Adult Neurogenesis Modulates the Hippocampus-Dependent Period of Associative Fear Memory

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SUMMARY

Acquired memory initially depends on the hippocampus (HPC) for the process of cortical permanent memory formation. The mechanisms through which memory becomes progressively independent from the HPC remain unknown. In the HPC, adult neurogenesis has been described in many mammalian species, even at old ages. Using two mouse models in which hippocampal neurogenesis is physically or genetically suppressed, we show that decreased neurogenesis is accompanied by a prolonged HPC-dependent period of associative fear memory. Inversely, enhanced neurogenesis by voluntary exercise sped up the decay rate of HPC dependency of memory, without loss of memory. Consistently, decreased neurogenesis facilitated the long-lasting maintenance of rat hippocampal long-term potentiation in vivo. These independent lines of evidence strongly suggest that the level of hippocampal neurogenesis play a role in determination of the HPC-dependent period of memory in adult rodents. These observations provide a framework for understanding the mechanisms of the hippocampal-cortical complementary learning systems.

INTRODUCTION

The hippocampus (HPC) is crucial for the formation of some types of declarative memories during the mnemonic process in humans (Burgess et al., 2002; Scoville and Milner, 1957; Squire et al., 2004). Many studies of retrograde amnesia in memory-impaired patients, neuroimaging with healthy volunteers, and experimental animals have shown that the recall of acquired memories is initially dependent on the HPC, but that HPC-dependency progressively decays over time, a process that is associated with a gradual increase in dependency upon an extra-hippocampal region, such as neocortex (Dudai, 2004; Frankland and Bontempi, 2005; McClelland et al., 1995; Squire and Bayley, 2007; Wiltgen et al., 2004). This is a common process observed in several different mammalian species (mice, rats, rabbits, cats, monkeys, and humans) (Anagnostaras et al., 2001; Kim and Fanselow, 1992; Squire and Bayley, 2007; Teng and Squire, 1999; Zola-Morgan and Squire, 1990). In rats and mice, some forms of associative and spatial memories follow a similar decay process (Anagnostaras et al., 2001; Frankland et al., 2004, 2007; Kim and Fanselow, 1992; Maviel et al., 2004; Takehara et al., 2003; Winocur, 1990). A gradual decay with a similar time course is also observed in hippocampal long-term potentiation (LTP) (Castro et al., 1989; Villarreal et al., 2002), a putative cellular model of learning and memory (Kandel, 2001; Morris et al., 2003; Okada et al., 2009). This gradual decay in the HPC dependency is proposed to play a role in clearing disused old memories once the memory has become dependent on the cortical networks, in order to preserve the learning capacity of the HPC (Willshaw and Buckingham, 1990). However, the mechanisms underlying the gradual decay of HPC dependency during the process of cortical permanent memory formation remain unknown.

In the hippocampal dentate gyrus (DG), new neurons are continuously generated in the subgranular zone (SGZ) throughout adulthood in many mammals, including mice, rats, monkeys, and humans (Altman and Das, 1965; Eriksson et al., 1998; Gould et al., 1999). The newly generated neurons form synapses and are functionally integrated into existing hippocampal neuronal circuits (Kee et al., 2007; Schmidt-Hieber et al., 2004; Toni et al., 2008; Toni et al., 2007; van Praag et al., 2002). The level of adult hippocampal neurogenesis is positively and negatively modulated by environmental conditions, neuronal activity, aging, and stress (Lledo et al., 2006; Ma et al., 2009; Ming and Song, 2005; Zhao et al., 2008). Many studies point

out the functional significance of adult hippocampal neurogenesis in antidepressant processes, CNS disorders, and learning and memory (Sahay and Hen, 2007; Zhao et al., 2008). Particularly, most research has focused on the functional roles played by neurogenesis in memory acquisition and in the early period of memory formation (1–3 day memory, recent memory) (Dupret et al., 2008; Imayoshi et al., 2008; Saxe et al., 2006; Shors et al., 2001; Winocur et al., 2006; Zhang et al., 2008; Zhao et al., 2008). There are several studies examining maintenance of memory (Imayoshi et al., 2008; Snyder et al., 2005), but whether the continuous integration of new neurons into hippocampal circuits modifies, impairs, or strengthens pre-existing memory information is not well understood. Because continuous integration of new neurons into pre-existing neural circuits would disturb the structure of pre-existing information (Lledo et al., 2006; Meltzer et al., 2005) and lead to the clearance of hippocampal memory trace (Feng et al., 2001), we hypothesized that hippocampal neurogenesis is involved in the gradual decay of hippocampal LTP and in the gradual decay of HPC dependency of associative memory over time (30 day memory, remote memory). Here, we examined this hypothesis by using DG LTP in freely moving unanaesthetized rats, from which stable in vivo recordings can be obtained for several weeks (Fukazawa et al., 2003), and the contextual fear conditioning in mice for taking advantage of transgenic mouse model (Ageta et al., 2008).

RESULTS

Irradiation Enhances the Retention of LTP in Rat Dentate Gyrus

We examined the effect of decreased hippocampal neurogenesis on the maintenance of dentate gyrus (DG) LTP in freely moving unanaesthetized rats. We used X-ray irradiation to inhibit neurogenesis in the subgranular zone of the DG (Monje et al., 2002; Santarelli et al., 2003) (see Figure S1 available online). Low-dose X-ray irradiation of the whole brain resulted in an 85% reduction in cell proliferation (measured by the number of BrdU$^+$ cells, $t_{12} = 12.12$, p < 0.0001) (Figure 1D) and a similar reduction in neurogenesis in rat DG (measured by the number of BrdU$^+$/NeuN$^+$ cells, $t_8 = 6.38$, p < 0.001; NeuN is a neuronal marker) (Figures 1A, 1B, and 1E). LTP was induced by delivering high-frequency stimulation (HFS) 11 days after the irradiation. HFS easily elicited LTP in both sham-irradiated (0 Gy) and irradiated (10 Gy) rats (Figures 1G and 1H). There was no significant difference in the magnitude of LTP between both groups at 1 day after HFS (0 Gy, 121 ± 8%; 10 Gy, 122 ± 6%, $t_{24} = -0.18$; p > 0.8) (Figure 1I). LTP in the sham group decayed gradually and returned to the basal level by 2 weeks (paired t test, 1 day versus 2 weeks; $t_{12} = 4.18$, p < 0.002) (Figures 1G and 1I). Surprisingly, X-ray irradiation led to sustained LTP for 2 weeks (paired t test, 1 day versus 2 weeks; $t_{12} = -0.12$, p > 0.9) (Figures 1H and 1I). There was a significant interaction between treatment (0 Gy and 10 Gy) and time course (1 day, 1 week, 2 weeks, and 3 weeks after HFS) (repeated-measures ANOVA; $F_{(3, 72)} = 3.82$, p < 0.02). These results indicate that the irradiation prolonged the maintenance of LTP. In addition, irradiation had no effect on the synaptic transmission at unpotentiated pathway at any timepoint (1 day, $t_{19} = 0.91$, p > 0.3; 1 week, $t_{19} = -0.38$, p > 0.7;

2 weeks, $t_{19} = -0.18$, p > 0.8; 3w, $t_{18} = 0.08$, p > 0.9) (Figure 1J), nor on the paired-pulse depression at perforant path-DG synapses (Figure S2), or on expression of immediate-early gene products (Zif268/Egr1 and c-Fos) following LTP induction (Matsuo et al., 2000) (Figure S3). Furthermore, maintenance of L-LTP in irradiated rats was protein synthesis-dependent, as was that of non-irradiated rats (Figure S4). These results suggest that the effect of X-ray irradiation was specific to LTP maintenance.

Notably, irradiation dramatically reduced the population of doublecortin$^+$ (DCX$^+$) cells in rat DG 1 day after irradiation (DCX is transiently expressed in ∼2 week-old-cells (Brown et al., 2003)) (Figure 1C). Similarly, irradiation reduced the number of BrdU$^+$ cells when animals were irradiated 7-10 days after BrdU incorporation, whereas irradiation did not affect the population of BrdU$^+$ cells when animals were irradiated 14 or 24 days after BrdU incorporation (Figure 1F). These results indicated that irradiation led to a reduction not only in the number of dividing cells, but also in the number of immature cells in the DG that were younger than 10 days old. Spine formation of newly born neurons starts around 3 weeks after cell division (Toni et al., 2007; Zhao et al., 2006). Given the timing of the irradiation, which preceded LTP induction by 11 days, almost no newborn neurons were integrated into the DG network after HFS delivery, because at the time of HFS delivery there were very few new neurons younger than 21 (10 + 11) days old. Thus, the integration of newly born neurons after LTP induction may promote the gradual decay of DG LTP.

Irradiation Extends the Hippocampus-Dependent Period of Memory

Electrophysiological results have implied that hippocampal neurogenesis is involved in the process of remote memory formation (30 day memory) rather than recent memory formation. First, we examined the effect of X-ray irradiation on the maintenance of remote memory in contextual fear conditioning, an associative learning between a chamber (context) and electrical footshocks that take place in that chamber, which requires the amygdala and the HPC including the trisynaptic pathway (Entorhinal cortex-DG-CA3-CA1) (Kim et al., 1993; Nakashiba et al., 2008; Phillips and LeDoux, 1992) (Figure 2). In memory retrieval tests, we measured the extent of "freezing" behavior as an index of successful retrieval of the conditioned fear memory (Figure S5). X-ray irradiation resulted in a 95% reduction of cell proliferation ($t_5 = 19.5$, p < 0.0001) and neurogenesis ($t_{10} = 11.7$, p < 0.0001) in mouse DG (Figures 2C–2H). X-ray irradiation also dramatically reduced the population of immature neurons (DCX$^+$ cells) in mouse DG 1 day after irradiation (Figures 2E). This effect lasted for at least 9 weeks after irradiation (Figure S6), and, thus, persisted at the time of behavioral testing without affecting the population of astrocytes, which is other type of proliferating cells, in the CA1 area and in the molecular layer of DG (Figure S7). We subjected the irradiated and sham wild-type (WT, C57BL6J) mice to contextual fear conditioning 5 weeks after irradiation (Figures S8–S10). No obvious effects of irradiation on the levels of freezing response were detected in retrieval tests of 1 day or 28 day memory, irrespective of the conditioning protocol used (irradiation slightly impaired the
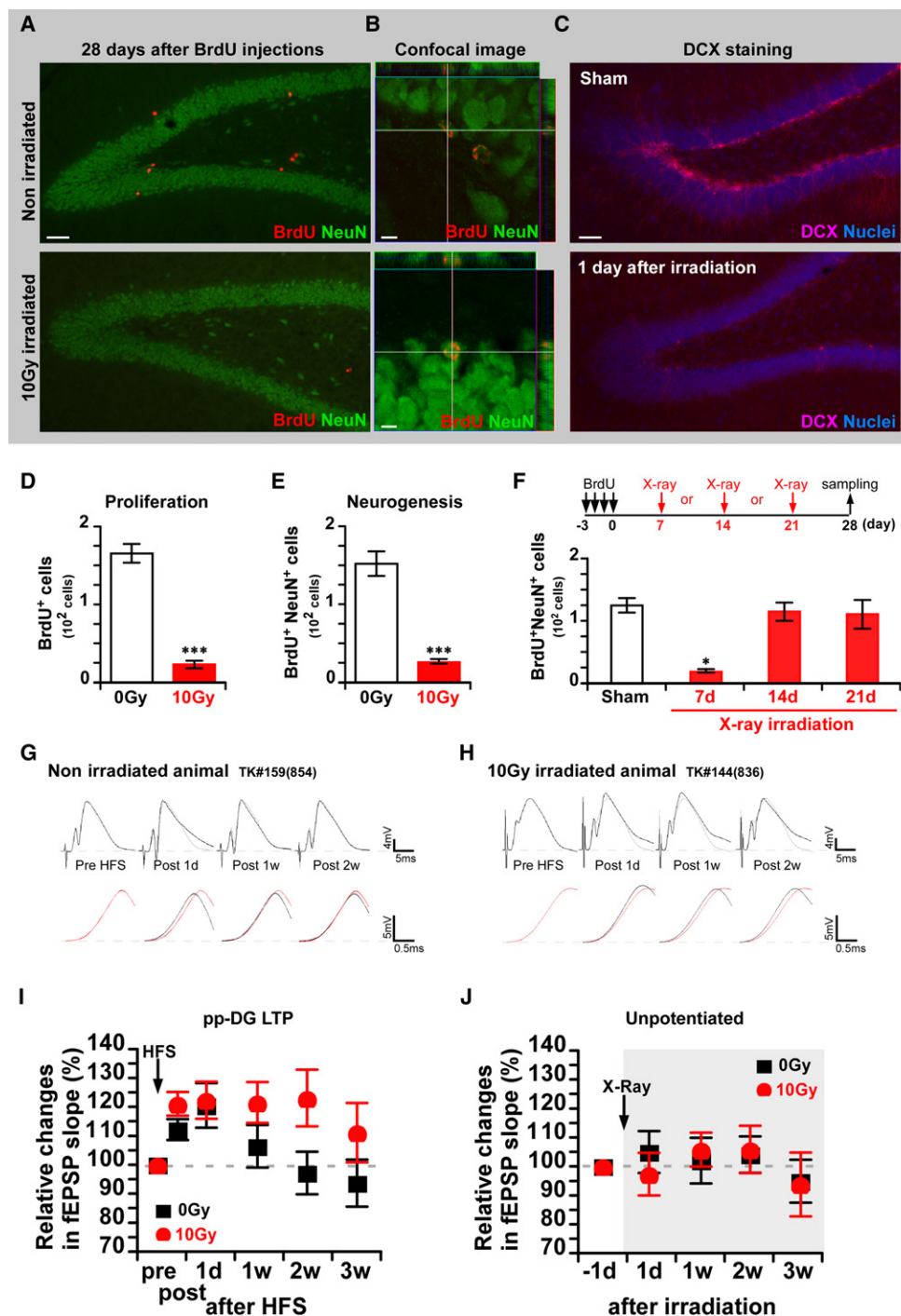
Figure 1. Irradiation Enhances the Retention of LTP in Rat Dentate Gyrus

(A) BrdU immunoreactivity in rat DG 28 day safter last BrdU injection. The scale bar represents 50 μm.

(B) Confocal orthographic images of cells double-labeled for BrdU (red) and NeuN (green) in DG. The scale bar represents 10 μm.

(C) DCX immunoreactivity in rat DG 1 day after irradiation. DCX, red; DRAQ5, blue. The scale bar represents 50 μm.

(D and E) Effect of irradiation on cell proliferation and neurogenesis in rat DG. BrdU was administered to 20- to 25-week-old rats 1 day after irradiation. Rats were sacrificed 2 hr (A) or 28 days (B) later. Irradiation markedly reduced the number of BrdU$^+$ cells (A) (n = 6–8 per group) and the number of BrdU$^+$NeuN$^+$ cells in DG (B) (n = 4–6 per group).

(F) Effect of irradiation on the survival of newly born cells. Rats were irradiated at day 7, 14, or 21, after BrdU injections, and sacrificed 28 days after the last BrdU injection. Irradiation at day 7 significantly reduced the number of BrdU$^+$NeuN$^+$ cells in DG at day 28, but irradiation at day 14 and day 21 did not (n = 3 per group). (F$_{(3, 11)}$ = 11.02, post-hoc Scheffe's test, p < 0.05).

retrieval of 1 day memory, but there were no subsequent differences in 28 day memory test in three footshock conditioning experiments) (Figures S8–S10). Irradiation had no effect in hybrid (C57BL6/129X1SvJ) mice (Figure S11). Irradiation also had no effect on c-Fos expression in the hippocampal CA1, CA3 and DG areas following contextual fear conditioning (Figure S12). These results indicate that the irradiation treatment does not affect the acquisition of memory or the maintenance of remote memory in contextual fear conditioning. Nevertheless, these results do not necessarily exclude the possibility of the involvement of hippocampal neurogenesis in memory acquisition. Neurogenesis ablation before learning, as performed by us and others (Dupret et al., 2008; Imayoshi et al., 2008; Saxe et al., 2006; Shors et al., 2001; Winocur et al., 2006; Zhang et al., 2008; Zhao et al., 2008), may pose the problem of compensation effects (e. g. by pre-existing old neurons, spared new neurons or other types of brain organization) that may mask the effect of inhibiting neurogenesis on memory acquisition. Thus, to clarify the roles of newborn neurons in memory acquisition, new neurons would need to be ablated after learning.

To assess the contribution of the HPC to the retrieval of recent memory and remote memory in irradiated mice, we next examined the transient and pharmacological inactivation of the neuronal activity of the HPC (Riedel et al., 1999). We implanted mice with microcannula into the dorsal HPC (Figure 2A). We subjected irradiated and sham WT (C57BL6J) mice to contextual fear conditioning (three footshocks, or FS × 3) 5 weeks after irradiation (Figure 2B). There were no differences between the groups in the pre-footshock travel activity and in the freezing behavior during training (Figures 2I and 2J). In retrieval test of 1 day memory, intrahippocampal infusion of tetrodotoxin (TTX, a sodium channel blocker) and 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, a selective antagonist of AMPA receptors) (henceforth we have called this treatment TTX-infusion) 30 min prior to the retrieval test significantly reduced the freezing response of both non-irradiated and irradiated mice, when compared with PBS-infused mice (0 Gy, $p < 0.04$; 10 Gy, $p < 0.04$, ANOVA, post-hoc Scheffe's test) (Figures 2K and 2M), indicating that the retrieval of 1 day memory in both groups was hippocampal neuronal activity-dependent (HPC-dependent). In contrast, TTX-infusion had no effect on the freezing response of non-irradiated mice in the retrieval test of 28 day memory ($p > 0.9$, ANOVA, post-hoc Scheffe's test) (Figures 2L and 2N), indicating that retrieval of 28 day memory in sham mice was completely independent of the HPC, as previously reported (Kim and Fanselow, 1992). Surprisingly, TTX-infusion significantly reduced the freezing response of irradiated mice in the retrieval test of 28 day memory, when compared with the PBS-infused group ($p < 0.03$, ANOVA, post-hoc Scheffe's test) (Figures 2L and 2N). There was a significant interaction in the

freezing response between irradiation treatment and TTX-infusion (two-way ANOVA; $F_{(1, 55)} = 4.02$, $p < 0.03$) (Figure 2N). To directly compare the effects of TTX-infusion in non-irradiated and irradiated animals, we calculated the ratio of freezing (Individual TTX_{Sham}/Average of PBS_{Sham} or Individual TTX_{X-Ray}/Average of PBS_{X-Ray}) in the same retrieval test. There was a significant difference in this ratio in the retrieval test of 28 day memory (0 Gy versus 10 Gy; $t_{29} = 2.78$, $p < 0.01$), but not in the retrieval test of 1 day memory (0 Gy versus 10 Gy; $t_8 = -0.19$, $p > 0.8$) (Figure 2Q). These results clearly indicate that the way how to retrieve remote memory in irradiated mice differs from that in non-irradiated mice, even if both groups successfully retrieved remote memory of contextual fear.

We obtained similar results from the analysis of activity suppression, performed as an alternative index of successful retrieval of the conditioned fear memory (Figures 2O and 2P). In the same animals, we calculated the extent of activity suppression (AS) by comparing activity levels before a shock delivered during training, with activity levels during the test, as described previously (Frankland et al., 2001). In the retrieval test of 1 day memory, TTX-infusion significantly enhanced the AS in both groups compared with PBS-infused mice (0 Gy, $p < 0.05$; 10 Gy, $p < 0.05$, ANOVA, post-hoc Scheffe's test) (Figure 2O). In the retrieval test of 28 day memory, TTX-infusion significantly enhanced the AS in irradiated mice ($p < 0.001$, ANOVA, post-hoc Scheffe's test), whereas TTX-infusion had no effect on AS in non-irradiated mice ($p > 0.7$, ANOVA, post-hoc Scheffe's test) (Figure 2P). There was a significant interaction in AS between irradiation treatment and TTX-infusion (two-way ANOVA; $F_{(1, 55)} = 4.01$, $p < 0.001$). These results support our conclusion obtained in the analysis of freezing response, described above.

We subsequently obtained similar results using a different conditioning protocol (eight footshocks, or FS × 8) (Figure S13). TTX-infusion experiment further revealed that the retrieval of 84 day memory was still partially dependent on hippocampal activity in hybrid WT mice (C57B6J/129X1SvJ) (Figure S14). Thus, the effects of X-ray irradiation were observed in different behavioral procedures and different mouse strains. Using fluorescently labeled muscimol (an agonist of $GABA_A$ receptor) (Allen et al., 2008), we analyzed the relationship between the freezing response and the spatial extent of inactivation. This analysis confirmed that inactivation of the HPC is indeed important in disrupting the retrieval of 28 days memory in irradiated mice (Figures S15–S17). These results show that the retrieval of remote memory in X-ray-irradiated mice partially depends on the HPC.

Irradiation Does Not Affect Hippocampus-Independent Memory Process

We subjected irradiated and sham WT mice to tone fear conditioning, an associative learning between a tone and electrical

(G and H) Superimposed traces show the fEPSP in pp-DG synapses recorded 15 min before HFS (pre), as well as 1 day, 1 week, and 2 weeks after HFS in non-irradiated and irradiated freely moving unanaesthetized rats. The lower traces show the expanded initial phase of fEPSP.

(I) Effect of irradiation on LTP persistence in pp-DG synapses. The average of the fEPSP slopes obtained 15 min before HFS served as the baseline (100%). Post indicates 15min after HFS. Graph shows the time-course of the relative changes in fEPSP slope following HFS in sham and irradiated animals (n = 13 per group).

(J) Effect of irradiation on basal transmission in pp-DG synapses. The average of the fEPSP slopes obtained 1 day before irradiation served as the baseline (100%). Graph shows the time-course of the relative changes in fEPSP slope following irradiation in sham and irradiated animals (n = 10–11 per group).

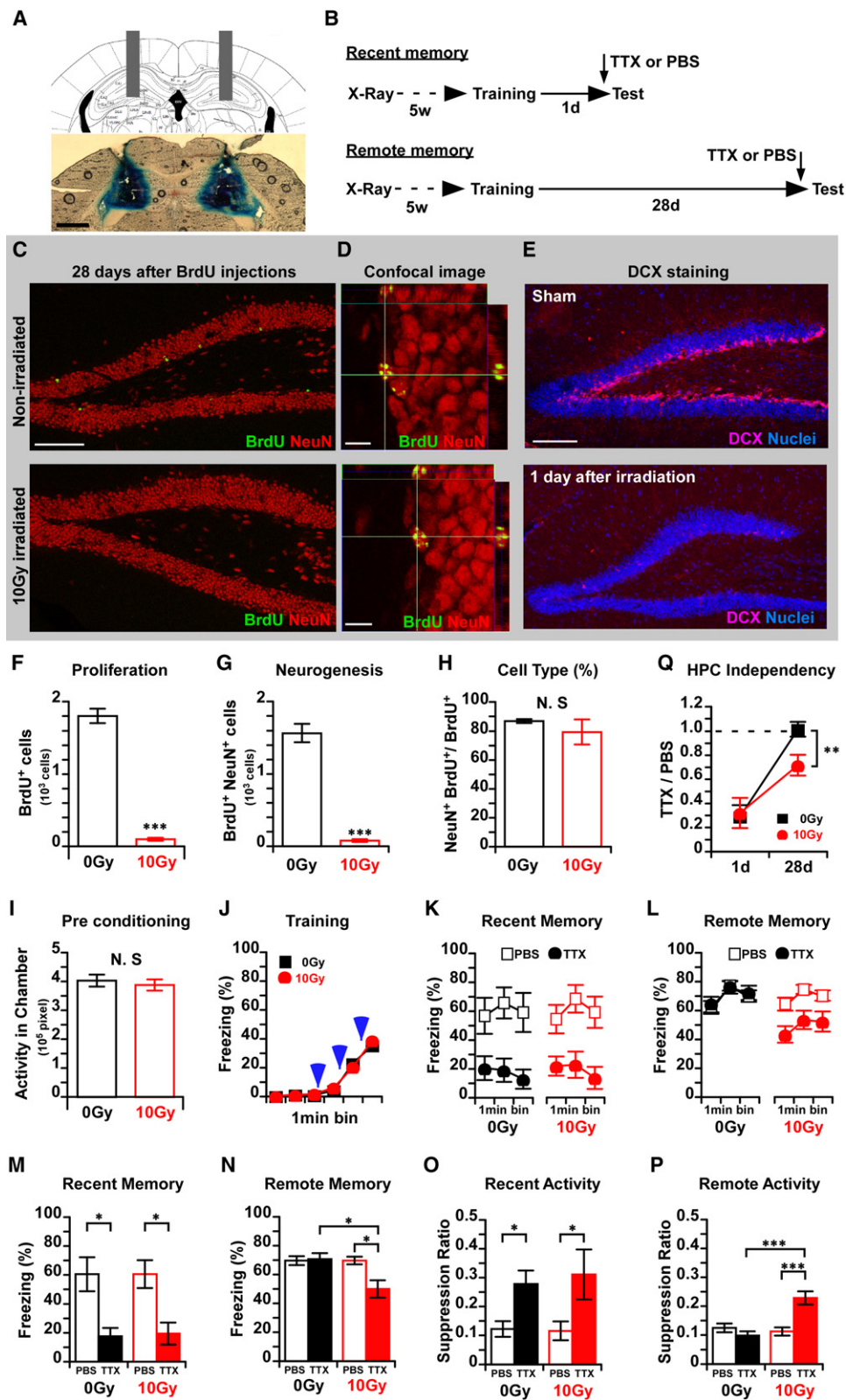***$p < 0.001$, *$p < 0.05$. Error bars indicate SEM.

Figure 2. X-Ray Irradiation Extends the Hippocampus-Dependent Period of Memory
(A) Microinjection of methylene blue solution into dorsal HPC. The scale bar represents 1 mm.
(B) Experimental design. Five-week-old mice were irradiated.

footshocks that take place in a chamber (Figure 3A). In contrast to the contextual fear conditioning, tone fear conditioning requires the amygdala but not the HPC (Anagnostaras et al., 1999; Phillips and LeDoux, 1992). There was no difference in the levels of freezing response during the 3 day-training sessions in both groups (Figure 3B). In retrieval test of 1 day memory, irradiated and sham mice showed freezing behavior in response to the tone stimulus (Pre versus Tone: 0Gy/PBS, p < 0.04; 0Gy/TTX, p < 0.001; 10Gy/PBS, p < 0.01; 10Gy/TTX, p < 0.001, paired t test), but no obvious effects of TTX-infusion on the levels of freezing response to the tone were detected ($F_{(3, 16)}$ = 0.11, p > 0.9, ANOVA) (Figure 3C), indicating that the retrieval of 1 day tone fear memory in both groups is completely HPC-independent. In retrieval test of 28 day memory, we observed higher levels of freezing response in pre-tone, when compared with that of 1 day memory in all groups (Figure 3C and 3D). This is classically called context generalization (the conditioned mouse freezes in a non-conditioned similar context as well as the conditioned context) (Figure S18) (Wiltgen and Silva, 2007; Winocur et al., 2007). However, we found higher levels of freezing response to the tone stimulus, when compared with that in the pre-tone, irrespective of TTX-infusion and irradiation (Pre versus Tone: 0Gy/PBS, p < 0.01; 0Gy/TTX, p < 0.003; 10Gy/PBS, p < 0.001; 10Gy/TTX, p < 0.01, paired t test) (Figure 3D) in this training protocol (Figures 3B and S19), indicating that the retrieval of 28 day tone fear memory in both groups is completely HPC-independent, similar to the 1 day tone fear memory.

Irradiation had no effect on the general activity, anxiety, pain sensitivity (Figures 3E–3H), and on c-Fos expression in the amygdala, somatosensory cortex, and hypothalamus following the fear conditioning (Figure S20). These results strongly suggest that X-ray irradiation specifically affects hippocampal functions and HPC-dependent memory processes.

FSM Mice Exhibit a Prolonged Hippocampus-Dependent Period of Memory

Although the irradiation-induced microglial activity was gradually suppressed over time, X-ray irradiation transiently caused severe changes in the microenvironment of the irradiated mouse tissue (Figure S21). We next assessed whether genetic suppres-sion of hippocampal neurogenesis prolongs the HPC-dependent period of contextual fear memory, as seen previously after physical inhibition (X-ray) of neurogenesis. We employed trans-genic mice that overexpress follistatin in a forebrain-specific manner (FSM mice) (Ageta et al., 2008). FSM mice have a severe deficit in hippocampal neurogenesis (t_5 = 4.06, p < 0.01) (Figures 4B–4D), caused by severe impairment in the survival of newly born neurons (Ageta et al., 2008), without affecting the microglial cell activity in the hippocampus (Figure S22). This deficit results in a relatively smaller DG than that of littermate WT mice (Figure S23). Follistatin is an inhibitor of the activin signaling pathway, which regulates differentiation and proliferation of numerous cell types, as well as dendritic spine morphology (Inokuchi et al., 1996; Shoji-Kasai et al., 2007). We subjected FSM mice and littermate WT mice to eight-footshock condi-tioning (Figure 4A). FSM mice successfully retrieved 1 day memory similarly to WT mice (p > 0.99, ANOVA, post-hoc Scheffe's test) (Figures 4E and 4G). TTX-infusion into the HPC disrupted the retrieval of 1 day memory in both WT and FSM mice (p < 0.01; p < 0.05, respectively, ANOVA, post-hoc Scheffe's test) (Figures 4E and 4G), indicating that 1 day memory retrieval in FSM mice was HPC-dependent. Importantly, TTX-infusion greatly disrupted the retrieval of 28 day memory in FSM mice (p < 0.005, ANOVA, post-hoc Scheffe's test) (Figures 4F and 4H). In contrast, TTX-infusion had no effect on the retrieval of 28 day memory in WT littermate mice (p > 0.2, ANOVA, post-hoc Scheffe's test) (Figures 4F and 4H). For the TTX/PBS ratio, there was a significant interaction between mouse genotype and delay (1 day, 28 day) (two-way ANOVA; $F_{(1, 17)}$ = 4.76, p < 0.05) (Figure 4I). Similar results were obtained in the analysis of AS during the retrieval test of 28 day memory (Figures S24). These results indicate that genetic suppression of hippocampal neurogenesis prolongs the HPC dependency of contextual fear memory, and further suggests that X-ray irra-diation exerts its effect on hippocampal memory through the inhibition of neurogenesis.

Running Wheel Exercise Speeds up the Decay Rate of Hippocampus Dependency of Memory

We examined the effect of enhanced neurogenesis on the gradual decay of HPC dependency of memory in WT mice

(C) BrdU immunoreactivity in mouse DG 28 days after the last BrdU injection. The scale bar represents 100 μm.
(D) Confocal orthographic images of cells double-labeled for BrdU (green) and NeuN (red) in DG. The scale bar represents 10 μm.
(E) DCX immunoreactivity in mouse DG 1 day after irradiation. DCX, red; DRAQ5, blue. The scale bar represents 100 μm.
(F and G) Effect of irradiation on cell proliferation and neurogenesis in mouse DG. BrdU was administered to 5-week-old mice 1 day after irradiation. Mice were sacrificed 2 hr (F) or 28 days (G) later. Irradiation markedly reduced the number of BrdU$^+$ cells (F) (n = 3–4 per group) and the number of BrdU$^+$NeuN$^+$ cells in DG (G) (n = 6 per group).
(H) Effect of irradiation on neuronal differentiation. Percentages of NeuN$^+$BrdU$^+$/BrdU$^+$ cells 28 days after the last BrdU injection are shown. The irradiation had no effect on differentiation (n = 6 per group).
(I) Activity during the initial 3 min exposure to the conditioning chamber.
(J) Contextual fear conditioning. Changes in freezing response during training. Arrowheads indicate the footshocks. There was no difference between groups (n = 39–40 per group).
(K and M) Retrieval test of 1 day memory. Effect of TTX-infusion and irradiation on changes in freezing response (K) and average freezing response (M) over the 3 min session (n = 5 per group).
(L and N) Retrieval test of 28 day memory. Effect of TTX-infusion and irradiation on changes in freezing response (L) and average freezing response (N) over the 3 min session (n = 13–16 per group).
(O and P) Activity suppression ratio in recent (O) and remote (P) memory tests (recent, n = 5 per group; remote, n = 13–16 per group).
(Q) Ratio of TTX/PBS in the 1 day and 28 day memory retrieval tests in the sham and irradiated groups (1 day, n = 5 per group; 28d, n = 15–16 per group).
*p < 0.05, **p < 0.01, ***p < 0.001. Error bars indicate SEM.
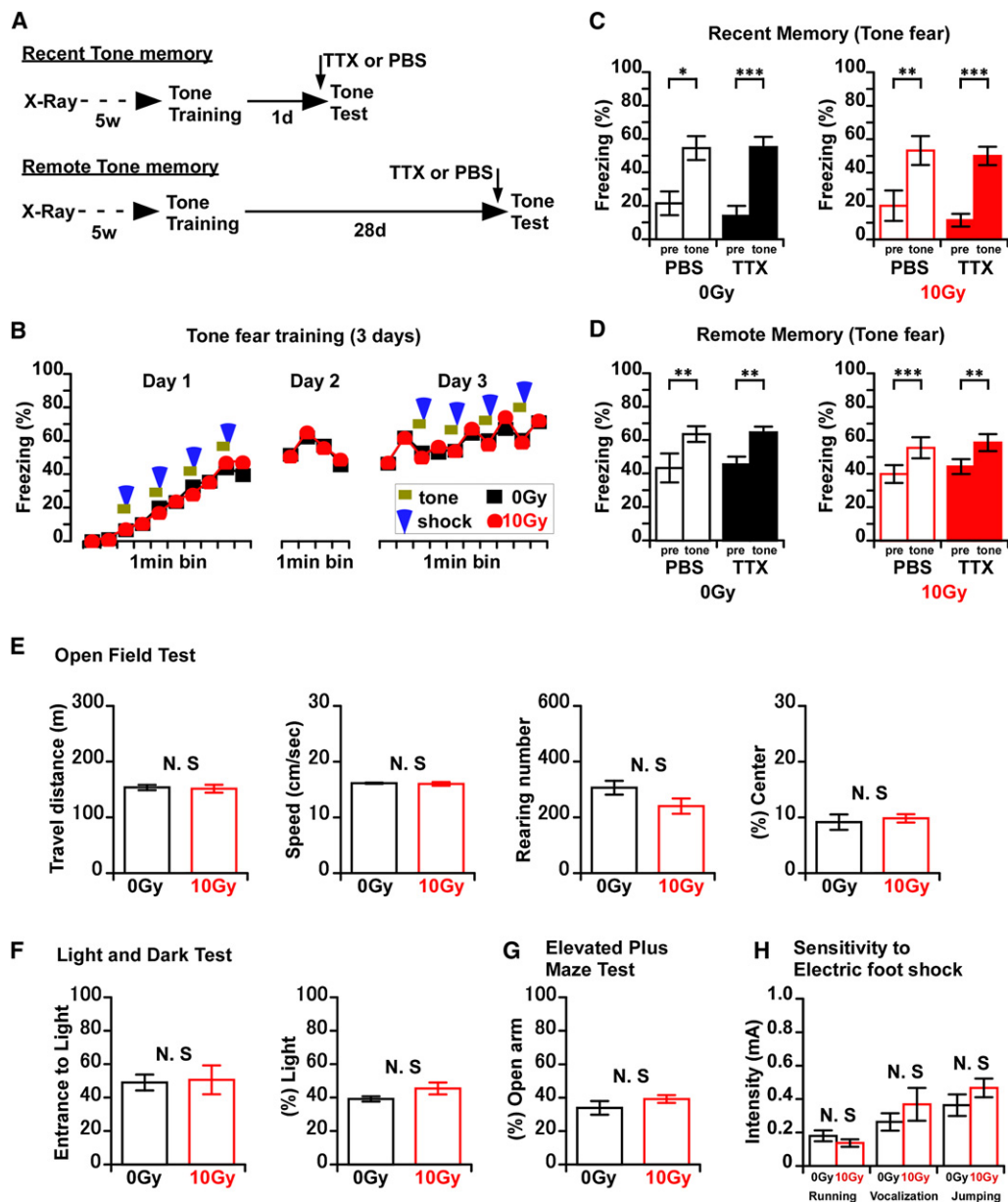
Figure 3. Irradiation Does Not Affect the Hippocampus-Independent Memory Process

(A) Experimental design. Five week-old mice were irradiated.

(B) Tone fear conditioning. Changes in freezing response during 3 days training. There was no difference between groups (n = 27–28 per group).

(C) Retrieval test of 1 day memory to tone. Effect of TTX-infusion and irradiation on average freezing response during pre-tone (2 min) and tone (2 min) (n = 5 per group).

(D) Retrieval test of 28 day memory to tone. Effect of TTX-infusion and irradiation on averages freezing response during pre-tone (over 2 min) and tone (over 2 min) (n = 7–10 per group).

(E) Open-field test. Travel distance, moving speed, rearing number and % time in the center during the 30 min test were analyzed.

(F) Light and dark test. The number of entries into the light side and the time spent in the light during the 30 min test were analyzed.

(G) Elevated plus-maze test. Time spent in the open arms during 10 min test was analyzed.

(H) Sensitivity to electrical stimulation test. The minimal levels of current required to elicit the stereotypical responses of running, jumping and vocalization were determined (n = 6–7 per group).

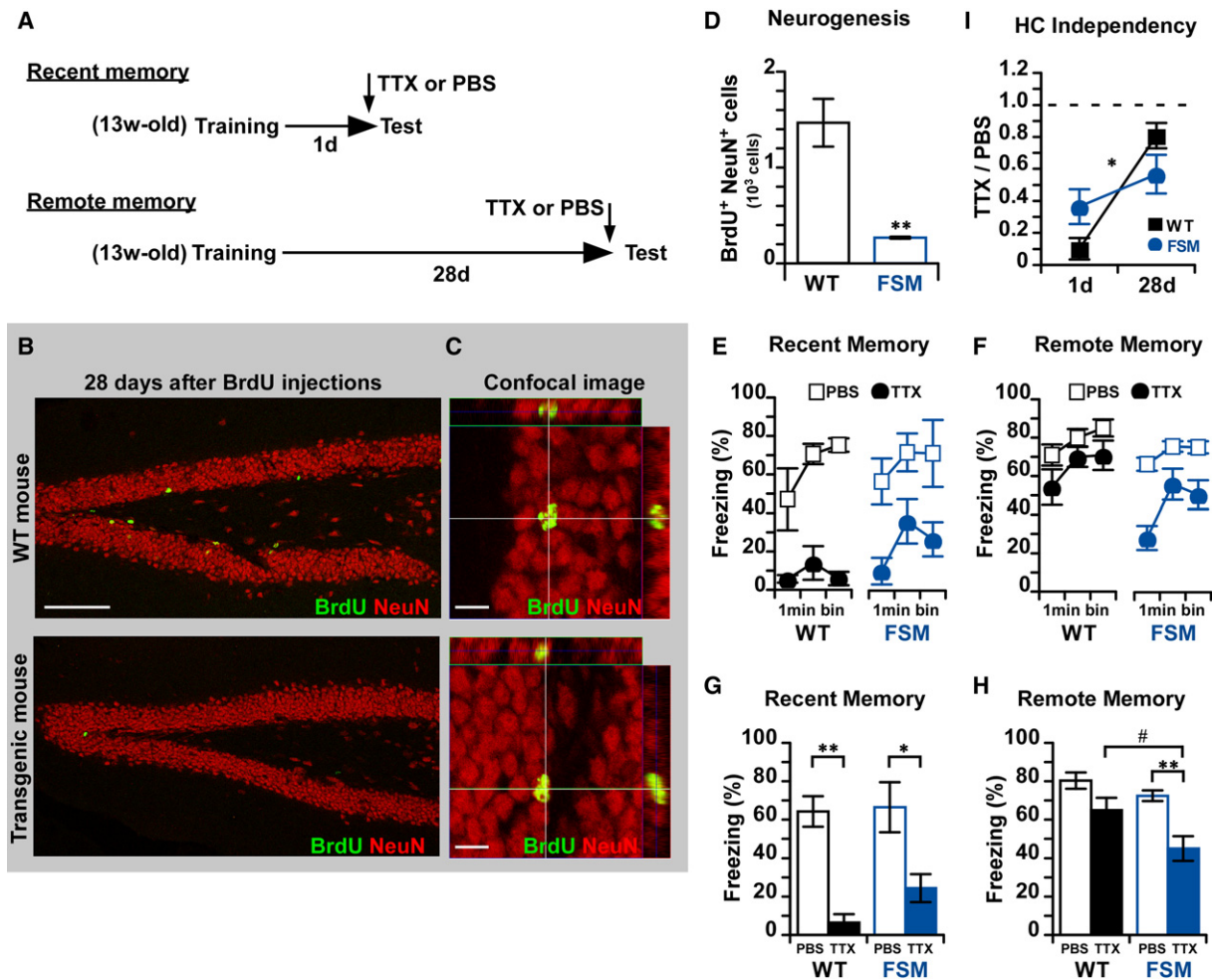*p < 0.05, **p < 0.01, ***p < 0.001. Error bars indicate SEM.

Figure 4. Genetic Suppression of Neurogenesis Extends the Hippocampus-Dependent Period of Memory

(A) Experimental design. Thirteen-week-old FSM and littermate WT mice were trained.

(B) BrdU immunoreactivity in mouse DG 28 days after the last BrdU injection. The scale bar represents 100 μm.

(C) Confocal orthographic images of cells double-labeled for BrdU (green) and NeuN (red) in DG. The scale bar represents 10 μm.

(D) Neurogenesis in DG of FSM and littermate WT mice. BrdU was administered to FSM mice at 5 weeks of age, and 28 days later mice were sacrificed. FSM mice had a severe reduction in the number of BrdU+NeuN+ cells in DG, compared with littermate WT mice (n = 3–4 per group).

(E and G) Retrieval test of 1 day memory. Effect of TTX-infusion on changes in freezing response (E) and average freezing response (G) over the 3 min session in WT and FSM mice (n = 3–4 per group).

(F and H) Retrieval test of 28 day memory. Effect of TTX-infusion on changes in freezing response (F) and average freezing response (H) over the 3 min session in WT and FSM mice (WT, n = 7–8 per group; FSM, n = 10 per group).

(I) Ratio of TTX/PBS in the 1 day and 28 day memory retrieval tests in WT and FSM mice (1 day, n = 3–4 per group; 28d, n = 7–10 per group).

#p = 0.08, *p < 0.05, **p < 0.01. Error bars indicate SEM.

(C57BL6J). Running wheel exercise for 2 weeks markedly increased cell proliferation and neurogenesis in the DG (Figures 5A–5F), as described previously (Kitamura et al., 2003; van Praag et al., 1999). We subjected mice to running wheel exercise 1 day after irradiation. There was no difference in the running distance between non-irradiated and irradiated 6–7-week-old mice (0 Gy, 7.15 ± 1.89 km/day; 10 Gy, 6.58 ± 1.69 km/day; t_8 = 0.23, p > 0.8, n = 5 per group). Five weeks after the onset of the exercise regimen we subjected these mice to three-footshock conditioning (Figure 5G). In the retrieval test of 7 day memory, TTX-infusion into the HPC significantly disrupted the retrieval of fear

memory in non-irradiated non-runner control mice (p < 0.01, ANOVA, post-hoc Scheffe's test) (Figures 5I and 5J). In contrast, TTX-infusions had no effect on the retrieval of 7 day memory in non-irradiated runner mice (p > 0.4, ANOVA, post-hoc Scheffe's test) (Figures 5I and 5J). Thus, the retrieval of 7 day memory in control mice was HPC-dependent, while that of non-irradiated runner mice was almost independent of HPC. Notably, the retrieval of 1 day memory in non-irradiated runner mice was HPC-dependent (Figure S25). The effect of the running wheel exercise regimen was cancelled by X-ray irradiation, as TTX-infusion significantly disrupted the retrieval of 7 day memory in
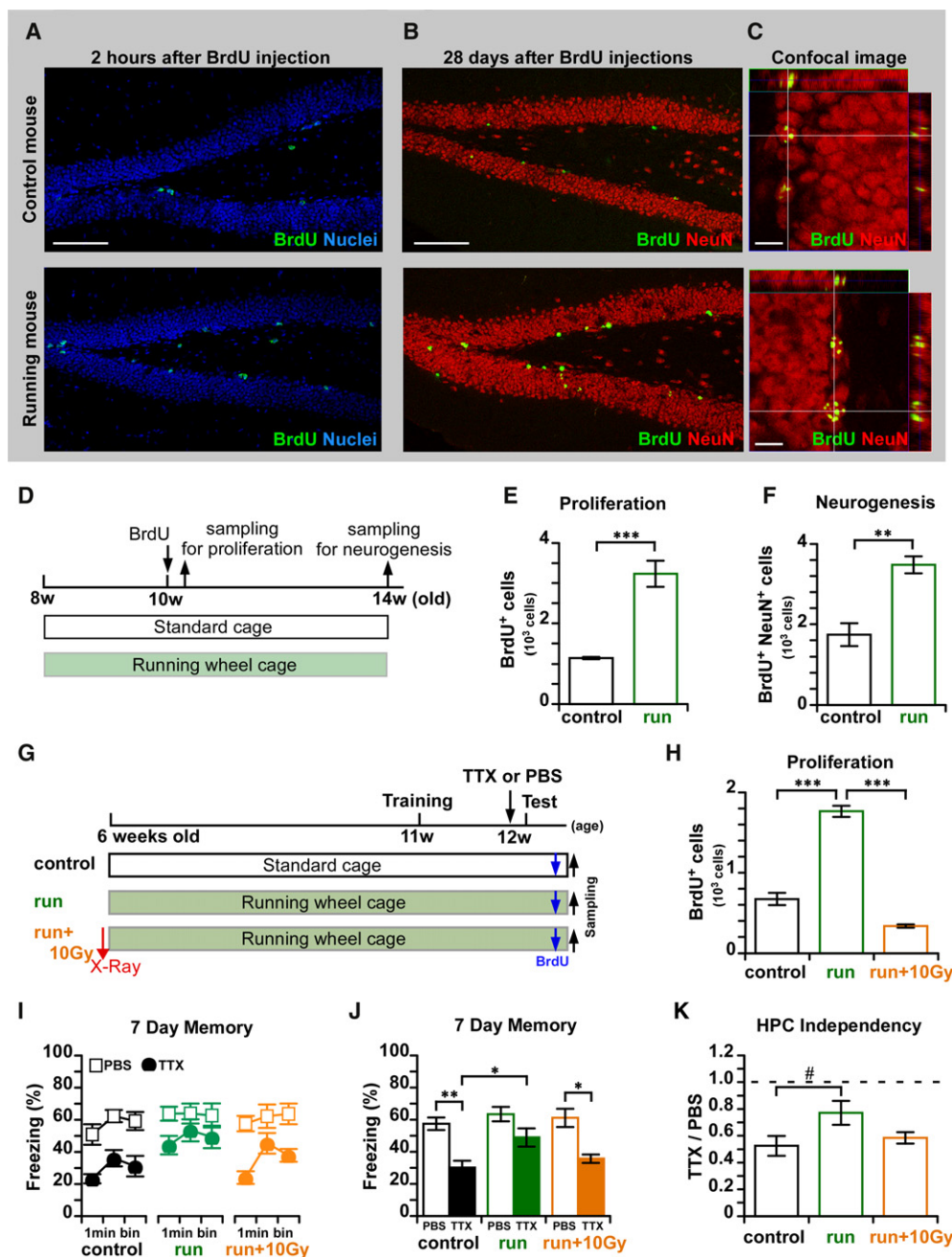
Figure 5. Enhancement of Neurogenesis Speeds up the Decay Rate of the Hippocampus Dependency of Memory

(A and B) BrdU immunoreactivity in mouse DG 2hrs (A) and 28 days (B) after the last BrdU injection. BrdU (green), NeuN (red) and DRAQ5 (blue). DRAQ5 stains nuclei. The scale bar represents 100 μm.

(C) Confocal orthographic images of cells double-labeled for BrdU (green) and NeuN (red) in DG. The scale bar represents 10 μm.

(D) Experimental design for quantification of proliferation and neurogenesis.

(E and F) Effect of running on cell proliferation and neurogenesis in mouse DG. Running exercises markedly enhanced the number of BrdU$^+$ cells (E) (n = 5–6 per group) and the number of BrdU$^+$NeuN$^+$ cells in DG (F) (n = 5 per group).

(G) Experimental design.

(H) Effect of long-term running wheel exercise on cell proliferation in DG. A 7-week running regimen markedly increased the number of BrdU$^+$ cells in DG, and this enhancement was cancelled by irradiation pre-treatment 1 day prior to running onset (n = 4–5 per group).

(I and J) Retrieval test of 7 day memory. Effect of TTX-infusion and running wheel exercise on changes in freezing response (I) and average freezing response (J) over the 3 min session in non-irradiated and irradiated WT mice (n = 10 per group).

(K) Ratio of TTX/PBS in the 7 day memory retrieval test in control, non-irradiated runner and irradiated runner group (n = 10 per group).

#p = 0.066, *p < 0.05, **p < 0.01, ***p < 0.001. Error bars indicate SEM.

irradiated runner mice (p < 0.02, ANOVA, post-hoc Scheffe's test) (Figures 5I and 5J). Importantly, there were no differences in the freezing response during the 7 day memory test between the three groups of PBS-infused mice ($F_{(2, 29)} = 0.40$, p > 0.6) (Figures 5I and 5J), whereas there was a significant difference in the freezing response between control and running groups of TTX-infused mice (p < 0.02, ANOVA, post-hoc Scheffe's test). There was a slight difference in the TTX/PBS ratio in the 7 day memory test between the control and the non-irradiated running groups (p = 0.066, ANOVA, post-hoc Scheffe's test), but not between the control and irradiated running groups (post-hoc Scheffe's test, p > 0.8) (Figure 5K). Similar results were obtained for the analysis of AS (Figure S26). To assess cell proliferation status, mice were administered BrdU after the behavioral test was completed, and 2 hr later they were perfused and analyzed. The 7 weeks of running exercise enhanced cell proliferation (control versus runner; post-hoc Scheffe's test, p < 0.0001) (Figure 5H), but this enhancement was cancelled by irradiation pre-treatment (runner versus irradiated runner; post-hoc Scheffe's test, p < 0.0001) (Figure 5H). Thus, exercise-induced neurogenesis speeds up the decay rate of HPC-dependency of memory, without affecting the expression of that memory.

Integration of New Neurons after Learning Is Important for the Gradual Decay of the Hippocampus Dependency of Memory

Finally, we examined whether the incorporation of newly born neurons after learning is critical for the gradual decay in the HPC dependence of memory. We subjected irradiated and sham WT mice to contextual fear conditioning 11 days after irradiation (Figure 6A). This irradiation protocol prevented the integration of newly generated neurons after the training (see LTP section above, Figure 1F). In the retrieval of 28 day memory, TTX-infusion disrupted the retrieval of 28 day memory in irradiated mice (p < 0.01, ANOVA, post-hoc Scheffe's test), but not in non-irradiated mice (p > 0.9, ANOVA, post-hoc Scheffe's test) (Figure 6B and 6C). There was a significant interaction in the freezing response between irradiation treatment and TTX-infusion (two-way ANOVA; $F_{(1, 36)} = 7.41$, p < 0.01) (Figure 6C). Similar results were obtained for the analysis of AS (Figure S27). Thus, this result suggests that the integration of new neurons after learning is important for the gradual decay of the HPC-dependency of memory as well as the gradual decay of LTP in rat DG (There was a slight difference in the time course of the gradual decay of LTP and that of the HPC dependency of memory (Figures 1 and 6). As one of the possibilities, we speculate that the difference may be due to the HFS protocols used for the LTP experiments). However, we cannot necessarily exclude the possibility that inhibition of neurogenesis may affect the learning mechanisms in such a way that it would be more difficult to become hippocampus-independent, because our irradiation protocol (Figure 1F) reduced the population of young new neurons (∼3 weeks of the cell age) at the learning phase. Therefore, the integration of new neurons needs to be inhibited specifically after learning, although methodologically difficult at present, to completely exclude this possibility.
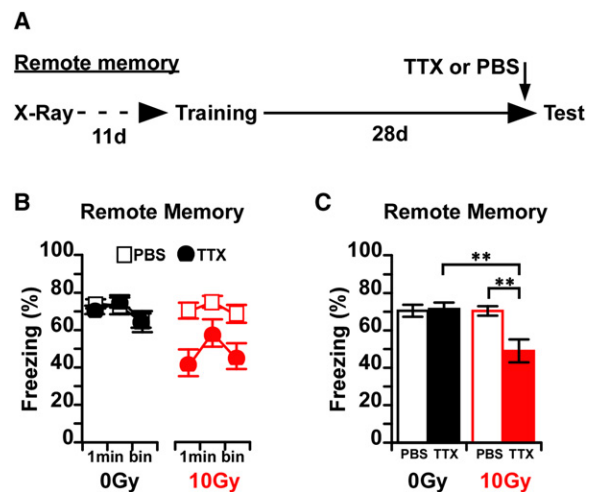

Figure 6. Integration of New Neurons after Learning Is Important for the Gradual Decay of the Hippocampus Dependency of Memory
(A) Experimental design. Eight week-old mice were irradiated.
(B and C) Retrieval test of 28 day memory. Effect of TTX-infusion and irradiation on changes in freezing response (B) and average freezing response (C) over the 3 min session (n = 10 per group).
**p < 0.01. Error bars indicate SEM.

DISCUSSION

In this study, we have shown that X-ray irradiation or genetic overexpression of follistatin, both of which severely impair neurogenesis, attenuate the loss of the HPC dependency of remote contextual fear memory, as assayed using TTX and CNQX infusions into the HPC prior to memory retrieval. Conversely, exercise on a running wheel, which promotes neurogenesis, increased the rate of loss of HPC dependency. Thus, the HPC-dependent periods for contextual fear memory are modulated by various treatments and conditions (Figure 7). Furthermore, the effect of running on the HPC dependency of memory is clearly cancelled by X-ray irradiation. Although these manipulations do not exclusively modulate the levels of hippocampal neurogenesis (Figures S21 and S23), these four independent lines of experiments, in which the levels of neurogenesis were modulated differentially, inhibiting or facilitating neurogenesis, strongly suggest a causal relationship between adult neurogenesis and the HPC-dependent period of associative fear memory. However, we did not observe the complete block of the decay progress of HPC-dependent period of memory (Figure 2, 4, and 6). This may be the result of a failure to completely ablate hippocampal neurogenesis by either physical or genetic treatments. Alternatively, hippocampal neurogenesis may play a role as a modulator, but not master regulator, of HPC dependence. In either case, our findings provide a framework for understanding the mechanism of the gradual decay of HPC dependency during cortical permanent memory formation.

Previous studies demonstrate a gradual decay of LTP in pp-DG (Castro et al., 1989; Villarreal et al., 2002). This is an active process because daily administration of an NMDA receptor antagonist after the induction of LTP blocks LTP decay (Villarreal
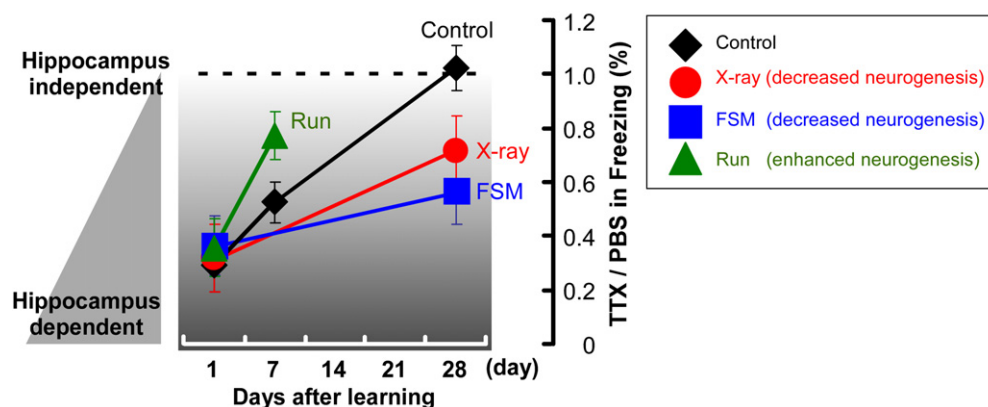
Summary

Figure 7. Summary of Experimental Results
The vertical axis shows the TTX/PBS ratio in the freezing response of each retrieval test, which indicates the HPC dependence of contextual fear memory. The horizontal axis shows the days after learning. The HPC dependence of contextual fear memory gradually decays with time in control mice. In contrast, X-ray irradiation or genetic overexpression of follistatin, both of which severely impair hippocampal neurogenesis, prolong the HPC-dependent periods of contextual fear memory, when compared to control mice. Conversely, the running wheel exercises, which promote hippocampal neurogenesis, speed up the decay rate of the HPC dependency of contextual fear memory, when compared to control mice. Error bars indicate SEM.

et al., 2002). The question of the mechanism of DG LTP decay arises from these studies. Our results clearly show that hippocampal neurogenesis, particularly the integration of new neurons, is a key factor in the gradual decay of DG LTP. Exposure of animals to an environmental enrichment accelerates the decay of LTP in DG (Abraham et al., 2002), at the same time enhancing hippocampal neurogenesis (Kempermann et al., 1997). Thus, our results suggest that enhanced neurogenesis caused by the environmental enrichment mediates the accelerated decay of DG LTP. A recent study using electron microscopic examination suggests that synaptic competition between old and new neurons occurs when newborn neurons form synaptic connections with pre-existing boutons in the DG (Toni et al., 2007). Specifically, newly generated neurons transiently (2∼6 weeks of the cell age) have the enhanced synaptic plasticity (Ge et al., 2007; Schmidt-Hieber et al., 2004), suggesting that new neurons may also transiently have strong ability to deprive pre-existing synapses of the presynaptic boutons. Therefore, the synaptic integration of new neurons into pre-existing neuronal circuits may actively interfere with LTP persistence, thus leading to a gradual decay in DG LTP.

There is a strong correlation between LTP and learning and memory. Physiological, pharmacological and genetic interventions that alter or occlude LTP are accompanied by impairments in learning and memory maintenance (Morris et al., 2003; Pastalkova et al., 2006). Furthermore, HPC-dependent learning induces LTP in the HPC (Whitlock et al., 2006). Our findings suggest that learning-induced LTP in the HPC may be gradually reversed by hippocampal neurogenesis, similar to tetanus-induced LTP. This notion implies that the hippocampal memory trace is potentially lost by interference brought on by neurogenesis. This body of evidence leads us to predict that the gradual decay of the HPC dependency of memory reflects the gradual erasure of the hippocampal memory trace mediated by hippo-

campal neurogenesis. Nevertheless, in the present stage, we cannot exclude the possibility that hippocampal neurogenesis contributes to the regulation of retrieval of HPC-dependent memory, because it is not clear to what extent the memory trace and its retrieval are coupled.

We showed that the running exercise speeds up the decay rate of the HPC dependency of memory, without the loss of memory expression (Figure 5). This result implies that the recall of the memory may depend on extra-HPC components acting in concert with the decay of HPC dependency; otherwise, the memory would simply be lost. This interpretation leads us to predict the possibility that there is a coupling mechanism between the decay of HPC dependence and the increase in neocortex dependence over time.

Mice tested for remote tone fear memory showed context generalization (Figure 3), which is proposed to reflect the transfer of hippocampus-dependent memories to the cortex (Wiltgen and Silva, 2007). However, we observed comparable levels of context generalization in both non-irradiated and irradiated mice. This result does not necessarily deny the proposed relationship between the hippocampus dependency and the levels of context generalization. Our result may simply indicate that under the experimental conditions used here for remote tone fear conditioning (such as context, footshock, etc.) irradiated mice showed a comparable level of context generalization. These mice might exhibit reduced context generalization under the different conditions. Another possibility is that shift of brain region that the memory depends on is not properly regulated in irradiated mice (for example, "slow decay of hippocampus-dependency and normal increment of cortex dependency," rather than "slow decay of hippocampus dependency and complementarily slow increment of cortex dependency").

Taken together, new neurons in the hippocampal DG may play multiple roles in learning and memory system, a contribution to

the formation of new memory (Dupret et al., 2008; Imayoshi et al., 2008; Kee et al., 2007; Saxe et al., 2006; Shors et al., 2001; Winocur et al., 2006; Zhang et al., 2008; Zhao et al., 2008), and the decay process of HPC dependency of memory over time (this study). Finally, the gradual decay in the HPC-dependency of memory is proposed to be important for maintaining learning capacity in the HPC (Willshaw and Buckingham, 1990). The gradual decay is also proposed to relate to the transformation of the memory from a precise (or detailed and contextually rich) form to a less precise (or generic and context-free) form (Wiltgen and Silva, 2007). We are now able to examine these hypotheses by modulating the progress of the HPC-dependency decay via the regulation of neurogenesis.

EXPERIMENTAL PROCEDURES

Animals
All procedures involving the use of animals were conducted in compliance with the guidelines of the National Institutes of Health and were approved by the Animal Care and Use Committee of the Mitsubishi Kagaku Institute of Life Sciences. See detailed description of experimental animals as well as productions of the transgenic mouse in the Supplemental Data.

Dentate Gyrus LTP in Unanaesthetized Freely Moving Rats
Measurement of DG LTP in unanaesthetized freely moving rats has been described previously (Fukazawa et al., 2003; Kato et al., 1997; Matsuo et al., 2000). For a detailed description, see the Supplemental Data.

X-Ray Irradiation
Five-week-old mice or electrode-implanted rats of approximately 20–25 weeks of age were anesthetized with pentobarbital (50 mg/kg of body weight for mice and 35 mg/kg of body weight for rats, i.p.) and the fully anesthetized animals were positioned in the X-ray irradiation apparatus (MBR-1505R, HITACHI) and irradiated at 150 kV and 5 mA. For a detailed description, see the Supplemental Data.

Cannulation and Drug Infusion
Mice were anesthetized with pentobarbital solution (80 mg/kg of body weight, i.p.) and the fully anesthetized mice were positioned in a stereotactic frame. Mice were implanted bilaterally with stainless-steel guide cannulae (Eicom) using the following stereotactic coordinates: AP = −2.0 mm, ML = ± 1.7 mm, V = −2.0 mm from the bregma. To transiently inactivate the HPC activity, we used TTX and CNQX, or fluorescently labeled γ-aminobutyric acid subtype A receptor agonist (FCM, fluorophore conjugated muscimol). Thirty minutes prior to the memory retrieval test, mice received infusions of 0.5 μl of a mixture of TTX (20 μM, Wako) and CNQX (3 mM, Tocris), or FCM (0.8 mM, Molecular Probe) or PBS alone, at a rate of 0.2 μl/min. For a detailed description, see the Supplemental Data.

Behavioral Experiments
All behavioral experiments including the contextual fear conditioning, tone fear conditioning, open field tests, light and dark test, elevated plus maze test, and sensitivity to electrical stimulation test were conducted during the light cycle in a dedicated soundproof behavioral room. See detailed description in the Supplemental Data.

Running Wheel
See detailed description of the housing of running wheel exercises in the Supplemental Data.

BrdU Labeling and Quantification
Procedures to quantify hippocampal cell proliferation and neurogenesis have been described previously (Ageta et al., 2008; Kitamura et al., 2003). See detailed description of the immunohistochemostry, BrdU-labeling and quantication in the Supplemental Data.

Statistical Analyses
All data are presented as mean ± SEM. The number of animals used is indicated by "n." Comparisons between two-group data were analyzed by unpaired Student's t test or paired t-test. If the data did not meet the assumptions of the t test, the data were analyzed using the non-parametric Mann-Whitney U-test. Multiple group comparisons were assessed using a one-way, two-way, or repeated-measures analysis of variance (ANOVA), followed by the post-hoc Scheffe's test when significant main effects or interactions were detected. The null hypothesis was rejected at the p < 0.05 level.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and 27 figures and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)01309-9.

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