

Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus

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Although hippocampal neurogenesis has been described in many adult mammals, the functional impact of this process on physiology and behavior remains unclear. In the present study, we used two independent methods to ablate hippocampal neurogenesis and found that each procedure caused a limited behavioral deficit and a loss of synaptic plasticity within the dentate gyrus. Specifically, focal X irradiation of the hippocampus or genetic ablation of glial fibrillary acidic protein-positive neural progenitor cells impaired contextual fear conditioning but not cued conditioning. Hippocampal-dependent spatial learning tasks such as the Morris water maze and Y maze were unaffected. These findings show that adult-born neurons make a distinct contribution to some but not all hippocampal functions. In a parallel set of experiments, we show that long-term potentiation elicited in the dentate gyrus in the absence of GABA blockers requires the presence of new neurons, as it is eliminated by each of our ablation procedures. These data show that new hippocampal neurons can be preferentially recruited over mature granule cells *in vitro* and may provide a framework for how this small cell population can influence behavior.

long-term potentiation | learning | memory

New neurons are born in the dentate gyrus (DG) of the hippocampus throughout the life of mammals (1) and derive from dividing progenitor cells located in the innermost part of the granule cell layer, a region called the subgranular zone. Young granule neurons integrate into the existing circuitry of the hippocampus, as evidenced by the development of functional synaptic inputs provided by the medial perforant path (MPP) and growth of axons to target cells in CA3 (2). Although a variety of environmental and pharmacological manipulations can affect neurogenesis (2, 3), it is unclear whether adult-born neurons provide a significant contribution to hippocampal function and, ultimately, how it might impact behavior.

Recent studies have shown that various strategies to disrupt neurogenesis produce a limited impairment in some hippocampal-dependent learning and memory tasks and in responses to antidepressant drugs (4–11). Unfortunately, the lack of spatial and cellular specificity provided by most ablation techniques has made it difficult to ascertain whether the consequent behavioral effects were caused by ablation of neurogenesis or other impairments. To circumvent these problems we have used two independent strategies of ablation. The first is a previously reported x-ray procedure that differs from similar methods in two ways: (i) the x-ray administration is restricted to a fraction of the brain containing the hippocampus and spares neurogenesis in the neighboring subventricular zone; and (ii) mice are allowed to recover for 3 months before testing to allow for the disappearance of markers of inflammation, such as reactive microglia (9). The second method of ablation is a genetic strategy that directly

targets dividing progenitors throughout the brain and avoids potential radiation-induced side effects. Therefore, the two strategies are complementary: irradiation affords spatial but not cellular specificity, whereas genetic ablation is specific to progenitor cells, but impacts both neurogenic regions of the brain.

Using these strategies, we examined whether loss of neurogenesis in adult mice altered hippocampal-dependent behaviors or changed electrophysiological responses within the hippocampus. Our results demonstrate a specific impairment in contextual fear conditioning and a loss of long-term potentiation (LTP) in the DG after ablation of neurogenesis. These findings show that new neurons make an essential contribution to a subset of hippocampal functions.

Results

Ablation of Neurogenesis Impairs Contextual Fear Conditioning. We have previously shown that a series of low-dose X irradiations targeted to the hippocampus of adult mice produces a lasting ablation of neurogenesis in the DG, while sparing cell proliferation in surrounding brain areas including the subventricular zone (4, 9). Although brain irradiation can induce a rapid inflammatory response, evidenced primarily by an increase in activated microglia (12, 13), we have shown that our procedure results in only a transient increase in inflammatory markers that subsides within 2–3 months (9). Therefore, animals were allowed to recuperate from irradiation for 3 months before functional analyses were performed.

Cued and contextual fear learning are forms of Pavlovian conditioning elicited by pairing a neutral conditioned stimulus (CS) with an aversive unconditioned stimulus (US) in a distinctive context. Acquisition of the CS–US association is known to require the amygdala (14, 15), whereas acquisition of a context–US association usually requires both the hippocampus and amygdala (16, 17). Sham and irradiated mice were trained in a cued conditioning protocol 3 months after x-ray treatment (Fig. 1A–C). Fig. 1D shows that irradiation did not alter freezing either before or during the CS in the cued conditioning test, suggesting that amygdala function is not grossly impaired by this

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Abbreviations: GFAP, glial fibrillary acidic protein; LTP, long-term potentiation; DG, dentate gyrus; MPP, medial perforant path; CS, conditioned stimulus; US, unconditioned stimulus; TK, thymidine kinase; GCV, ganciclovir; EPSP, excitatory postsynaptic potential; TG, transgenic.

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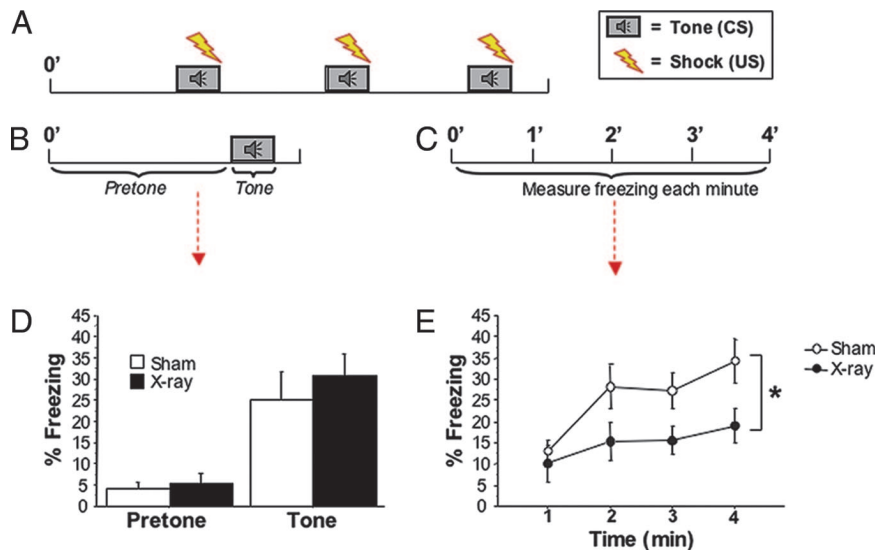


Fig. 1. Irradiation impairs contextual but not cued fear conditioning. (A–C) Schematic diagram of the fear conditioning procedure ($n = 14$ per group). (A) Day 1: training (context A). (B) Day 2: cued test (context B). (C) Day 3: context test (context A). (D) Conditioned freezing during the tone test. Percent time freezing during the 60 s before tone presentation (pretone) and during the 20-s tone presentation (Tone). Data were subjected to a 2 (treatment) \times 2 (period: pretone vs. tone) ANOVA ($n = 14$ per group). Mice froze significantly more during the tone than during the pretone period [$F(1,26) = 38.7, P < 0.001$]. There was no effect of X irradiation [$F(1,26) = 0.3$] and no treatment \times period interaction [$F(1,26) < 1$]. (E) Percent time freezing during the 4 min of the context test. Data were subjected to a 2 (treatment) \times 4 (minute) ANOVA. Irradiated mice spent significantly less time freezing than sham mice [$F(1,26) = 5.4, P = 0.03$]. Error bars represent ± 1 SEM.

procedure. However, irradiated mice froze significantly less than shams in the test of context-elicited fear (Fig. 1E).

The fact that tone-elicited freezing was normal in irradiated mice suggests that the deficit in context-elicited freezing was not caused by an impairment in the motor control of freezing or a reduction in shock sensitivity. We also sought to confirm that the reduction in context-elicited freezing in these animals was not caused by a general reduction in anxiety-like behaviors. Therefore, irradiated and sham mice were subjected to two tests of anxiety-like behavior: the elevated-plus maze and the light–dark choice task. Each test examines the exploratory behavior of animals within an apparatus that contains anxiogenic areas. The number of entries and time spent in the anxiogenic areas are increased by treatment with classical anxiolytic drugs (18–20). As shown in Fig. 4 A–C, which is published as supporting information on the PNAS web site, there was no effect of irradiation on activity in either the open versus closed arms (elevated-plus maze) or the light versus dark compartments (light–dark choice task).

Spatial Memory Is Normal in Irradiated Mice. The performance of sham and irradiated mice was compared by using several water maze tasks: the visible and hidden platform tasks and the delayed matching-to-place task (21, 22). As shown in Fig. 5 A–C, which is published as supporting information on the PNAS web site, irradiation resulted in no detectable impairments in either the visible platform task or the training phase of the hidden platform task, which assesses the ability to use extra-maze spatial cues to locate a submerged platform. Likewise, sham and irradiated mice displayed similar memory for the hidden platform location in probe trials conducted on the final day of training (Fig. 5D Left) and 2 weeks after the end of training (Fig. 5D Right).

The delayed matching-to-place task assessed the ability to rapidly acquire and reacquire new hidden platform locations. Mice received four trials per day with a new platform location each day. The performance of sham and irradiated mice was compared over a period of 4 weeks. Again no effect of irradiation was observed (Fig. 5E). Sham and irradiated mice were also

tested in a place recognition task, the Y maze (23). Mice are allowed to freely explore two arms of a three-armed maze for 10 min and, after a 30-min delay, are returned to the maze with all arms open for a 5-min recognition test. During this test, intact rodents typically make more visits to the novel unexplored arm overall. Fig. 4D shows that novel arm preference was unaffected by irradiation.

Fear Conditioning in Glial Fibrillary Acidic Protein (GFAP)-Thymidine Kinase (TK) Transgenic (TG) Mice. Because the impairment of contextual fear conditioning was the only significant effect of our irradiation procedure on learning and memory processes, we sought to confirm this result by using an independent method to ablate neurogenesis. TG mice that express herpes virus TK under the regulation of the mouse GFAP promoter were generated as described (24). TK is expressed by both proliferating and non-proliferating GFAP-positive cells in the brains of these mice; however, delivery of the antiviral prodrug ganciclovir (GCV) only kills proliferating TK-positive cells. Because neurons in the adult hippocampus derive from GFAP-positive progenitor cells, neurogenesis is ablated by chronic delivery of GCV (25, 26).

To determine whether the impairment in contextual conditioning observed after irradiation was caused by a loss of young neurons, WT and GFAP-TK TG mice were treated with GCV for 6 weeks to ablate neurogenesis and then subjected to the fear conditioning procedure. GCV-mediated ablation of neurogenesis did not alter cued fear conditioning, but produced a significant reduction of freezing during the contextual conditioning test (Fig. 2). Similar to our observations in irradiated mice, GCV-treated TG mice displayed normal levels of anxiety-related behaviors in several conflict tests (data not shown).

Neurogenesis-Dependent Plasticity in the DG. The physiological consequences of ablating neurogenesis were examined in two hippocampal subregions that exhibit LTP, the MPP/DG connection and the Schaffer collateral commissural projections to CA1 (SC/CA1). In the first experiment, slices derived from mice 3 months after x-ray treatment were used for extracellular

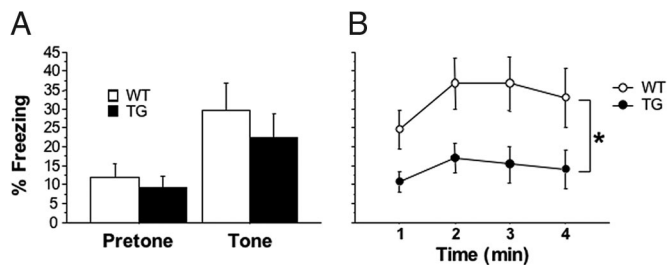


Fig. 2. GCV treatment impairs contextual but not cued fear conditioning in GFAP-TK TG mice. (A) Conditioned freezing during the tone test. Percent of time freezing during the 60 s before tone presentation (pretone) and during the 20-s tone presentation (tone) is shown. Data were subjected to a 2 (treatment) \times 2 (period: pretone vs. tone) ANOVA [$n = 19$ (WT) and 11 (TG)]. Mice froze significantly more during the tone than during the pretone period [$F(1,28) = 14.1, P < 0.001$]. There was no effect of genotype [$F(1,28) = 0.5$] or genotype \times period interaction [$F(1,28) < 1$]. (B) Percent time freezing during the 4 min of the context test. Data were subjected to a 2 (treatment) \times 4 (minute) ANOVA. Irradiated mice spent significantly less time freezing than did sham mice [$F(1,28) = 4.7, P = 0.04$].

recordings. The slope of excitatory postsynaptic potentials (EPSPs) evoked by stimulation of the MPP was examined by placing an extracellular recording electrode in the molecular layer of the upper blade of the DG. Because no difference was detected in either the input–output relationships (Fig. 6A, which is published as supporting information on the PNAS web site) or paired-pulse depression (Fig. 6B) in the DG of irradiated animals, we next investigated long-term plasticity in this synaptic connection.

LTP of the MPP/DG connection has proven difficult to evoke in slice preparations because of the prominent GABAergic innervation of this hippocampal subfield (27); yet recent studies in rats have identified a small but stable form of LTP that can be elicited with a 100-Hz stimulation protocol and is disrupted by cranial gamma-irradiation (28, 29). To confirm these findings in our experimental system, we elicited LTP by using this 100-Hz protocol and compared responses evoked in the DG of sham and irradiated mice. Fig. 3A shows that a stable potentiation of $\approx 15\%$ was observed in sham mice, but was completely absent in irradiated animals. In contrast, stimulation of MPP afferents in the presence of the GABA blocker picrotoxin produced a larger potentiation ($\approx 200\%$) in the DG that was not affected by irradiation (Fig. 3B). These results support previous reports that young neurons in the DG exhibit a lack of GABAergic inhibition and are, therefore, more receptive to the induction of LTP (28–31).

To determine whether the effects of irradiation on hippocampal physiology were specific to the DG, we also examined plasticity in the CA1 subfield, a region of the hippocampus that is devoid of neurogenesis in adulthood. We found that sham and irradiated slices displayed similar input–output relationships and paired-pulse facilitation (Fig. 6C and D) and robust LTP in the Schaffer/CA1 synaptic connection induced by a similar 100-Hz stimulation protocol (Fig. 3C). Thus, irradiation does not impair synaptic plasticity in the CA1 region but completely abolishes LTP evoked in the DG. We next sought to confirm these findings by repeating the above experiments by using the genetic model of ablation.

WT and GFAP-TK TG mice were treated with GCV for 6 weeks before we collected hippocampal slices for extracellular field recordings. Similar to our findings using irradiation, this method of ablating neurogenesis abolished LTP evoked in the DG without picrotoxin (Fig. 3D). However, no difference in DG LTP was observed between WT and TG mice when picrotoxin was added to the bath solution (Fig. 3E). To determine whether

the effects of GCV treatment were specific to plasticity associated with young neurons, additional comparisons between GCV-treated WT and TG mice were performed. As shown in Fig. 7, which is published as supporting information on the PNAS web site, we detected no differences between WT and TG mice in input–output relationships and paired-pulse depression in the MPP/DG connections or in input–output and paired-pulse facilitation in the Schaffer/CA1 connections. Finally, LTP in the Schaffer/CA1 connection was normal in GCV-treated TG mice (Fig. 3F). Thus, genetically targeted disruption of neurogenesis causes an impairment of LTP within the DG without altering synaptic plasticity in the CA1 subfield, in good agreement with our observations using irradiation. It is noteworthy that, after induction of LTP in the DG, posttetanic potentiation is significantly reduced in irradiated mice, but not in GCV-treated TG mice. It is possible that this difference is because the loss of neurogenesis is not as profound in TG mice when compared with irradiated mice. Alternatively, non-neurogenesis-dependent mechanisms may contribute to the posttetanic potentiation changes observed in irradiated mice.

Discussion

New Neurons Contribute to LTP in the DG. Recent studies have identified a form of DG LTP that can be elicited in the absence of GABA_A blockers and is disrupted by gamma irradiation (28, 29). In keeping with these results, we observed a small but stable LTP in response to stimulation of the MPP that did not require the use of GABA blockers. The fact that this LTP was abolished by both of our ablation paradigms indicates that it requires neurogenesis. In contrast, LTP evoked in the presence of a GABA blocker was larger in magnitude and, importantly, was not altered by ablation of neurogenesis.

Single-cell recordings have demonstrated that young neurons are more excitable than mature granule cells because of properties such as an elevated resting membrane potential, high input resistance, and low-threshold calcium channel expression (29, 31). In addition, young neurons (2–4 wk old) are not inhibited, and can in fact be depolarized, by GABAergic activity (30, 32). These properties may explain why young granule neurons are more receptive to the induction of LTP. We suspect that in the presence of GABA blockers both young and mature neurons are recruited, resulting in a larger LTP. In this case, LTP is not affected by ablation because the new neurons represent only a small fraction of the total number of granule cells responding to stimulation (33) or, alternatively, because the excitatory influence of GABA on new neurons is blocked.

Role for Hippocampal Neurogenesis in Contextual Fear Conditioning.

As in the case of the electrophysiological studies, we examined animal behavior after ablation of neurogenesis with two different strategies. Although we found no deficit in spatial learning or memory as assessed in several water maze paradigms or in novel place recognition in the Y maze, each ablation method produced a significant impairment of contextual fear conditioning. The effect of ablation was specific to contextual fear conditioning because conditioned responses to the tone alone were not altered. Moreover, the reduction in context-elicited freezing does not appear to arise from reduced basal levels of anxiety or fear, as we observed no differences in classic tests of anxiety-like behaviors such as the elevated-plus maze or light–dark choice task.

Our finding of impaired contextual fear conditioning conflicts with previous studies reporting a reduction in hippocampal-dependent trace conditioning after ablation of neurogenesis by systemic antimetabolic treatment in rats (5), but no change in contextual conditioning (6). Our results also conflict with a report that whole-head irradiation in rats impairs long-term memory in the water maze (10). One explanation for the

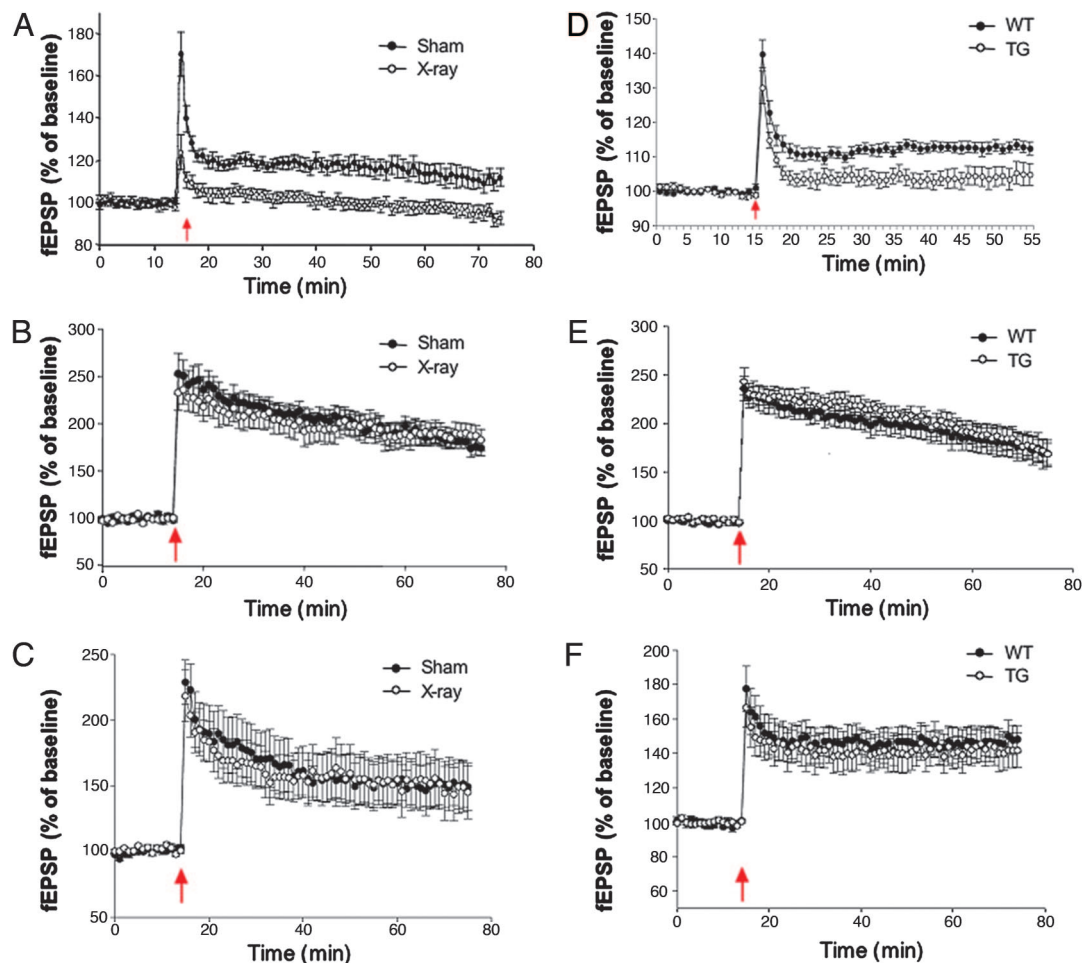


Fig. 3. Ablation of neurogenesis prevents a form of LTP in the DG. Slices for physiological experiments were derived either 3 months after x-ray treatment (A–C) or immediately after a 6-week GCV treatment was completed (D–F). (A) MPP/DG LTP in sham ($n = 8$) and irradiated ($n = 8$) mice 3 months after treatment. Arrow indicates the delivery of the 100-Hz stimulation. Repeated-measures ANOVA performed on the last 10 min of recording confirmed a significant difference between sham and x-irradiated animals [$F(1,14) = 11.2, P < 0.001$]. (B) LTP evoked in the DG after the addition of 50 μM picrotoxin. Stimulation parameters are the same as above. There is no difference in LTP between sham and irradiated mice [$F(1,14) < 1$]. (C) LTP in the Schaffer collateral to CA1 connection was not altered by irradiation [$F(1,14) < 1$]. (D) Six weeks of GCV treatment caused a loss of LTP in the DG in TG ($n = 9$) but not WT ($n = 15$) mice. Repeated measures ANOVA on the last 10 min confirmed a significant difference between WT and TG animals [$F(1,24) = 7.2, P = 0.01$]. (E) GCV treatment did not alter DG LTP elicited in the presence of 50 μM picrotoxin [$F(1,24) < 1$]. (F) LTP in Schaffer collateral/CA1 connections was also unaffected by GCV [$F(1,24) < 1$]. Error bars represent ± 1 SEM.

discrepancies may be the greater spatial specificity of our ablation method. Another possibility is that the behavioral significance of neurogenesis differs between mice and rats. However, a recent study reported that contextual fear conditioning was impaired in rats after whole-head gamma irradiation (11). Integrating these findings will require further information on whether extrahippocampal neurogenesis impacts these tasks and how subtle methodological variations in the tasks might impact their neurogenesis dependence.

It is intriguing that ablation of hippocampal neurogenesis reduced contextual fear conditioning but did not affect performance in spatial maze tasks that are also known to be sensitive to hippocampal lesions. One explanation may come from reports that spatial maze learning and contextual conditioning use different molecular signaling pathways within the hippocampus (34) and, importantly, require different extrahippocampal circuitry. Lesioning the amygdala, for example, does not impair spatial maze learning but disrupts fear conditioning, and activation of the amygdala during strong emotional stimuli like those experienced in fear conditioning can enhance hippocampal-dependent learning (35, 36). Therefore, one interpretation of our results is that new

neurons may influence learning and memory primarily in tasks that involve significant emotional arousal or coactivation of the hippocampus and the amygdala.

An alternative hypothesis is suggested by experiments showing that exposure to novel environments increases GABAergic tone in the DG and facilitates the generation of LTP (37–39). Because young neurons are not inhibited by GABA, they may be preferentially recruited under such conditions. If so, the effects of blocking neurogenesis would be largest in tasks that take place in a novel environment. This would explain why loss of neurogenesis impairs context conditioning but not water maze performance because contextual conditioning occurs in a single exposure to a novel environment, whereas learning in the water maze requires repeated exposure to an environment over multiple days. It is also interesting to note that our previous studies have revealed a role for neurogenesis in the behavioral response to antidepressant drugs (4). The principal test used in these experiments (the novelty-suppressed feeding paradigm) relies on the fear elicited by placement of the animal in a novel environment.

In summary our studies reveal that, under certain conditions, the activity of young neurons may be preferentially recruited

