conditioning were similar irrespective of which baseline was used to evaluate changes in movement. MAM altered movements during the trace interval after the first CS when movements during exposure to the novel context on testing day were the baseline measure [$F(1,15) = 4.94$; $P < 0.05$]. Saline-treated animals reduced their movements during the trace interval by ~70%, whereas animals treated with MAM reduced their movement by ~35%. During the first three testing trials, rats treated with MAM exhibited less of a reduction in movements during the time when the US would have occurred [$F(1,15) = 4.27$; $P = 0.05$], again suggesting that they had acquired less fear after trace conditioning.

Effects of MAM on Movement and Contextual Fear Conditioning

Treatment with MAM did not alter number of gross movements in the various contexts [$F(1,29) = 2.41$; $P = 0.13$] (Fig.

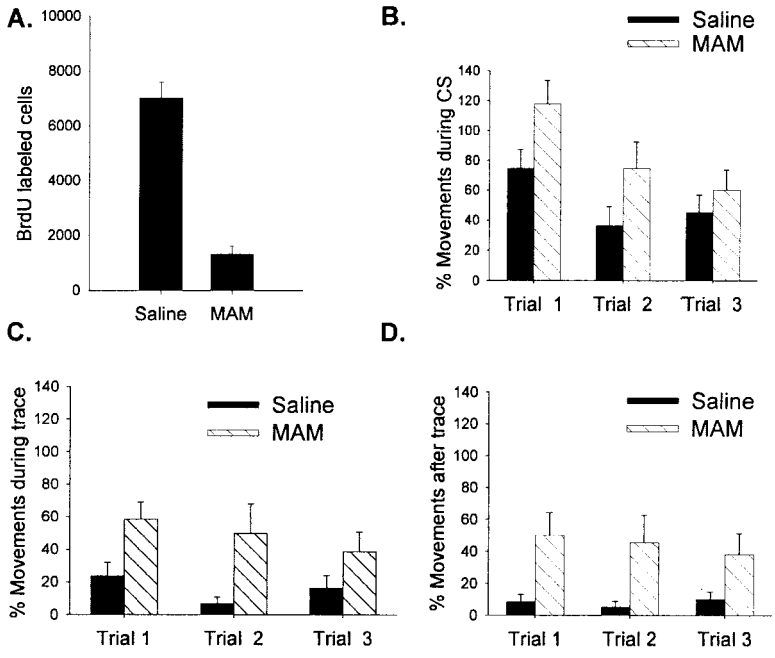

FIGURE 3. Depletion of adult-generated neurons reduces trace fear conditioning. A: Total number of BrdU-labeled cells in the dentate gyrus after 2 weeks of treatment with saline or methylazoxymethanol acetate (MAM). Bars represent mean ±SEM. B: Movement during the first three conditioned stimulus (CS) periods (15-s) 24 h after exposure to 10 trials of trace fear conditioning. Movement is expressed as a percentage of the baseline movements measured during acclimation to the training context (mean ±SEM). C: Percentage movements during the 30-s trace interval on the first three CS-alone trials. D: Percentage movements during the 30-s period during and after when the unconditioned stimulus (US) would have occurred during trace conditioning.

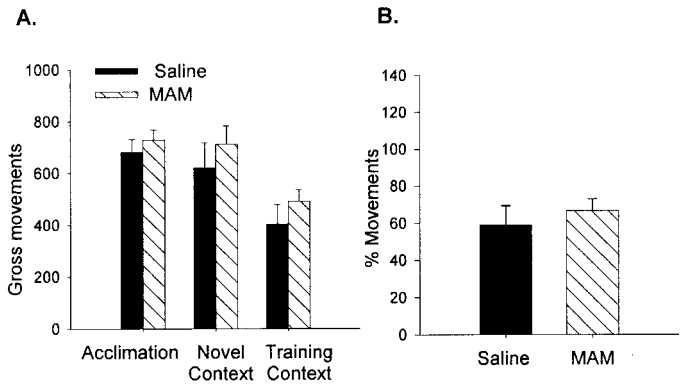

FIGURE 4. Treatment with methylazoxymethanol acetate (MAM) does not affect the expression of contextual fear or the exploration of novel environments. A: MAM treatment did not affect movements during acclimation to the training context, during exposure to a novel context, after training or reexposure to the training context. Movements were recorded during a 3-min period and bars represent mean ±SEM. B: Percentage movements in the context in which fear conditioning occurred. Movement is expressed as a percentage of the baseline movements during initial exposure to the training context.

4A). Relative to movements during acclimation, rats emitted less in the training context ($P < 0.0005$) but responded similarly in the novel context after training ($P = 0.15$). MAM treatment did not differentially affect movements in animals that were reexposed to the context in which trace conditioning had occurred [$F(1,15) = 0.86$; $P = 0.37$] (Fig. 4B). Under these conditions, MAM treatment did not affect contextual fear conditioning.

Effects of MAM on Anxiety-Related Behavior

Treatment with MAM did not affect behavior in the plus maze with respect to time in the closed versus open arms [$F(1,15) = 0.16$; $P = 0.69$] (Fig. 5A) or the number of entries into the closed ($P = 0.82$) or open arms ($P = 0.43$) (Fig. 5B). Groups treated with MAM or saline spent ~80% of their time in the closed arms and <20% in the open arms.

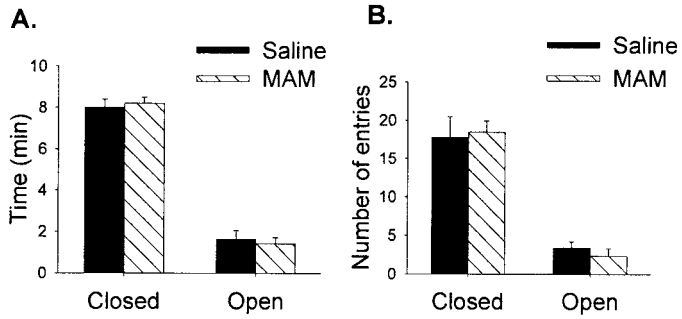

FIGURE 5. Treatment with methylazoxymethanol acetate (MAM) does not alter anxiety-related behavior in the plus maze. A: Time (mean ±SEM) spent in closed or open arms during 10 min in the maze. B: Number of entries into closed versus open arms.

DISCUSSION

There has been considerable speculation about the function of neurons generated in the adult and in particular about their role in memory formation. In this report, we present data suggesting that the depletion of neurons in the adult hippocampus is associated with learning deficits in some tasks that are dependent on the hippocampus but not others. Specifically, a substantial reduction in cell number over 2 weeks was associated with impaired acquisition of a fear-conditioned response using a trace paradigm in which a temporal interval is placed between the associated stimuli (Figs. 2 and 3). This task is dependent on an intact hippocampus, as are other trace conditioned responses (Solomon et al., 1986; Weiss et al., 1999; McEchron et al., 1998, 2000; Beylin et al., 2001). Moreover, it was previously reported that a similar reduction in cell proliferation impairs trace conditioning of the eyeblink response (Shors et al., 2001). Thus, the present results are consistent with previous reports that newly generated neurons are somehow involved in the formation of trace memories. The detrimental effect of MAM treatment on trace conditioning was not directly attributable to effects of MAM on movement or response to novelty. All groups displayed similar levels of activity when presented with a novel context, both before and after exposure to the training stimuli (Fig. 4). The groups also showed similar behaviors in the plus maze, suggesting that depletion of new neurons did not alter generalized anxiety or exploration (Fig. 5). Thus, treatment with a drug that reduces the number of newly generated cells in the adult brain is associated with a deficit in the formation of trace memories involving fearful stimuli.

To this point, the data that we have discussed are consistent with the idea that newly generated neurons in the adult hippocampus are involved in the acquisition of memories that are dependent on the structure. However, the data in the present report on spatial navigation learning contradict this idea. In particular, depletion of the newly generated neurons did not affect subsequent acquisition of spatial memories in the Morris water maze task (Fig. 1), which is dependent on the hippocampus (Galani et al., 1998; Riedel et al., 1999). Also, treatment with MAM did not affect contextual fear conditioning (Fig. 4), another task that is reportedly dependent on an intact hippocampus (Kim and Fanselow, 1992; Holt and Maren, 2000; but see Good and Honey, 1991; McNish et al., 1997). What are the likely reasons for these apparently contradictory findings? One possibility is all types of hippocampal-dependent learning involve new neurons for acquisition but that spatial navigation learning can still occur with a very small percentage of new neurons. This possibility is supported by reports that only a small piece of the hippocampus, a "dorsal minislab," is necessary for spatial navigation learning to remain intact (Moser et al., 1995). A second possibility is that only certain types of hippocampal-dependent learning require new cells and spatial navigation learning is not one of those types. Similar arguments can be made with regard to the lack of an association between neuron depletion and contextual fear conditioning: either this type of learning involves these new cells but acquisition can occur with a reduced

population or acquisition of the task does not depend on their presence. It should be noted that in no case has neuron depletion resulted in an absolute prevention of learning (Shors et al., 2001). A final possibility is that MAM treatment may have adversely affected processes other than neurogenesis that in turn impair certain types of learning and not others.

Despite some dissociations between hippocampal neurogenesis and learning in general, it appears that the newly generated cells may be particularly sensitive to learning when a trace interval separates the associated stimuli. During trace conditioning, the animal must maintain a memory trace of the CS in order to associate it with the US that occurs later in time, hence the term "trace" conditioning. However, there are other differences between trace and delay conditioning that are unrelated to learning about temporal intervals or maintaining memory traces. For example, learning a trace conditioned response is generally more difficult than learning a delay conditioned response; i.e., the rate of acquisition is slower. Thus, one could propose that the hippocampus does not play a unique role in trace conditioning but rather becomes critically engaged as task demands increase. We recently tested this hypothesis and found that hippocampal lesions impair delay conditioning if the task is rendered sufficiently difficult to acquire, i.e. the rate of acquisition is similar between trace and delay conditioning (Beylin et al., 2001). Thus, it may be the case that learning a more difficult training regime of delay eyeblink conditioning (e.g., very long interstimulus intervals) may engage the newly generated neurons. Obviously, more studies will be necessary to resolve the potential relationships between task difficulty, hippocampal dependence and neurogenesis.

It was shown previously that the hippocampus plays a time-limited role in the acquisition of memory. During training with delay-nonmatching to sample tasks as well as contextual fear and trace eyeblink conditioning, lesions to the hippocampus are less effective as more time elapses between acquisition and recollection of the memory (Kim et al., 1995; Squire and Zola, 1996). The observation that most adult-generated neurons die within several weeks of their generation may be consistent with a time-limited role in learning processes. Moreover, it may explain why depletion of the adult-generated population did not affect spatial maze learning. This type of learning is apparently dependent on the hippocampus not only for acquisition but also for various stages in retrieval and long-term storage (Riedel et al., 1999), and thus a more stable population of neurons may be involved, such as those in areas CA3 and CA1 (Hampson and Deadwyler, 1999; Lever et al., 2002). It would be interesting to evaluate the effect of neuron depletion on spatial memory tasks that require the hippocampus but for more temporally limited operations, such as those that engage working memory (Galani et al., 1998).

The idea that new neurons in the brain contribute to learning has been considered for some time, especially in avian species (Barnea and Nottebohm, 1994; Wilbrecht et al., 2002). There are also numerous indirect associations between neurogenesis and learning. Experience-related changes that affect neurogenesis such as stress or environmental enrichment also affect learning (Gould et al., 1997; Nilsson et al., 1999; van Praag et al., 1999; Lemaire et al., 2000). Hormonal changes that occur across the female estrous cycle affect both neurogenesis and learning, as do drug-induced changes such as those that occur in response to opiate and antidepressant administration (Cameron and Gould, 1994; Shors et al., 1998; Tanapat et al., 1999; Eisch et al., 2000; Malberg et al., 2000; Wood et al., 2001). One might expect that changes in overall numbers of neurons over an extended period of time could indirectly affect memory formation. Our previous data suggest that hippocampal-dependent memories target neurons that are more than 1 week of age, without necessarily affecting proliferation after the conditioned response has been acquired (Gould et al., 1999a). Whether these affected cells are also involved in the learning process remains to be determined; indeed, the present data suggest that those cells affected by spatial maze learning are not necessarily involved in performance of that response. Moreover, the present data suggest that a relationship between neurogenesis and learning, whether direct or indirect is specific to certain types of learning—those in which the conditioning stimuli do not intersect in time. In previous studies, we found that hippocampal lesions do not affect expression of the trace memory if animals were previously trained on a task in which the same stimuli intersect in time (Beylin et al., 2001). Thus, it would appear that the hippocampus is not necessary for acquiring or expressing a trace memory once the association is established. Given the relative naivete of the newly generated neurons in the hippocampus, it is conceivable that they play some role in maintaining new stimulus representations over time until these types of associations have been established. If new granule neurons do participate in this type of learning, studying their properties, particularly at an immature stage, may elucidate the mechanism by which this occurs.

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