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

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DOI 10.1016/j.cell.2009.10.020

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; Wittgen 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

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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 unanesthetized 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 unanesthetized 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, $t_{6} = 6.38, p < 0.001$; 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 significant LTP decay 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: $F_{(2, 72)} = 3.82, p < 0.02$). 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 time point (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 (ZIP26/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 ($t_{6} = 10.5, p < 0.0001$) 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, C57BL/6J) 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 days after 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).

Cell 139, 814–827, November 13, 2009 ©2009 Elsevier Inc.
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 TTXSham/Average of PBSSham or Individual TTXX-Ray/Average of PBSX-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_{20} = −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 mouse (C57BL6J/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 stimulation, an associative learning between a tone and electrical stimulation (Allen et al., 2008), we analyzed the relationship between the freezing response, describe above.

(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 hoc indicates 15 min 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) (Wilgen 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 < 0.01; 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 suppression of hippocampal neurogenesis prolongs the HPC-dependent period of contextual fear memory, as seen previously after physical inhibition (X-ray) of neurogenesis. We employed transgenic mice that overexpress follistatin in a forebrain-specific manner (FSM mice) (Agata et al., 2008). FSM mice have a severe deficit in hippocampal neurogenesis (t = 4.06, p < 0.01) (Figures 4B–4D), caused by severe impairment in the survival of newly born neurons (Agata 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 conditioning (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).

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.
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 average 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 % time in the center 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.
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 non-irradiated non-runner control mice (p < 0.01, ANOVA, post-hoc Scheffe’s test) (Figures 5I and 5J).
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.

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
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 hippocampal 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 immunohistochemistry, BrdU-labeling and quantification 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.

ACKNOWLEDGMENTS

We thank T. Hino, R. Migishima, and K. Nakamura from the Mouse Genome Technology Centre at Mitsubishi Kagaku Institute of Life Sciences (MITILS) for in vitro fertilization and embryo transfer; F. Hayashi for technical assistance with X-ray irradiation; R. Okubo-Suzuki for programming for the data analysis; and S. Kamijo and M. Matsuo for maintenance of the transgenic mice. We also thank N. Ohkawa for reading of the manuscript and discussion, and all members of the Inokuchi laboratory for daily discussion and advice; and P.W. Frankland, B.-K. Kaang, and A.J. Silva for critically reading the manuscript. This work was supported by the Core Research for Evolutional Science and Technology (CREST) program of Japan Science and Technology Agency (JST) to K.I., a Grant-in-Aid for Scientific Research in Priority Area in “Molecular Brain Science” to K.I., and by a Grant-in-Aid for Young Scientists B to T.K. T.K. was a fellow of the Japan Society for the Promotion of Science.

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

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Supplemental 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. For WT experiments, male C57BL/6J mice 5 weeks of age were purchased from CLEA Japan Inc. For experiments using mutant mice (follistatin over expressing transgenic mice), we produced the progeny for each line using in vitro fertilization (IVF) and embryo transfer (ET) techniques, to produce a number of animals sufficient for behavioural testing. We carried out the tests using age- and gender-matched littermates. All behaviour experiments were conducted in a blind fashion. Food and water were provided ad libitum.

Dentate gyrus LTP in unanaesthetized freely moving rats. For LTP experiments we examined male Wistar ST rats approximately 20–30 weeks of age. We used a surgical procedure described previously, with a slight modification (Kato et al., 1997). Briefly, a bipolar stimulating electrode and a monopolar recording electrode made of tungsten wire were positioned stereotaxically so as to selectively stimulate medial perforant pathways (MPP) and projections, while recording in the DG. The electrode stimulating the MPP fibers was positioned 8.7 mm posterior, 5.3 mm lateral and 5.3 mm inferior to the bregma. A recording electrode was implanted ipsilaterally 4.0 mm posterior, 2.5 mm lateral and 3.8 mm inferior to the bregma. Rats were allowed to recover for at least 2 weeks in individual home cages. Following recovery, input/output (I/O) curves reflecting evoked field EPSP (excitatory postsynaptic potential, fEPSP) as a function of current intensity (0.1–1.0 mA) were collected for each animal. We used current intensities that evoked 60% of the maximum population spike amplitude for all stimulation, and kept them constant throughout the experiment. Test stimuli were delivered at 20 sec intervals to record the fEPSP. LTP experiments on freely moving animals were performed as described previously, with the following modifications (Matsuo et al., 2000) (Fukazawa et al., 2003): we used HFS (500), a strong tetanic stimulus
(biphasic square wave form, 200 μs pulse width). HFS (500) consisted of ten trains with 1 min inter-train intervals. Each train consisted of five bursts of ten pulses at 400 Hz, delivered at 1 sec interburst intervals. The initial EPSP slopes are shown as a percentage of the mean value obtained in the 15 min immediately prior to delivery of the HFS. For daily recordings, we measured the relative changes of the slope of fEPSP by averaging 45 evoked responses collected at 20-sec intervals.

**Dentate Gyrus LTP in Urethane-Anesthetized Animals and Drug Delivery.** Wistar ST rats at 20 weeks of age were irradiated, and 11 days later were used for the analysis of DG LTP under urethane-anesthetesia (Inokuchi et al., 1996). The stimulating electrode and the recording electrode were implanted unilaterally after the animal had been anesthetized by urethane (i.p., 1.0 g/kg body weight). All the stimuli were biphasic square wave pulses (200 μs width), and their intensities were set at the current that evoked 40% of the maximum population spike amplitude. After monitoring a stable basal transmission for at least 30 min, the solution (saline or CHX: Cycloheximide 90 ug) was injected into the lateral ventricule. This usually occurred 3-4 hr after the electrode implantation. LTP was induced by HFS (500) 15 min after the injection of solution. Test stimuli were delivered at 20 s intervals to monitor fEPSP. The body temperature of the animals was kept at 37°C throughout the LTP experiments by the Animal Blanket System MK-900 (Muromachi Kikai Co., Tokyo, Japan).

**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. Animals were exposed to the irradiation with a lead shield covering their entire body except the head (Figure S1). The corrected dose rate was approximately 0.35 Gy/min at a source-to-skin distance of 13 cm. The procedure lasted about 30 min, delivering a total of 10 Gy.

**Cannulation and Drug infusion.** Mice were anesthetized with pentobarbital solution (80 mg/kg of body weight, i.p.) and the fully anesthetized mice were set in a stereotactic frame. Vaseline was placed over the eyes of the animals to prevent them from drying. 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. Mice were allowed to recover for at least 10 days in individual home cages. To transiently inactivate hippocampus activity, we used tetrodotoxin (TTX, a sodium channel blocker) and 6-cyano-7-nitroquinoxaline (CNQX, a selective antagonist of AMPA receptors), or fluorescently labelled γ-aminobutyric acid subtype A receptor agonist (FCM, a fluorophore conjugated muscimol). Mice were
briefly anesthetized with isoflurane to facilitate insertion of the injection cannula (28-gauge). 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. The injection cannula was left in place for 2 min following the infusion.

**Contextual fear conditioning.** Mice were housed individually in plastic cages with laboratory bedding at least 2 weeks before behavioural analyses, and maintained on a 12:12 h light:dark cycle. Mice were handled daily for seven days before behavioural analyses, with the exception of cannulated animals. Training and testing were conducted during the light cycle in a dedicated soundproof behavioural room (Room A). The conditioning chamber had a plexiglass front and gray side- and back-walls (width × depth × height; 175 × 165 × 300 mm), and the chamber floors consisted of 26 stainless steel rods with a diameter of 2 mm diameter placed 5 mm apart. The rods were connected to a shock generator via a cable harness (Chamber A). All experiments were conducted and analyzed by scientists blind to the genotypes of the animals.

During the training phase, mice were placed in the conditioning chamber. After 3 min, animals were subjected to 1, 3 or 8 unsignalled footshocks (2-sec duration, 0.5 mA, 1 min apart; namely FS × 1, FS × 3 and FS × 8, respectively). After the last shock, mice remained in the chamber for 1 min, and were then returned to their home cages. During the testing phase, mice were placed back into conditioning chamber A for 3 min, unless otherwise noted.

At the end of each session, mice were returned to their home cages and the chambers were cleaned with 70% ethanol. All experiments were conducted using a video tracking system (Muromachi Kikai) to measure the activity and freezing behaviour of the animals. Freezing was defined as a complete absence of movement, except for respiration. We started scoring the duration of the freezing response after 1 sec of sustained freezing behaviour. For each testing session, freezing (%) was then averaged among mice within a particular treatment or genotype group. Activity suppression (AS) ratios were calculated by comparing activity levels during the first 3 min of training with activity levels during the first 3 min of testing, according to the formula: $\frac{\text{activity}_{\text{test}}}{\text{activity}_{\text{train}} + \text{activity}_{\text{test}}}$. Activity levels were estimated by cumulative area of movement (pixel size) per 0.1 sec in the first 3 min of training or testing.

**Tone fear conditioning (for Figure S10).** We used different chambers for each auditory fear conditioning training and testing session (chamber A in room A, chamber B in room B). Mice were placed in
conditioned chamber A for 2 min, and then received three pairings of tone (20 s, 80 dB, 4 kHz) and a co-terminating shock (2 s, 0.5 mA), with inter-trial intervals of 2 min. After the last shock, mice remained in the chamber for 1 min, and were then returned to their home cages. For tone testing, we used a white-coloured conditioning chamber forming a triangular enclosure (‘190 x 190 x 180’ x 400 mm) with a grid floor covered by paper towel (Chamber B) in soundproof behavioural Room B. Mice were put into the chamber B for 2 min and then received tone (120 s, 80 dB, 4 kHz). In retrieval test, freezing behaviour to the tone was monitored.

**Tone fear conditioning for remote memory test.** We used different chambers for each auditory fear conditioning training and testing session (chamber A in room A, chamber C in room C). Day 1 for training, mice were placed in conditioned chamber A for 2 min, and then received four pairings of tone (20 s, 80 dB, 4 kHz) and a co-terminating shock (2 s, 0.5 mA), with inter-trial intervals of 2 min. After the last shock, mice remained in the chamber for 1 min, and were then returned to their home cages. Day 2 for training, mice were placed back into conditioning chamber A for 4 min. Day 3 for training, mice were placed back into conditioning chamber A, and conditioned by same protocol as Day 1 training. For tone testing, we used a gray-coloured larger conditioning chamber (width x depth x height; 280 x 250 x 300 mm) with a floor covered by paper towel (Chamber C) in soundproof behavioural Room C. One day or 28 day after training, mice were put into the chamber C for 2 min and then received tone (120 s, 80 dB, 4 kHz). In retrieval test, freezing behaviour to the tone was monitored.

**Running wheel.** One 6-week-old mouse was placed in a cage (width x height x depth: 28 x 13 x 15 cm) equipped with a running wheel (diameter, 12 cm; width, 5 cm) for 6 weeks. To analyse the usage of the wheel, an infrared sensor and an electronic preset counter (Keyence) were used to count the number of wheel rotations, as previously described (Kitamura et al., 2003).

**Immunohistochemistry.** Animals were anesthetized with an overdose of pentobarbital solution and the fully anesthetized animals were perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Brains were stored in fixative (4% PFA in PBS) for 3 h at 4°C, and then incubated overnight in 30% sucrose, followed by immersion in dry ice powder. A cryostat was used to collect mouse brain sections of 14 μm thickness or rat brain sections of 20 μm thickness. Sections were blocked with 0.1% BSA or 3% goat serum in PBS containing 0.1% Tween-20 (PBS-T) at room temperature for 1 h. After blocking, tissues were incubated with blocking solution containing rabbit polyclonal anti-Zif 268 (1:250; Santa Cruz) antibody, rabbit polyclonal anti-c-fos (1:250; Santa Cruz) antibody, mouse monoclonal anti-glial fibrillary acidic protein (GFAP) (1:200; Chemicon)

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antibody, goat polyclonal anti-DCX (1:500; Santa Cruz) or rabbit polyclonal anti-Iba-1 (1:2000; Wako) antibody. Sections were then incubated with anti-rabbit IgG-AlexaFluor 546, anti-mouse IgG-AlexaFluor 488 or anti-goat IgG-AlexaFluor 546 secondary antibodies and counterstained with DAPI, propidium iodide or DRAQ5. For BrdU immunohistochemistry, sections were boiled for 10 min, treated with 2M HCl for 30 min, rinsed in 0.1 M boric acid (pH 8.5) for 10 min, and then blocked at room temperature for 1 h. After blocking, tissues were incubated with blocking solution containing rat monoclonal anti-BrdU (1:250; Accurate) and mouse monoclonal anti-NeuN (1:200; Chemicon) antibodies. Sections were then incubated with anti-rat IgG-FITC, anti-rat IgG-Cy3, anti-mouse IgG-AlexaFluor 488 or anti-mouse IgG-AlexaFluor 546 secondary antibodies and counterstained with DAPI, propidium iodide, or DRAQ5.

**BrdU labelling and quantification.** Procedures to quantitate hippocampal cell proliferation and neurogenesis were as described previously (Kitamura et al., 2003) (Ageta et al., 2008). Briefly, to analyze cell proliferation in mice, animals were injected with BrdU in 0.9% NaCl solution (100 mg/kg, i.p., Sigma), and were perfused 2 h later. For analysis of neurogenesis in mice, animals were injected with BrdU three times per day for three consecutive days, and were perfused 28 days later. For analysis of cell proliferation in aged rats, animals were injected with BrdU, and were perfused 2 h later. For analysis of neurogenesis in aged rats, animals were injected with BrdU twice a day for four consecutive days, and were perfused 28 days later. For quantification analysis of proliferation and neurogenesis in mice, sampling of BrdU-positive cells was conducted throughout the dentate gyrus in its rostro-caudal extension (Ageta et al., 2008). Three consecutive sections (14 µm each) out of a total of 13 sections were used for counting of BrdU⁺ cells and BrdU⁺NeuN⁺ cells (about 45 sections per mouse), using a ×40 objective (BX41, OLYMPUS), in a genotype- and treatment-blinded manner. The number of positives per a dentate gyrus was obtained by multiplying the value by 13/3. For quantification analysis of proliferation and neurogenesis in rats, sampling of BrdU-positive cells was conducted in dorsal dentate gyrus (bregma from -3.0mm to -5.0mm). Every other coronal sections (20 µm each) were used for counting of BrdU⁺ cells and BrdU⁺NeuN⁺ cells (totally 50 sections per rat), using a ×40 objective (BX41, OLYMPUS), in a treatment-blinded manner. In rat study, we used the counting number of BrdU⁺ cells and BrdU⁺NeuN⁺ cells.

**Authors’ Contributions** This study was conceived and designed by T.K. and K.I. Electrophysiological experiments were conducted by Y.S. Behavioural experiments were conducted by T.K., N.T. and A.M. Microinjection techniques were introduced into the laboratory by Y.N. Histological experiments were conducted by A.M. Transgenic mice were provided by H.A. The original concept of this study was
conceived by T.K., Y.S., H.A., H.S. and K.I. The manuscript was written by T.K and K.I. The entire project was supervised by K.I.

Supplemental References


Rat and mice were anesthetized using an intraperitoneal injection of pentobarbital solution, and the head of fully-anesthetized animals were irradiated in a Hitachi X-irradiation apparatus (MBR-1505R, HITACHI, Tokyo, Japan). Bodies were shielded with lead.
Figure S2.
Irradiation does not affect short-term synaptic plasticity in pp-DG synapses.

(A) Experimental design. Electrode-implanted 20-25 week-old rats were irradiated. Pre, 20 min before HFS; post, 20 min after HFS; PPD, paired-pulse depression. (B) Sample traces show the first and second fEPSP with paired-pulse inter-stimulus intervals of 30 msec. PPD of fEPSP were easily observed in both sham and irradiated rats. (C and D) fEPSP slope (C) and population spike amplitude (D) in response to paired-pulse stimulation were recorded at pre-HFS, post-HFS, 1d, 1w, 2w, 3w and 4w after HFS, in sham and irradiated rats (n = 7 per group). There were no differences in PPD between sham and irradiated rats for 4 weeks after irradiation. Repeated measures ANOVA revealed that there was no significant interaction between {treatment vs. delay} and the main effects. (Slope: treatment, \( F_{(1, 96)} = 3.13, P > 0.1 \), delay, \( F_{(6, 96)} = 1.54, P > 0.1 \); (Spike: treatment, \( F_{(1, 96)} = 0.64, P > 0.4 \), delay, \( F_{(6, 96)} = 0.58, P > 0.7 \).
Figure S3. Irradiation does not affect gene expression following LTP induction.

(A) Experimental design. Electrode-implanted 20-25 week-old rats were irradiated. (B and C) Immunohistochemistry of Zif268/Egr1 and c-Fos in hippocampus of sham and irradiated rats. The immunoreactivity of Zif268 and c-Fos was markedly increased by HFS in ipsi-lateral DG soma (LTP side), but not in contra-lateral DG soma (control side), irrespective of irradiation (n = 2 per group). (D) We examined phalloidin staining (a specific probe for F-actin) as an index of LTP, because LTP is accompanied by an upregulation of F-actin in spines, which is essential for LTP maintenance (Fukazawa et al., 2003). Phalloidin reactivity in the middle molecular layer of ipsilateral DG was increased by HFS, irrespective of irradiation (n = 2 per group). Scale bar represents 500 μm. DG, dentate gyrus; Mo, molecular layer.
The maintenance of L-LTP in irradiated rats is protein synthesis-dependent, as is that of non-irradiated rats.

The effect of the injection of cycloheximide (CHX) on the maintenance of L-LTP in non-irradiated and irradiated rats. The average of the fEPSP slopes obtained 15 min before HFS served as the baseline (100%). Graph shows the time-course of the relative changes in fEPSP slope following HFS in sham and irradiated animals. In non-irradiated rats, a high frequency stimulation (HFS500) produced a long-lasting L-LTP that persisted for 12 h (0Gy saline group, n=5). However, when the CHX, protein synthesis inhibitors, was pre-injected into the lateral ventricle, LTP induced by HFS gradually decayed and returned to basal levels by 9 h (0Gy CHX, n=5). Importantly, the decay time course in irradiated rats injected with CHX was similar to that of non-irradiated rats (10Gy CHX, n=3), indicating that the maintenance of L-LTP in irradiated rats is protein synthesis-dependent as that of non-irradiated rats. These results suggest that the maintenance of L-LTP of irradiated rats is normal.
Figure S5.
In the retrieval test of 1-day memory, the freezing response is specifically elicited by exposure to the fear conditioned context, but not by exposure to a different context.

(A) Experimental design. Mice at 10 weeks of age were subjected to contextual fear conditioning. During training, mice were placed in the conditioning chamber (context A). After 3 min they were subjected to 3 footshocks (2-sec duration, 0.5 mA, 1 min apart; namely FS x 3) or no footshock (no-training). After the last shock, mice were kept in the chamber for 1 min. In the retrieval test of 1-day memory, mice were placed back into the conditioning context (context A) or into a new context (context B) for 2 min. (B) The left-hand panel shows changes in the freezing response during the 2-min memory retrieval test, in which the time spent in the freezing phase was calculated. The right-hand panel shows the average freezing response during the 2-min memory retrieval test. (n = 5-7 per group). In retrieval test of 1-day memory, untrained mice showed no freezing response in the conditioning context (context A), whereas trained mice exhibited the freezing response in the conditioning context (context A) (post-hoc Scheffe's test, \( P < 0.001 \)). Importantly, trained mice showed a significantly lower freezing response in a different context (context B) than that in the conditioning context (context A) (post-hoc Scheffe's test, \( P < 0.005 \)). This result shows that the freezing response is specifically elicited by exposure to the conditioned context in the retrieval test of 1-day memory, and that the extent of freezing behaviour is suitable as an index of successful retrieval of associative contextual fear memory. **\( P < 0.01 \), ***\( P < 0.001 \). Context A consisted of Chamber A in Room A. Context B consisted of Chamber B in Room A (see the Experimental Procedures).
Figure S6.
Irradiation causes long-lasting inhibition of cell proliferation in mouse DG.

(A) Experimental design. Five-week-old mice were irradiated. At seven weeks or nine weeks after irradiation, mice were injected with BrdU, and animals were perfused 2 h later. (B) Seven weeks after irradiation, cell proliferation in hippocampal DG was still extremely reduced (n = 6 per group) (t_{10} = 9.27, P < 0.0001). (C) Nine weeks after irradiation, cell proliferation in DG was still significantly reduced (n = 4 to 6 per group) (t_{8} = 7.42, P < 0.0001).
Figure S7.
Irradiation does not affect the population of astrocytes in the hippocampus.

(A) Experimental design. Five week-old mice were irradiated. Mice were perfused 1 day, 5, 7 or 9 weeks after irradiation. (B-D) Immunohistochemistry of GFAP (glial fibrillary acidic protein; a marker of astrocytic glia) in hippocampus (CA1 area and dentate gyrus) of sham and irradiated mice. PI, propidium iodide (nuclear stain). There were no significant differences in the immunoreactivity of GFAP between sham and irradiated mice (n = 2 per group). (B), Hippocampal CA1 area. Apical, apical dendrite area; basal, basal dendrite area. (C), Hippocampal DG. Mo, molecular layer.
Figure S8.
Irradiation does not affect the retrieval of recent (1 day) and remote (28 day) memory of contextual fear conditioning.

(A) Experimental design. Five week-old mice (C57BL6J) were irradiated. During training, mice were placed in the conditioning chamber. After 3 min they were subjected to 1, 3 or 8 unsignalled footshocks (2-sec duration, 0.5 mA, 1 min apart; namely FS X 1, FS X 3 and FS X 8). After the last shock, mice remained in the chamber for 1 min. During testing, mice were placed back into the conditioning context for 6 min. (B-D) The two left-hand panels show changes in freezing response during the 6-min memory retrieval test, in which the time spent in the freezing phase was calculated. The two right-hand panels represent the average of freezing response during the 6-min memory retrieval test. The right-most panels show the AS ratio. (B) In the FS x1 conditioning protocol there were no significant differences in freezing response and AS ratio in 1-day and 28-day memory retrieval tests (n = 12 per group) (freezing recent, $t_{22} = 0.25$, $P > 0.8$; freezing remote, $t_{22} = 0.26$, $P > 0.8$) (AS recent, $t_{22} = -0.28$, $P > 0.2$; AS% remote, $t_{22} = 0.25$, $P > 0.8$). (C) In the FS x3 conditioning protocol there was a significant difference in freezing response, but not in AS, in 1-day and 28-day memory retrieval tests (n = 12 per group) (freezing recent, $t_{22} = 2.25$, $P < 0.05$; freezing remote, $t_{22} = 0.47$, $P > 0.6$) (AS% recent, $t_{22} = -0.40$, $P > 0.65$; AS% remote, $t_{22} = -0.37$, $P > 0.7$). (D) In the FS x8 conditioning protocol there were no significant differences in freezing response and AS in 1-day and 28-day memory retrieval tests (n = 9 to 10 per group) (freezing recent, $t_{17} = 0.30$, $P > 0.75$; freezing remote, $t_{17} = 0.57$, $P > 0.5$) (AS recent, $t_{17} = -1.70$, $P > 0.1$; AS remote, $t_{17} = -0.16$, $P > 0.8$). N.S., not significant.
Figure S9.
Irradiation does not affect the retrieval of the recent memory of contextual fear conditioning when weak conditioning is used.

(A) Experimental design. Five-week-old mice (C57BL6J) were irradiated. During training, mice were placed in the conditioning chamber. After 1 min they were subjected to a 1 footshock (2-sec duration, 0.5 mA). After a shock, mice were kept in the chamber for 30 sec. During testing, mice were placed back into the conditioning context for 3 min. (B) The left-hand panel shows changes in the freezing response during the 3-min memory retrieval test. The right-hand panel shows the average of the freezing responses during the 3-min memory retrieval test. There was no difference in the freezing response between no-irradiated and irradiated mice (n = 5 to 11 per group) (t_{14} = 0.26, P > 0.7).
Figure S10.
Irradiation does not affect the retrieval of recent memory of the tone and context associative fear conditioning.

(A) Experimental design. Five week-old mice (C57BL6J) were irradiated. During training, mice were placed in the conditioning chamber (Context A). After 2 min they were received three pairings of tone (20 s, 80 dB, 4 kHz) and a co-terminating shock (2 s, 0.5 mA), with inter-trial intervals of 2 min. After the last shock, mice remained in the chamber for 1 min. During test 1, mice were placed back into the conditioning context for 3 min. During test 2, mice were placed back into the another context (context B) for 2 min, and then received tone for 2 min (120 s, 80 dB, 4kHz). (B) The left-hand panel shows the average of the freezing responses during the 3-min memory retrieval test 1. The right-hand panel shows the average of the freezing responses during pre tone 2 min and post tone 2 min in memory retrieval test 2. There was no difference in the freezing response between no-irradiated and irradiated mice (n = 9 to 12 per group) (Test 1; $t_{19} = 0.19$, $P > 0.85$, Test 2 (pre tone); $t_{19} = -0.26$, $P > 0.79$, Test 2 (post tone); $t_{19} = 0.07$, $P > 0.94$). Context A consisted of Chamber A in Room A. Context B consisted of Chamber B in Room B (see the Experimental Procedures).
Figure S11.
Irradiation does not affect the retrieval of recent (1 day) and remote (28 day) memory of contextual fear conditioning in 129X1SvJ/C57B6J hybrid mice.

(A) Experimental design. Five weeks-old mice were irradiated. (B) Graphs show the changes in freezing (%) during 6min test, and the average of freezing (%) in 6min test. In FSx8 conditioning protocol, there were no difference in freezing (%) and AS (%) in 1-day and 28-day memory test (n = 3 to 13 per group) (freezing recent; \( t_{11} = 0.29, P > 0.75 \), freezing remote; \( t_{23} = 1.08, P > 0.2 \)) (AS% recent; \( t_{11} = 0.06, P > 0.9 \), AS% remote; \( t_{23} = -0.47, P > 0.6 \)).
Figure S12.
Irradiation has no effect on c-Fos expression in the hippocampal CA1, CA3 and DG areas following contextual fear conditioning.

(A) Immunohistochemical staining for c-Fos 30 mins after electroconvulsive seizure treatment (ECS). The Fos immuno-reactivity (IR) in the dentate gyrus clearly increased by ECS treatment. The ECS treatment also increased Fos-IR in CA1 and CA3 area as well as the entire cortical regions. (B) Immunohistochemistry for c-Fos 30 mins after the contextual fear conditioning (FSx3). (C-E) Graphs show the number of c-Fos positive cells per section in the dentate gyrus (C), CA3 (D) and CA1 area (E). In both groups, the number of c-Fos positive cells were increased by the conditioning. (n = 3 per group) (***P < 0.001. *P < 0.05; ANOVA, followed by post-hoc Scheffe's test) (HC: homecage, FC: fear conditioning)
Figure S13.
Irradiation extends the hippocampus-dependent period of memory in an alternative conditioning protocol (eight footshocks).

(A) Experimental design in recent (1-day) and remote (28-day) memory retrieval test. Five week-old mice (C57BL6J) were irradiated. (B) In 1-day memory retrieval test, intrahippocampal infusion of TTX 30 min prior to memory retrieval test significantly reduced the freezing response of non-irradiated and irradiated mice, compared with PBS-infused animals (n = 4 to 11 per group) (0 Gy, $t_{17} = 3.67, P < 0.002$; 10 Gy, $t_{7} = 5.10, P < 0.002$). (C) TTX-infusion 30 min prior to 28-day memory retrieval test significantly reduced the freezing response of irradiated mice, compared with PBS-infused animals (n = 10 per group) ($t_{18} = 2.77, P < 0.02$), but not of non-irradiated mice (n = 10 per group) ($t_{18} = -0.81, P > 0.4$). (D) Ratio of (TTX/PBS) during the 1-day and 28-day memory retrieval tests, in sham and irradiated groups (1d; n = 4 to 11 per group, $t_{14} = 0.02, P > 0.9$, 28d; n = 10 per groups, $t_{18} = 3.40, P < 0.004$). (E and F), AS in the recent and remote memory retrieval tests in each group (recent; n = 4 to 11, remote; n = 10 per group). *$P < 0.05$, **$P < 0.01$. 

*Figures and graphs show the results of statistical analyses performed using Microsoft Excel. 

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Figure S14.
Irradiation extends hippocampus-dependent period of memory in an hybrid mouse strain (129X1SvJ/C57B6J hybrid mice).

(A) Experimental design. Five week-old mice (129X1SvJ/C57B6J hybrid mice) were irradiated. (B and C) In 28-day memory retrieval test (test 1), there were no differences in freezing response and AS between non-irradiated and irradiated mice (n = 5 to 6 per group) (freezing; $t_9 = 0.26, P > 0.7$, AS; $t_9 = -0.07, P > 0.9$). In 84-day memory retrieval test (test 2), TTX-infusion into hippocampus 30 min prior to the test significantly reduced the freezing response of irradiated mice, compared with the non-irradiated TTX-infused group (n = 5 to 6 per group) ($t_9 = 2.67, P < 0.03$). Similarly, in 84-day memory retrieval test, TTX-infusion also enhanced the AS of irradiated mice, compared with the non-irradiated TTX-infused group (AS; $t_9 = -2.3, P < 0.05$). *$P < 0.05$. 
Figure S15.
Distribution of fluorophore-conjugated muscimol (FCM) after injection into the dorsal hippocampus.

FCM was infused in the hippocampus, and 30 min later brain was removed. Photographs of the fifteen brain section from anterior to posterior are shown. FCM is clearly infused into the dorsal hippocampus as well as the adjacent cortical areas.
Figure S16. FCM infusion into the hippocampus disrupts the retrieval of 28-day memory in irradiated mice.

(A) Experimental design of recent (1-day) and remote (28-day) memory retrieval test. Five-week-old mice (C57BL6J) were irradiated. (B) In 1-day memory retrieval test, intrahippocampal infusion of FCM 30 min prior to memory retrieval test significantly reduced the freezing response of non-irradiated mice, compared with PBS-infused animals (n = 4 to 5 per group) (t = 5.42, P < 0.001). The left-hand panel shows changes in the freezing response during the 3-min memory retrieval test and the right panel shows the average freezing response. (C) FCM-infusion 30 min prior to 28-day memory retrieval test significantly reduced the freezing response of irradiated mice, compared with FCM-infused non-irradiated mice (n = 10 per group) (t = 3.86, P < 0.002). The left-hand panel shows changes in the freezing response during the 3-min memory retrieval test and the right panel shows the average freezing response. (D) The photographs of FCM-infused brain sections from 0 Gy and 10 Gy irradiated mice, with the individual’s score for average freezing response indicated at the lower right. **P < 0.01, ***P < 0.001.
Figure S17.
FCM infusion into the cortex adjacent to the HPC did not disrupt the retrieval of 28-day memory in irradiated mice.

(A) Experimental design of remote (28-day) memory retrieval test. Five week-old mice (C57BL6J) were irradiated. (B) Photographs of brain section showing the FCM-infused cortical area. (C) FCM-infusion 30 min prior to 28-day memory retrieval test did not affect the freezing response of irradiated mice, compared with FCM-infused non-irradiated mice (n = 5 per group) (t₀ = 0.53, P > 0.6). The left-hand panel shows changes in the freezing response and the right panel shows average freezing response for the 3-min memory retrieval test.
Figure S18.
Fear generalization increases over time.

(A) Experimental design. Mice at 10 weeks of age were subjected to contextual fear conditioning (FS x3) and tested 1 day or 28 days later in the training context (context A) or a novel context (Context B). (B, C) Changes in the freezing response during the 3-min memory retrieval test (B), and average freezing response during the 3-min memory retrieval test (C) (n = 8 per group). In retrieval test of 1-day memory, mice showed higher freezing response in context A, when compared with context B (A vs B; n = 8 per group, \( t_{14} = -3.01, P < 0.01 \)). In retrieval test of 28-day memory, mice showed the same freezing response in both contexts (A vs B; n = 9 to 10 per group, \( t_{17} = 0.14, P > 0.8 \)). Context A consisted of Chamber A in Room A. Context B consisted of Chamber B in Room A (see the Experimental Procedures).
Figure S19.
Search for experimental conditions that are suitable to examine remote tone fear memory.

In our standard protocol, mice showed a high level of freezing response in the novel context in the retrieval test of 28-days memory that was comparable to that in training context (Figure S18). Therefore, to test 28-day tone fear memory, we searched for conditions in which mice show less freezing responses than in the standard remote memory test. (A) Experimental designs of a series of tone fear conditioning. Training was carried out in Chamber A in Room A. In order for mice to more readily discriminate the contexts, we used larger chamber (width x depth x height, 280 x 250 x 300 mm) (Chamber C) and different soundproof room (Room C) in the retrieval test to the tone fear. (B) Retrieval tests of 28-day memory for tone. We found that the training protocol Group 3 was suitable for the remote tone fear memory test (n = 8 per group), because freezing response in post-tone was significantly higher than pre-tone (P < 0.01, t-test). See Experimental Procedures for details. Protocol Group 3 was used to evaluate the effect of irradiation on remote tone fear memory (Figure 3).
Figure S20.
Irradiation has no effect on c-Fos expression in the somatosensory cortex, amygdala, and hypothalamus following the contextual fear conditioning.

Five week-old mice (C57BL6J) were irradiated and 5 weeks later we subjected irradiated and non-irradiated mice to contextual fear conditioning (FS X3). The brains were removed 30 min after conditioning and c-Fos expression was examined by immunohistochemistry. Fear conditioning dramatically increased the number of c-Fos positive cells, irrespective of irradiation treatment.
Figure S21.
Irradiation transiently caused the enhancement of microglial activity in the hippocampus.

(A) Experimental design. Five week-old mice were irradiated. Mice were perfused 1 day, 4 weeks or 9 weeks after irradiation. (B) Immunohistochemistry of Iba-1 (a marker for microglia: red) in the hippocampus. We observed the activated microglia (right panel) in mouse irradiated tissue 1 day after irradiation, but the Iba-1 staining is normal (left panel) in non-irradiated tissue. DRAQ5 (blue) as nuclear stain. Scale bars, 2 μm. (C) Iba-1 immuno-reactivities dramatically increased in the dentate gyrus 1 day after irradiation, but it returned to the initial level 4 weeks later. This indicates that irradiation transiently enhanced microglial activities in the hippocampus 1 day after irradiation, but 4 weeks later the enhancement was suppressed. Scale bars, 200 μm. (D) Higher magnification micrographs in the dentate gyrus of non-irradiated and irradiated tissue 1 day after irradiation. Scale bars, 20 μm. There were Iba-1 immunoreactivities like phagocytosis. (E) Four examples of the Iba-1 immunoreactivity like phagocytosis. The Iba-1 immunosignal enfolds several condensed nuclei in the dentate gyrus. Scale bars, 2 μm.
Figure S22. Microglial activities in the hippocampus of FSM mice and running mice.

(A) Immunohistochemistry for Iba-1 (red) of FSM mouse, running mouse and the age-matched control (10 weeks old) (Blue; nuclei). Scale bars, 200 μm. (B) Magnified photographs in the dentate gyrus of FSM mouse, running mouse. There were no differences in Iba-1 immunoreactivities between three groups. Scale bars, 20 μm.
Figure S23.
FSM mice have smaller dentate gyrus than that of littermate WT mice.

(A, B) A series of photographs of dentate gyrus from anterior to posterior in WT (A) and FSM (B) mouse.  
(C) Estimated volumes of dentate gyrus of WT and FSM mice (n = 4 to 6 per group) (t₈ = 3.59, P < 0.01). To evaluate the total volume of dentate gyrus, the 20-21 sections, 84 μm apart, containing dentate gyrus, were obtained. The dentate gyrus was stained with DAPI, and the area size of the dentate gyrus was stereologically evaluated (Adobe Photoshop program). The total volume of the dentate gyrus was calculated from the dentate area size and the thickness of the sections.
Figure S24.
FSM mice showed prolonged hippocampus-dependent period of memory as indicated by the activity suppression ratio.

(A) Experimental design in recent (1-day) and remote (28-day) memory retrieval test. (B) In 1-day memory retrieval test, intrahippocampal infusion of TTX 30 min prior to memory retrieval test significantly enhanced the suppression ratio of WT and FSM mice, compared with PBS-infused groups (n = 3 to 4 per group) (WT, $t_5 = -5.15, P < 0.001$; FSM, $t_7 = -2.01, P = 0.09$). (C) TTX-infusion 30 min prior to 28-day memory retrieval test significantly enhanced the suppression ratio of FSM mice, compared with PBS-infused animals (n = 10 per group) ($t_{18} = -5.63, P < 0.001$), but not of WT mice (n = 7 to 8 per group) ($t_{13} = -1.22, P > 0.2$). #P = 0.09, ***P < 0.001.
Figure S25.
Retrieval of 1-day memory in runner mice is hippocampus-dependent.

(A) Experimental design. We subjected 6 week-old mice to running wheel exercise regimen. After 5 weeks of running, we subjected running mice to contextual fear conditioning. One day later we performed a 1-day memory retrieval test. (B and C) In 1-day memory retrieval test, intrahippocampal infusion of TTX 30 min prior to the test significantly reduced the freezing response of runner mice, compared with the PBS-infused animals (n = 5 per group) (t8 = 4.27, P < 0.003). In addition TTX-infusion significantly enhanced the AS in runner mice (n = 5 per group) (t8 = -3.13, P < 0.02). **P < 0.01, *P < 0.05.
Figure S26.
Enhancement of neurogenesis shortens the hippocampus-dependent period of memory as indicated by the activity suppression ratio.

TTX-infusion 30 min prior to a 7-day memory retrieval test significantly enhanced the suppression ratio of control, non-irradiated running and irradiated running groups, compared with PBS-infused animals (n = 10 per group) (P < 0.001, ANOVA, post-hoc Scheffe’s test). Furthermore, there was a significant difference in the suppression ratio between control and running groups of TTX-infused mice (P < 0.001, ANOVA, post-hoc Scheffe’s test). ***P < 0.001. **P < 0.01.
Figure S27.
Integration of new neurons after learning is important for the gradual decay of hippocampus-dependency of memory, as indicated by the activity suppression ratio.

(A) Experimental design in remote (28-day) memory retrieval test. Eight week-old mice (C57BL6J) were irradiated. (B) TTX-infusion 30 min prior to 28-day memory retrieval test significantly enhanced the suppression ratio of irradiated mice, compared with PBS-infused animals (n = 10 per group) (P < 0.001, ANOVA, post-hoc Scheffe's test), but not of non-irradiated mice (n = 10 per group) (P > 0.9, ANOVA, post-hoc Scheffe's test). ***P < 0.001.