Female rats learn trace memories better than male rats and consequently retain a greater proportion of new neurons in their hippocampi

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Learning increases the survival of new cells that are generated in the hippocampal formation before the training experience, especially if the animal learns to associate stimuli across time [Gould E, Beylin A, Tanapat P, Reeves A, Shors TJ (1999) Nat Neurosci 2:260–265]. All relevant studies have been conducted on male rats, despite evidence for sex differences in this type of learning. In the present study, we asked whether sex differences in learning influence the survival of neurons generated in the adult hippocampus. Male and female adult rats were injected with one dose of bromodeoxyuridine (BrdU; 200 mg/kg), to label one population of dividing cells. One week later, half of the animals were trained with a temporal learning task of trace eyeblink conditioning, while the other half were not trained. Animals were killed 1 day after training (12 days after the BrdU injection). Hippocampal tissue was stained for BrdU and a marker of immature neurons, doublecortin. Both sexes learned to emit the conditioned eyeblink response during the trace interval. As a consequence, more new neurons remained in their hippocampi than in sex-matched controls. Individual animals, the number of surviving cells correlated positively with asymptotic performance; those that expressed more learned responses retained more new neurons. However, animals that learned very well retained even more new cells if they required many trials to do so. Because females emitted more learned responses than males did, they retained nearly twice as many new cells per unit volume of tissue. This effect was most evident in the ventral region of the hippocampal formation. Thus, sex differences in learning alter the anatomical structure of the hippocampus. As a result, male and female brains continue to differentiate in adulthood.

The studies addressing the effects of learning on neuronal survival have been conducted exclusively in male rodents, despite evidence for sex differences in associative learning and hippocampal neurogenesis, both of which are enhanced when estrogen levels are high (18–22). In the present study, we asked whether sex differences in trace conditioning would be evident even when females were trained when estrogen levels are relatively low (diestrus). Sex differences were expressed, and as a consequence females retained proportionately more new neurons in their hippocampi than did males.

Results

Females Outperform Males During Training with Trace Eyeblink Conditioning. Before training, males and females expressed similar levels of spontaneous blinking (P > 0.05; data not shown). They responded similarly to a white-noise stimulus before any training occurred (P > 0.05; 1–2 sensitized blinks out of 10) and did not respond to the conditioned stimulus (CS; white noise) on the first trial of training (P > 0.05; one animal in each group emitted a response during the trace interval of the first trial). Upon presentations of paired stimuli (Fig. 1B), both males and females learned to associate the conditioned stimulus with the unconditioned stimulus (US; eyelid stimulation) and emitted conditioned responses (CRs) during the trace interval in anticipation of the US (effect of trials of training: F(11, 176) = 54.6, P < 0.001).

Overall, females emitted more CRs across the 800 trials of training than did males (sex difference effect: F(1, 16) = 4.7, P < 0.05) (Fig. 3A). This effect was pronounced during the first session of training (the first 200 trials). Females also emitted late CRs sooner than did males. Late CRs were those blinks that occurred within 250 ms of the US (late CRs on the first day, sex difference effect: F(1, 16) = 7.49, P = 0.01; percent late CRs in males: 8 ± 2; females: 22 ± 5). Because late blinks more accurately predict the onset of the US, they are considered adaptive (23, 24). Minimally, the present results indicate that females learned to time the CR sooner in training than did males. As a group, the percentage of eyeblinks continued to increase in males, even during the last session of training, indicating that some animals had not reached asymptote. There was no sex difference in responding at the end of training (P > 0.05). However, more females than males reached a criterion of 60%

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Learning Increases Survival of the New Cells in the Hippocampus. The overall effect of training on the survival of new neurons was similar in males and females. The number of cells labeled with BrdU after training was increased in animals that were trained versus those that were not trained ($F_{(1, 31)} = 6.2, P < 0.05$). In previous studies, the increase in cell survival after training was preferentially expressed in animals that learned, defined as those animals that expressed CRs on at least $60\%$ of the trials in a session. The increase in survival did not occur in animals that were exposed to the same number of trials but did not learn to express the CR ($8$). In the present study, we replicated this finding and extend it to include females; the number of surviving cells was elevated in animals emitting CRs on at least $60\%$ of the trials within a session ($n = 7$ males and $n = 7$ females) ($F_{(1, 27)} = 7.4, P = 0.01$) (Fig. $2A$). As previously reported, the number of new cells remaining in the hippocampi of animals that did not learn ($n = 4$) was not different from the number in those that were not trained ($P > 0.05$; data not shown) ($8$).

In the next analysis, we only considered animals that learned the CR. We considered animals to have learned if they expressed $60\%$ CRs during training. In those animals, we then determined the trial at which each animal reached its highest level of responding ($25, 26$). This trial, hereafter referred to as the “asymptotic trial,” was the trial at which an animal reached $95\%$ of its asymptotic performance. This trial correlated positively with the number of cells labeled with BrdU after training ($r = 0.54, P < 0.05$) (Fig. $2B$). To verify this relationship, we used a more traditional measure of learning during eyeblink conditioning: the trial on which an individual animal emitted $8$ out of $9$ CRs on consecutive trials. This trial was also correlated with the number of BrdU-labeled cells in the hippocampus ($r = 0.533; P < 0.05$). Thus, both analyses suggest that animals that learn and require more trials to do so also retain more of the new neurons in their DG. It is important to reiterate that animals that learn retain more new cells after training than animals that do not learn, but within those animals that learn well, those that require more trials to do so retain the most new cells. This relationship between new cell survival and trials to criterion has also been demonstrated before in males ($13$).

Learning Increases the Density of New Neurons More in Females than in Males. In the next analyses, we compared the effects of training on the absolute number versus density of surviving cells. This analysis was necessary because the female hippocampus is significantly smaller in volume than the male hippocampus ($27, 28$). To conduct the analysis, all animals were included, regardless of their performance on the trace memory task. The analysis revealed significant main effects of training and sex differences on the absolute number of BrdU-labeled cells that were detected in the DG ($F_{(1, 31)} = 6.2, P < 0.05$; $F_{(1, 31)} = 10.1, P < 0.01$, respectively), with no interaction (BrdU-labeled cells; males with no training: $4,056 \pm 333$; males with training: $4,908 \pm 338$; females with no training: $3,114 \pm 89$; females with training: $3,834 \pm 383$). Next, we assessed the effects of training on the proportion of new cells that survive in the male versus the female hippocampus. This was conducted to account for $2$ sex differences in the hippocampal structure. First, $12$ days after the one injection of BrdU, females that were not trained (left in home cage) possessed fewer BrdU-labeled cells ($3,114 \pm 89$) than did males that were not trained and left in their home cage ($4,056 \pm 333$). It is during this time period that many of the new cells die ($11, 13$). Thus, more cells may die in females than in males across time. The second issue is that the female hippocampus is smaller than the male hippocampus ($27, 28$). To account for these sex differences, we calculated the density of new cells in the DG (cells per mm$^3$). Indeed, the volume of the DG [granular cell layer/subgranular zone (GCL/SGZ) and hilus] was smaller in females than in males (sex effect: $F_{(1, 31)} = 7.7, P = 0.01$). Training with trace conditioning did not alter the volume of the
DG in either sex (males with no training: 11.1 ± 0.3 mm³; males with training: 11.6 ± 0.5 mm³; females with no training: 10.7 ± 0.2 mm³; females with training: 9.9 ± 0.4 mm³). Nonetheless, training with the trace-conditioning memory task increased the density of BrdU-labeled cells in the DG of both trained males and females (main effect of training: F(1, 31) = 6.7, P = 0.01; with no main effect of sex: F(1, 31) = 3.0, P > 0.05). The density of new cells in the animals that were not trained was lower in females than in males (1-way ANOVA: F(1, 15) = 0.4, P < 0.05), but was not different between males and females that were trained (P > 0.05) (Fig. 3B). As a result of training, twice as many new cells per unit volume survived in the female (34%) than in the male hippocampus (17%) (see Fig. 3B). The effect size was similar when the analysis was limited to animals that learned (reached 60% CRs). In this case, there was a 40% increase in the proportion of new cells that remained in the DG of females versus 22% in males.

Nearly 80% of the cells labeled with BrdU also expressed the immature neuronal marker doublecortin (DCX) (16). The percentage of BrdU-labeled cells that coexpressed DCX was similar in males and females, as reported previously (19). These data suggest that the by end of training, the vast majority of new cells have already begun to differentiate into neurons and the sex of the animals does not alter that process (Fig. 3E).

Recent data suggest that dorsal and ventral regions of the hippocampus differentially contribute to learning the trace eyeblink response (29, 30). If this is true, new cells generated in these two regions may also respond preferentially to trace conditioning (31). To examine this possibility, we estimated the density of the new cells in the dorsal versus the ventral hippocampus, based on methods described previously (32) (Fig. 4A and B). In response to training alone, the density of new cells that survived in the GCL/SZ of the dorsal hippocampus of male and female rats was similar. However, the effect of training on the density of surviving cells was more pronounced in the ventral part of the female hippocampus. Training increased the density of BrdU-labeled cells in the ventral hippocampus (F(1, 31) = 4.7, P < 0.05), with an interaction between training and sex differences (F(1, 31) = 3.8, P = 0.05). This effect was exacerbated by the fact that fewer new cells survived in the ventral hippocampus of females that were not trained versus males that were not trained (P < 0.05) (Fig. 4C). As before, the density of BrdU-labeled cells correlated positively with the highest level of responding that an animal achieved by the end of training (“asymptote”) (Fig. 4D) (8). The correlation was stronger in females than in males (females: r = 0.63, P = 0.09; males: r = 0.39, P = 0.2), probably because more females than males learned well.

**Discussion**

Learning rescues new neurons from death in the adult hippocampus (8, 11–13, 17). Here, we report that sex differences in learning predict how many new cells will survive to become neurons after the training experience is over. Using a task that
is known to enhance new cell survival, trace eyelink conditioning, we observed that females learned to accurately time the conditioned response sooner than did males and, as a result, more of them reached a learning criterion. As a further consequence, twice as many new cells per unit volume survived in the female than in the male hippocampus, when both sexes were compared to animals that were not trained. In both sexes, the degree to which each animal expressed the learned response related in a positive way to the number and density of the new cells that survived. Consistent with previous studies, individual differences in learning predicted how many new cells would survive after training (8–10, 12, 13, 16, 33). However, and perhaps more importantly, in those animals that did learn, those that required more trials to reach the learning criterion retained more of the new neurons. Together, these data indicate that at least 2 factors are essential to rescue the new cells from death. First, the animals must learn and learn well, consistently expressing the conditioned response across hundreds of trials. Second, animals that learn well and require more trials to do so tend to retain most of the new neurons (present study and ref. 13).

How this effect of learning on neurogenesis occurs at the neuronal level is not known. It is well-established that the mature and established neurons in the hippocampal formation, especially the pyramidal cells in area CA1 of the hippocampus, become more active during acquisition of the trace eyelink conditioning (29, 34). Also, individual differences in learning predict in a positive way the degree of the activity that occurs (35). Thus, increases in neuronal activity throughout the hippocampus during training may contribute to the preservation of these new neurons (7, 13). In animals that do learn, those that maintain an increase in cellular activity across more trials of training may retain more new cells after the learning experience has occurred.

The present study shows for the first time that sex differences in learning influence the survival of new neurons in the adult hippocampus. Females learned better than males and more of them reached the criterion of 60% CRs (good learners). As a result, females retained a higher percentage of new cells per unit volume at the end of training. Because the new cells were labeled with BrdU 1 week before training began, the effects are not likely to reflect changes in the production of new cells (proliferation). Rather, because some of the new cells have already begun to die when the training begins, the effect reported here reflects an increase in new cell survival (7). Why females learn the trace memory better than males and what it is about their learning that increases cell survival is not known, but there are clues. Overall, females emitted more learned responses early in training. During this period, they also emitted CRs closer to the onset of the US. These so-called “late” CRs indicate that an animal has accurately learned to predict the onset of the US. Thus, females emitted more well-timed responses than did males, suggesting that they more readily learned to time the CR. Some studies suggest that the hippocampus is involved in the precise timing of the CR (23, 34). Thus, timing of the CR may influence the effect of learning on cell survival. Finally, it is important to note that before training, females did not emit more spontaneous blinks or sensitized responses to the CS than did males.

Sex differences during eyelink conditioning occur in other species, including humans (36). In rats, they are most evident when females begin training in proestrus, when estrogen levels are elevated (18, 20, 22). In the present study, the differences in trace conditioning were not limited to stages of estrus when estrogen levels are elevated. Rather, females began training in diestrus, a phase of the estrous cycle characterized by lower levels of estrogen and progesterone. As far as neurogenesis is concerned, females produce more new cells in proestrus than in estrus (19). In the present study, we purposely injected all females with BrdU during a phase of the cycle (i.e., estrus) when the numbers of proliferating cells in the hippocampus are similar between sexes (19). However, at the time of their being killed (12 days later), control females that were not trained possessed fewer of the BrdU-labeled cells than did males that were trained. Thus, without stimulation, fewer new cells seem to survive in naïve females than in males. Perhaps the presence of testosterone enhances cell survival in males (37). In any event, we do not know exactly how many new cells were available to rescue at the beginning of training in these experiments. In males, virtually all cells that were available to rescue at the beginning of training were rescued from death by trace conditioning, again provided that the animal learned slowly and well (13). Thus, it is possible that in the present study more new cells were available to rescue in females than in males at the beginning of training, and that in the absence of training, more of those cells died. It is further noted that females also learn delay conditioning better than do males (21, 22), but since delay conditioning does not enhance new cell survival (11), there should be no consequence for neurogenesis per se.

In humans, some sex differences in learning are related to gender and thus influenced by social and environmental factors (38). Other sex differences in learning are mediated by the presence of sex hormones and sex differences in the structure of the brain (27, 28, 39–41). As presented here, the total volume of the dentate gyrus is greater in males than in females (27, 42). This sex difference is dependent on the organizational effect of gonadal hormones because it can be reversed if females are masculinized with testosterone at birth (27). Similarly, sex differences in trace eyelink conditioning are organized, at least in part, by the presence of gonadal hormones (43). Females that are masculinized at birth by the presence of testosterone acquire the trace memory at a similar rate to males when trained as adults (43). Ultimately then, the neurogenic response to learning in males versus females is likely organized by the presence of gonadal hormones during early brain development. However, the present results demonstrate the influence of learning on brain anatomy; they do not address the anatomy that underlies sex differences in learning.

Recently, a great deal of focus has been placed on distinguishing the dorsal from the ventral region of the hippocampus. These two regions have differing degrees of input fromafferent structures, as well as differences in efferent connections (31, 44, 45). During trace conditioning, mature neurons in both regions are activated (29), but they possibly play distinctive roles in the learning process (29, 30, 46, 47). In the present study, we found suggestive evidence that new neurons generated in the ventral hippocampus may be especially responsive to learning. In females, the effect of trace conditioning on new cell survival was preferentially expressed in the ventral hippocampus. During spatial learning, mature neurons in the dorsal hippocampus are primarily activated, whereas the immature neurons are more active in the ventral regions (48). It was suggested that the cells in the ventral hippocampus respond more to emotional aspects of learning (49). Trace eyelink conditioning is a form of aversive and, by association, emotional learning (50). In cycling females, these emotional processes may be especially influential. Females possess a greater density of estrogen receptors in the ventral than in the dorsal hippocampus (51). Because sex differences in eyelink conditioning are mediated, at least in part, by the presence of estrogen, estrogen receptors in this region may be involved in the enhanced new cell survival in females (18, 21). On the other hand, the preferential expression of new cells in the ventral region of females may simply reflect the fact that more females learned well than did males.
To summarize, females outperformed males while learning to associate stimuli across time; as a consequence, they retained a greater percentage of adult-generated neurons in their hippocampi than did males. The number of new cells, most of which went on to become neurons, correlated in a positive way with the expression of the trace memory. Thus, no differences in learning are not only mediated by sex differences in brain anatomy, they can also induce sex differences in that anatomy.

Materials and Methods

Subjects. Experiments were approved by the Rutgers University Animal Care and Facilities Committee. Adult (70–90 days old) male (300–350 g) and female (250–350 g) Sprague–Dawley rats were individually housed and maintained on a 12-hour light/dark cycle. Stages of estrus were determined in female rats with daily vaginal smears, as described (20, 52). Only adult females that were on a 12-hour light/dark cycle. Stages of estrus were determined in female rats maintained on isoflurane and oxygen. Two pairs of electrodes were attached to a headstage and implanted through the upper eyelid (8). Naive male (n = 9) and female (n = 9) rats were kept undisturbed in their home cages.

Following recovery, animals were injected i.p. with one dose of BrdU (200 mg/kg), to label 1 population of dividing cells 1 week before training (8, 11, 53) (see Fig. 1A). BrdU is incorporated into the DNA of dividing cells during the S-phase of the cell cycle (53, 54). All females were injected with BrdU during the same phase (estrus) of the cycle, to control for differences in proliferation across the estrous cycle (19). Two operated females and 1 naive female were excluded from the study before training because of irregular cycles. Seven days after the one BrdU injection, rats in the training groups were given 45 min to acclimate (no stimuli presented) to the conditioning environment. Twenty-four hours after acclimation and 8 days after the BrdU injection, male and female rats were placed again in the same conditioning chambers. For 20 min, no stimuli were presented while the numbers of spontaneous blinks were recorded. Then, all rats were exposed to 10 trials with the white-noise stimulus alone (82 db, 250 ms). Blinks in response to the noise were used to determine whether either sex emitted sensitized responses to the CS before training. Immediately after, they were exposed to 800 trials of trace eyelid conditioning (200 trials/day). A white-noise generator attached to a speaker administered a white noise as a CS and a shock generator delivered an eyelid shock (0.65 mA) as the US. Each block of conditioning consisted of 100 trials with every 10-trial sequence composed of 1 CS-alone presentation, 4 paired presentations of the CS and US, 1 US-alone presentation, and 4 paired presentations of the CS and US. The intertrial interval was 25 ± 5 s. A 250-ms CS was followed by a 500-ms trace interval which was followed by a 100-ms US (Fig. 18). Eyeblinks that occurred during the trace interval were considered conditioned responses (CRs) and were detected by changes in eyelid electromyographic activity, as described (8, 12) (See supporting information (SI) Methods for details). We also assessed how well the animals learned to time the CR. To do that, we counted late CRs, defined as those that occurred within 250 ms of the US.

Immunohistochemistry for BrdU. Animals were perfused 24 h after the last training session (12 days after BrdU injection), along with their respective naive controls. For killing, rats were deeply anesthetized with sodium pentobarbital (100 mg/kg) and intracardially perfused with 0.1 M phosphate buffer. Brains were extracted, postfixed, and coronal sections (40 μm) were cut through the entire DG of one hemisphere of the brain. For BrdU peroxidase staining, one of every twelfth section was collected and stained as before (8, 13) (See SI Methods for details). The primary mouse anti-BrdU was purchased from Becton Dickinson ImmunoSys. After BrdU staining, slides were counterstained with cresyl violet.

Quantitative Analysis and Volume. Estimates of total numbers of BrdU-labeled cells were made using a modified unbiased stereology protocol (8, 11, 55, 56). This was accomplished by counting the number of BrdU-labeled cells in the SGZ, GCL, and hilus in every twelfth unilateral section on 2 balance side of the DG (21.88 to 24.52 mm anteroposterior) (57). Slides were coded and the cells were counted blind to the experimental conditions, avoiding cells in the outermost focal plane. The tissue was magnified by 1,000 with a Nikon Eclipse E400 light microscope. The number of cells was multiplied by 24 to obtain an estimate of the total number of BrdU-labeled cells in the hippocampus. To account for sex differences in the size of the hippocampus, we also assessed the total volume of the DG (GCL, SGZ, and hilus) from cross-sectional area measurements obtained with Scion Image software (19, 58). The area of the DG was calculated from digital pictures of every twelfth unilateral section throughout the rostrocaudal hippocampus. To calculate the volume of the DG, the Cavalieri’s principle was used (59) (See SI Methods for details). BrdU counts were expressed as density of BrdU-labeled cells per volume of the total DG or GCL/SGZ alone (number of BrdU-labeled cells/mm³). Finally, the density of BrdU-labeled cells was examined separately in the rostral (interaural 3.70 to 6.88 mm) and caudal (interaural 2.28 to 3.70 mm) hippocampus (57), as described in detail elsewhere (32). The dorsal hippocampus is associated with the rostral area, whereas the ventral area is more caudal.

Double Labeling: BrdU with DCX. Double labeling for BrdU and DCX was performed in separate hippocampal free-floating sections slices, as before (16). Primary antibodies were goat anti-DCX (Santa Cruz Biotechnology), and mouse anti-BrdU (Becton–Dickinson ImmunoSys). Secondary antibodies were rhodamine red-X anti-goat (Jackson Immunoresearch) and Fluo 488 anti-mouse (Molecular Probes). The number of cells that expressed both markers was determined using a confocal laser scanning microscope (16). Twenty cells per subject (n = 4) were counted on random sections throughout the hippocampus (See SI Methods for details). The percentage of the new cells that differentiated into neurons (~80%) was similar to that reported in previous studies (11–13, 16, 17, 19).

Behavioral Analysis. All results are presented as means ± one standard error. The percentage of CRs across training trials was analyzed using repeated measures ANOVA with sex as the between-subjects independent variable and trials as the within-subjects independent variable. A closer look at the individual data suggested that many subjects did not display a gradual acquisition curve. Instead, vigorous conditioned responding often appeared abruptly, after a period of no appreciable responding, the duration of which varied from rat to rat. To identify when these changes in responding occurred, we used an algorithm developed by Gallistel that detects changes in the slope of the cumulative record (25, 26). The slope of the cumulative record indicates a momentary rate of responding (61). Therefore, abrupt changes in performance are often indicated by similarly abrupt changes in the slope (See SI Methods for details). To characterize acquisition, we defined 2 measures of learning: (i) the estimate of the asymptote for each rat, which was defined as the mean percentage of CRs during the last 2 sessions of training (even though some animals continued to increase their responding) and (ii) the asymptotic trial, which was the trial of the change point which led to a slope of 95% or more of the asymptote. This measure provided an estimate of how many trials of training were necessary to reliably express the trace CR.

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