

ber. Overall WBC counts, lymphocytes, and monocytes remained significantly associated with mating partner number ($n = 8$ contrasts, $P = 0.01$ to 0.04), whereas both neutrophils and body mass increased with transitions to greater terrestrial substrate use ($n = 5$ contrasts, $t = 2.87$, $P = 0.02$ and $t = 2.81$, $P = 0.02$, respectively). However, multivariate analysis of these variables using the CRUNCH algorithm (27) provided no significant results ($n = 23$ contrasts, $P = 0.09$ to 0.11). Finally, we tested whether the allometric relationship with neutrophils reflects an underlying life history correlate. In particular, a stronger immune system might be required in species with a longer life-span. However, longevity did not account for neutrophil counts when holding body mass constant in multiple regression [$b = -0.23$, $F(1,34) = 1.82$, $P = 0.19$]. No significant results were found in allometric analysis of other WBC types, although a negative slope for lymphocytes approached significance ($b = -0.11$, $F(1,38) = 3.66$, $P = 0.06$).

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19. In males, overall WBC counts were highly associated with female counts in contrasts analysis [$b = 0.64$, $F(1,38) = 24.07$, $P < 0.0001$], as were particular WBC types ($P < 0.05$ in all tests). This makes sense in the case of sexually transmitted disease: when one sex experiences increased risk, then the other sex should experience a corresponding increase (32). Male overall WBC ($t = 3.14$, $P = 0.007$), neutrophils ($t = 3.15$, $P = 0.007$), and lymphocytes ($t = 1.87$, $P < 0.05$) increased significantly over evolutionary transitions in female promiscuity (monocytes, $t = 1.61$, $P = 0.07$). Patterns of male promiscuity are difficult to analyze because there is less detailed information on male partner number, and, within species, greater variance in male mating success (i.e., sexual selection) may weaken patterns across species. Thus, analyses of discrete transitions to increased partner number in males produced significant results for neutrophils ($n = 4$ contrasts, $t = 2.66$, $P = 0.04$) when using the same set of species, as in analyses of females. But other analyses were not significant, including those using a wider range of species to give more contrasts. In multivariate contrasts analysis, however, residual testes mass accounted for variation in male WBC in excess of that explained by female WBC counts [$b = 0.08$, $F(1,20) = 4.86$, $P = 0.04$]. Although other explanations are possible, this relation is consistent with male mating promiscuity affecting basal WBC counts.
20. First, we compared human standard reference WBC counts (33) to values for nonhuman primates among the discrete mating categories. For overall WBC, neutrophils, and lymphocytes, ranges for humans closely matched equivalent ranges in monogamous species, while human monocyte values best matched ranges for the intermediate category of "1+ mates" (see Fig. 1 caption). Second, we used the midpoint of human reference values in a hierarchical cluster analysis of the apes. We found that humans align most closely with the gorilla (*Gorilla gorilla*), a polygynous species with low sperm competition (17), and secondarily with a monogamous gibbon (*Hylobates lar*).
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NMDA Receptor-Dependent Synaptic Reinforcement as a Crucial Process for Memory Consolidation

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The hippocampal CA1 region is crucial for converting new memories into long-term memories, a process believed to continue for week(s) after initial learning. By developing an inducible, reversible, and CA1-specific knockout technique, we could switch *N*-methyl-D-aspartate (NMDA) receptor function off or on in CA1 during the consolidation period. Our data indicate that memory consolidation depends on the reactivation of the NMDA receptor, possibly to reinforce site-specific synaptic modifications to consolidate memory traces. Such a synaptic reinforcement process may also serve as a cellular means by which the new memory is transferred from the hippocampus to the cortex for permanent storage.

The hippocampus is critical for converting short-term memories into long-term memories (1–7). The NMDA receptor in the CA1 region serves as a gating switch for the modification of major forms of synaptic plasticity (8–13) and is required for certain types of learning (14–17). Despite both gain-of-function and loss-of-function genetic evidence linking the NMDA receptor to memory formation (18–20), its role in memory consolidation, which occurs over the days and weeks after initial learning, has not been well studied (5). The lack of coherent effort may be in part due to the general knowledge that activation of the NMDA receptor is required for induction, but not maintenance of synaptic plasticity. This has led to the popular belief that consolidation at the synaptic level is the result of molecular cascades initiated by a single long-term potentiation (LTP)-like event triggered during learning. However, this time scale of a single LTP-like molecular event (e.g., protein synthesis and gene expression) may not be adequate to account for the long-term memory consolidation process

that is known to continue many days and weeks after initial learning experience.

To examine the role of the NMDA receptor in long-term memory consolidation, we used the third-generation knockout technique [see supplementary Web material (21)] and generated the inducible, reversible, and CA1-specific NR1 knockout mice (iCA1-KO) (22, 23). Our overall strategy is to use both tTA (24–26) and Cre/loxP system (27) to achieve CA1-specific, tetracycline-regulated expression of the NR1-GFP transgene (28), thereby restoring the CA1 NMDA receptor function in the CA1-specific NR1 knockout mice (14, 16). However, feeding the iCA1-KO mice with drinking water containing doxycycline (doxy), a tetracycline analog with higher permeability through the blood-brain barrier, will switch off NR1-GFP transgene expression and return the mice to the NR1 knockout state in the CA1 region. Furthermore, removal of doxy from the water restores NR1-GFP expression in the CA1 region. Using a green fluorescent protein (GFP)-specific antibody (29), we found that the level of NR1-GFP protein was mostly restricted to the CA1 region of untreated iCA1-KO mice (Fig. 1, A and B), whereas the doxy treatment (1 mg/ml) suppressed NR1-GFP expression in the CA1

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region within 3 to 5 days (Fig. 1, C and D). This duration may indicate the possible turnover rate for NMDA receptor complex in the brain. NR1-GFP started to reappear within 5 days after the withdrawal of doxy from the drinking water.

We also measured the CA1 field excitatory postsynaptic potentials (EPSPs) in hippocampal slices prepared from mice treated with doxy or vehicle (30) and observed no obvious change in α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated synaptic transmission between control littermates and iCA1-KO mice (either doxy-treated or vehicle-treated, $n = 4$ mice each group). Moreover, we found that NMDA receptor-mediated EPSPs were indistinguishable between those of controls or vehicle-treated iCA1-KO mice, suggesting the functional rescue of NMDA receptor activity in CA1. However, upon the treatment of doxy, CA1-NMDA currents in iCA1-KO mice were completely abolished in most slices (five out of seven slices, seven mice) (Fig. 1E, top panels), although in two slices we could still elicit NMDA currents at high intensities of stimulation. This indicates the presence of residual NMDA receptors at a few CA1 synapses despite the fact that we could no longer see NR1-GFP staining in the CA1 region.

To study LTP in the hippocampal slices, we applied two 1-s trains (100 Hz, test strength, spaced 10 s apart) in Schaffer-collateral CA1 pathway. LTP was readily produced in both vehicle-treated iCA1-KO and control littermates ($210.0 \pm 19.2\%$, $n = 5$ control mice; $227.6 \pm 16.3\%$, $n = 5$ iCA1-KO mice). However, no LTP was observed in doxy-treated iCA1-KO mice ($n = 5$; $105.1 \pm 6.9\%$) (Fig. 1, E, bottom panels, and F), suggesting the loss of NMDA-dependent plasticity after doxy treatment.

To examine the acute effect of switching-off CA1 NMDA receptors on learning, we first used a hidden-platform water maze (31). We tested three groups of young adult animals: iCA1-KO mice treated with vehicle, iCA1-KO mice treated with doxy, and control mice treated with doxy (1 mg/ml in the drinking water, starting 5 days before the training). We found that doxy-treated iCA1-KO mice exhibited longer escape latency than doxy-treated control or vehicle-treated iCA1-KO mice [$F(2, 38) = 17.88$, $P < 0.001$] (Fig. 2A). The deficits in doxy-treated iCA1-KO mice were further confirmed in the transfer test (Fig. 2B), indicating the requirement of CA1-NMDA plasticity for spatial learning and memory.

We then used a fear-conditioning task (32) with the same doxy treatment paradigm (1 mg/ml, starting 5 days before the training began), and a retention test was carried out 24

hours after training (Fig. 2, C and D). In contextual conditioning, a hippocampal-dependent task (33, 34), vehicle-treated iCA1-KO mice exhibited similar freezing responses to those of control mice, indicating that the expression of NR1-GFP has also rescued the contextual memory deficits in CA1-KO mice. However, iCA1-KO mice treated with doxy showed significantly fewer freezing responses compared with those in either control or vehicle-treated iCA1-KO mice (Fig. 2C). To further assess the behavioral specificity in iCA1-KO mice, we conducted the hippocampal-independent, cued-fear conditioning. As expected from the CA1-specific genetic manipulation, we did not find any significant difference in freezing responses among these three groups (Fig. 2D). Our additional observations further suggested the behavioral specificity of iCA1-KO mice (35).

Memory formation consists of at least

three major distinct stages: acquisition, consolidation and storage, and retrieval (3–6, 36, 37). To examine whether reactivation of CA1-NMDA receptors is required for long-term memory consolidation or retrieval, we first examined the consolidation of long-term spatial memory by using the hidden-platform water-maze task. We trained both control and vehicle-treated iCA1-KO mice with a seven-session training protocol (31). Both control and iCA1-KO mice exhibited similar learning curves (Fig. 3A). After the seven-session training, both groups of mice were treated with doxy for the first week (1 mg/ml, posttraining days 1 to 7) and then with water from the second week (days 8 to 14) (Fig. 3A). Retention tests were carried out on the 15th day after training, and transfer tests were performed 24 hours later. A significant difference in the latency between doxy-treated control

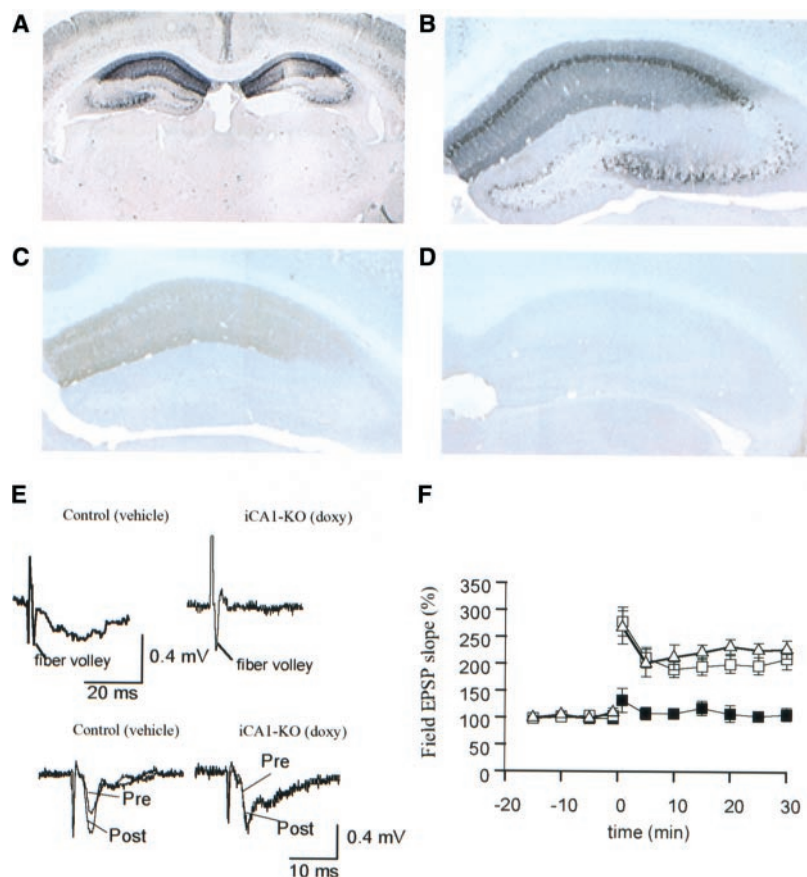
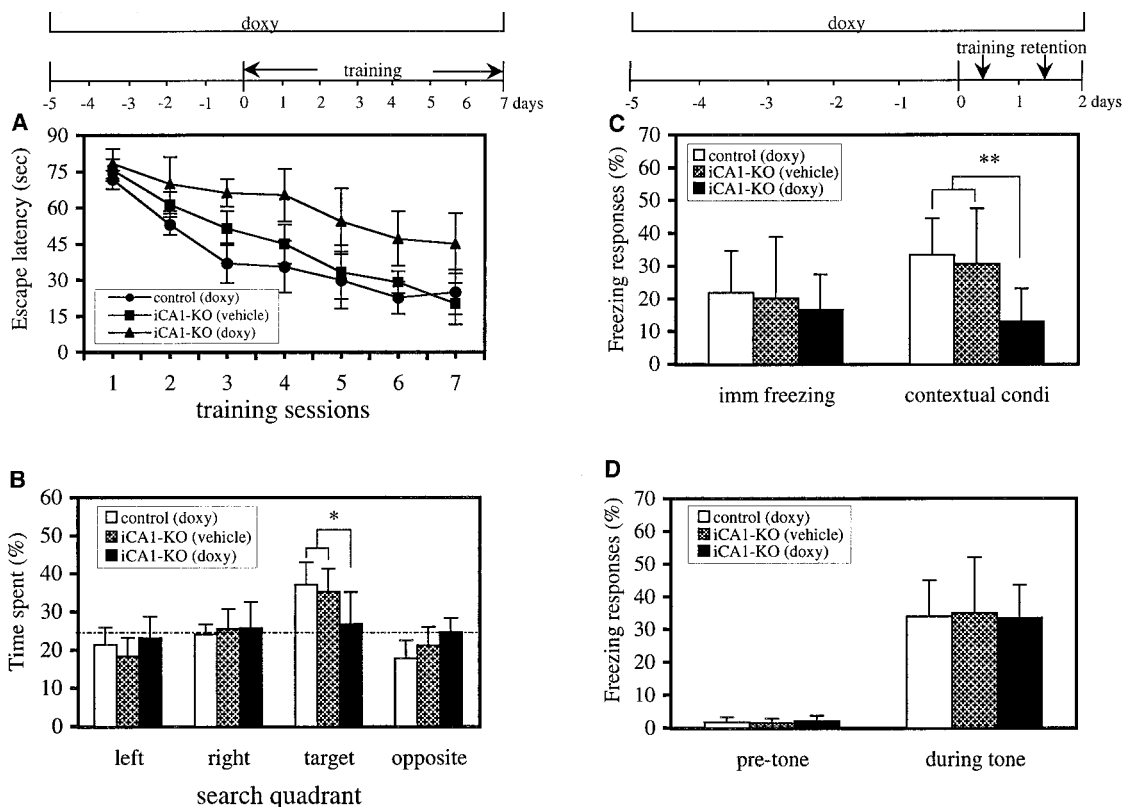


Fig. 1. Characterization of iCA1-KO mice: (A to D) expression of NR1-GFP fusion protein revealed by GFP immunohistochemistry. (A) iCA1-KO mice have high expression in CA1 in the absence of doxy (5 \times magnification). (B) At higher magnification (40 \times), high expression of NR1-GFP in dendrite, axons, and cell bodies of CA1 pyramidal cells in vehicle-treated iCA1-KO mice. (C) iCA1-KO mice treated with doxy (1 mg/ml) for 3 days, showing only residual NR1-GFP in CA1. (D) iCA1-KO mice treated with doxy for 5 days, showing no detectable GFP signals. (E) Normal NMDA receptor-mediated EPSPs in the presence of the AMPA/kainate receptor antagonist CNQX in controls (not shown) or vehicle-treated iCA1-KO mice, but lack of CA1 NMDA currents in doxy-treated mice (top panels). Single EPSP traces before and after LTP induction in control or doxy-treated iCA1-KO (bottom panels). (F) Normal CA1-LTP (averaged) in hippocampal slices from vehicle-treated iCA1-KO (Δ , $n = 5$ mice) or control mice (\square , $n = 5$ mice), but no LTP could be induced in doxy-treated iCA1-KO mice (\blacksquare , $n = 5$ animals).

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Fig. 2. Loss of CA1-NMDA receptors before training impairs learning in iCA1-KO mice. **(A)** Escape latency (mean \pm SEM) in water-maze training in doxy-treated control ($n = 14$), vehicle-treated iCA1-KO ($n = 12$), and doxy-treated iCA1-KO ($n = 15$) mice. Repeated ANOVA analysis indicated a significant difference between control or vehicle-treated iCA1-KO mice and doxy-treated iCA1-KO mice. A posthoc test indicated the significant difference between doxy-treated control or vehicle-treated iCA1-KO mice and either doxy-treated control or vehicle-treated iCA1-KO mice ($P < 0.05$ between sessions 3 and 7). **(B)** Place preference in the transfer test conducted at the end of training session. Both doxy-treated control and vehicle-treated iCA1-KO mice spent more time in the target quadrant than other quadrants, compared with doxy-treated iCA1-KO mice. **(C)** Contextual conditioning (condi) in doxy-treated control ($n = 10$), vehicle-treated iCA1-KO ($n = 11$), and doxy-treated iCA1-KO ($n = 10$) mice. Although there is no significant difference in immediately freezing (imm freezing), a significantly lower freezing response was found in doxy-treated iCA1-KO mice, compared with either doxy-



treated control ($P < 0.01$, Student's t test) or vehicle-treated iCA1-KO ($P < 0.01$, Student's t test) mice. **(D)** Cued conditioning in the same groups of mice. No significant difference was found between any two groups of these animals.

and doxy-treated iCA1-KO mice was found in the retention test (Fig. 3A). This deficit was further confirmed by the transfer test (Fig. 3B), indicating that the reactivation of CA1 NMDA receptor-mediated synaptic plasticity week(s) after initial learning is required for the consolidation of short-term into long-term spatial memory.

To assess the role of NMDA receptor in memory retrieval and/or the late stages of consolidation, we trained another two groups of mice using the 7-day training as above. Control and vehicle-treated iCA1-KO mice exhibited similar learning curves, and there was no significant difference in the latency in finding the platform between the two groups (Fig. 3C). At the end of the 7 days of training, the mice were returned to their home cages, continuously treated with regular water for 9 more days, and then switched to doxy for the last 6 days (1 mg/ml, posttraining days 10 to 15). We found no significant difference in latency between control and doxy-treated iCA1-KO mice in the retention test conducted on posttraining day 15 (Fig. 3C). A transfer test on day 16 further confirmed the normal preference toward the target quadrant in iCA1-KO mice (Fig. 3D). Thus, our results suggest that the requirement of CA1-NMDA receptor for memory consolidation is time-

dependent and both the retrieval and the late-stage consolidation of long-term spatial memory do not require CA1-NMDA receptor activity.

We next examined whether the NMDA receptor is also involved in the consolidation of emotional memory using a fear-conditioning paradigm that is capable of producing long-lasting memories in a single training (within seconds). Previous studies have shown that lesions of the hippocampus at either 7 or 14 days but not 28 days after fear conditioning can still produce significant retrograde amnesia of contextual fear memory (38). This suggests that the hippocampus is important for the initial storage and consolidation of the contextual fear memory during the following weeks after learning but with the passage of time memories are transferred elsewhere, such as the cortex.

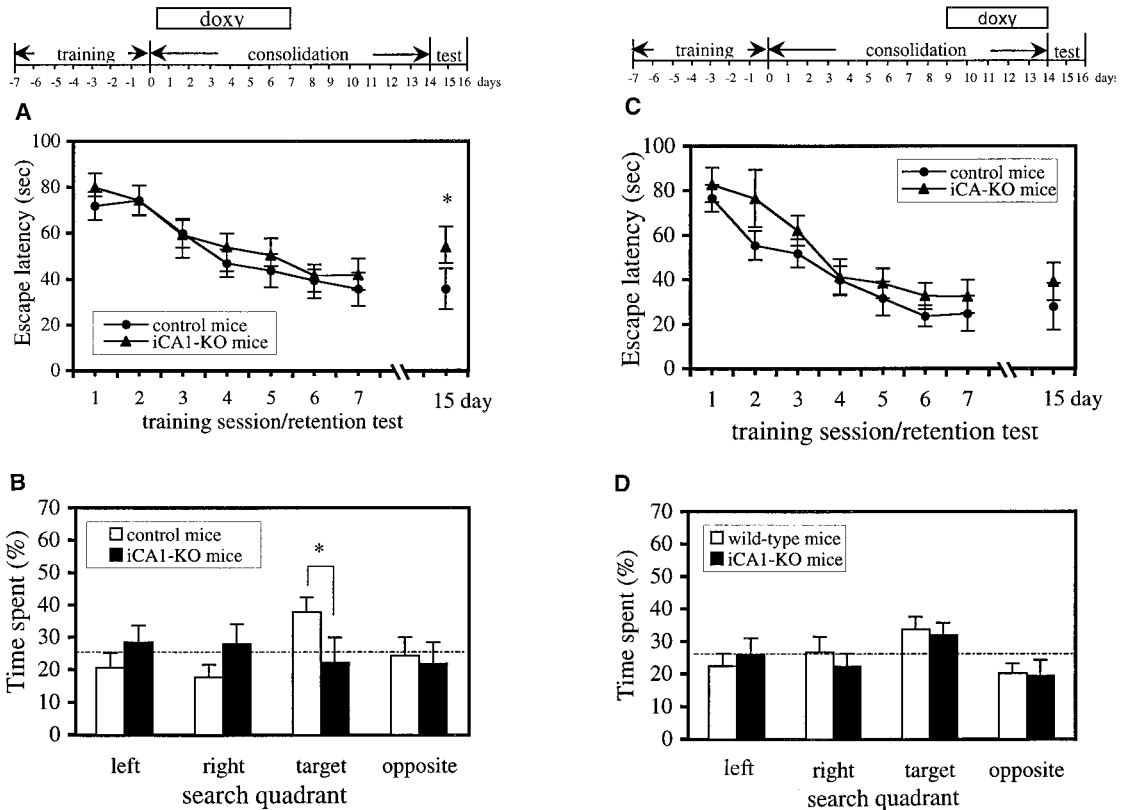
We used a 1-month retention fear-conditioning protocol (32). We found that iCA1-KO mice, receiving posttraining doxy treatment (1 mg/ml, days 1 to 14), exhibited a significant impairment at the 1-month retention test for the contextual memory (Fig. 4A) despite normal freezing in response to the tone (Fig. 4B). These results suggest that reactivation of CA1 NMDA receptor in the following days and week(s) after learning is

indeed crucial for the formation of the long-term contextual fear memory. We further found that treatment of doxy in the last 8 days (days 22 to 29) has no effects on either the contextual or cued retention tests (Fig. 4, C and D), suggesting that the CA1 NMDA receptors have a time-dependent role in memory consolidation but are not required for the retrieval of fear memory.

Because the CA1 NMDA receptor is not involved in basal synaptic transmission (11–13), the reactivation of the NMDA receptor in CA1 is likely to act as a triggering mechanism for modifying synaptic efficacy. Therefore, our above results indicate that memory consolidation may require multiple rounds of site-specific synaptic modifications, possibly to reinforce plastic changes initiated during learning, thereby making memory traces stronger and more stable. Recent studies report that the learning-induced correlation states among CA1 neurons are reactivated spontaneously in the postlearning period (37, 39, 40). Such a coactivation of these neurons might suggest the existence of the natural condition within the hippocampus by which the recurrent synaptic strengthening can occur during memory consolidation.

We hypothesize that such a synaptic reen-

Fig. 3. Memory consolidation and retrieval in the hidden-platform water maze. **(A)** Slower escape latency (mean \pm SEM) in doxy-treated iCA1-KO mice during the retention test. Both control ($n = 12$) and iCA1-KO ($n = 15$) mice received a 7-day training without doxy treatment. Animals learned equally well. After training, mice were fed with doxy for 7 days and then fed water for another 7 days. A retention test was carried out on posttraining day 14. A significant difference was found as measured by escape latency ($*P < 0.05$, Student's t test). **(B)** A transfer test carried out on day 15 confirmed the significant deficits in spatial memory in iCA1-KO mice. **(C)** Escape latency in retrieval test. Both control ($n = 13$) and iCA1-KO ($n = 14$) mice received regular water during the 7-day training, and this continued for up to 9 additional days after training. From day 10, the mice were treated with doxy for 6 days. A retention test was carried out on posttraining day 15. No significant difference was found during the training procedures or the retention test. **(D)** A transfer test carried out on posttraining day 16 confirmed the normal memory in both doxy-treated iCA1-KO and control mice.



try reinforcement (SRR) process can also be applied to explain how the hippocampus transfers newly created memories to the cortex for permanent storage. As the hippocampus undergoes reactivation during consolidation, it may also act as a coincidence regenerator for activating neurons in the cortical areas such as the association cortex. This would allow cortical neurons previously corresponding to the different sensory modules to be reactivated together, leading to the strengthening of the connections between them through SRR. Indeed, such a coordinated reactivation of hippocampal-cortical neurons after learning has been observed recently (41). Once these cortical connections are fully consolidated and stabilized, the hippocampus itself becomes dispensable for the retrieval of the "old memory" (3–6, 42). Therefore, we postulate that the hippocampus, by serving as a coincidence regenerator, may induce the reinforcement of synaptic connection within the cortex during memory consolidation as the cellular means to convert short-term memories into long-term memories.

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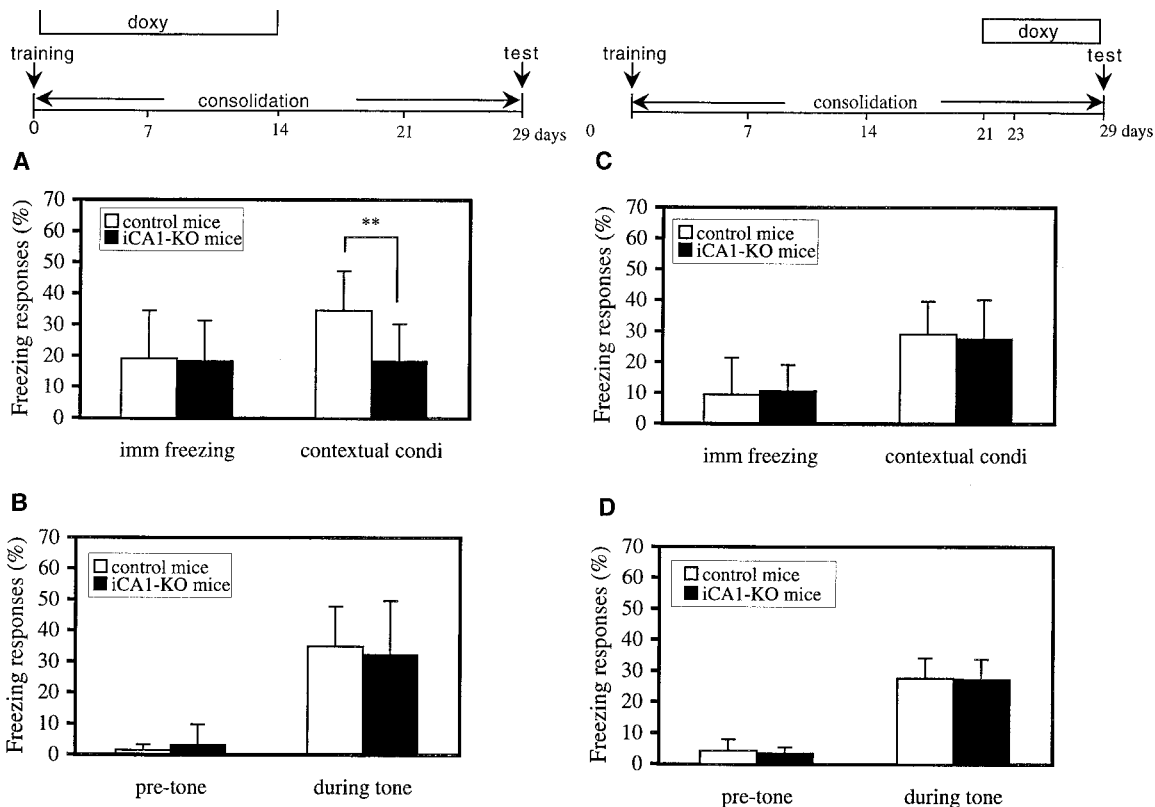
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21. Web fig. 1 is available at www.sciencemag.org/feature/data/1054469.shl.
22. Our design for the generation of inducible CA1 NR1 knockout mice was composed of three steps [see Web fig. 1 (21)]. First, we generated tTA transgenic lines in which the tTA transgene was driven by the β -actin promoter. The translation of tTA was prevented by an inserted translational "stop" sequence that is flanked by loxP sites (27). Second, we generated tetO-NR1 transgenic mouse lines in which the NR1-GFP transgene was under the control of the tetO minimal promoter. Third, we crossed each line of tTA and tetO-NR1 transgenic lines with the CA1-KO mice in a systematic manner. In the progeny, some of the mice will carry triple transgenes (Cre, tTA, and NR1 transgenes) and homozygous floxed NR1. These mice

were termed the inducible CA1 NR1 knockout mice, or simply iCA1-KO. In the iCA1-KO mice, expression of the tTA transgene in the CA1 region was achieved by the Cre/loxP-mediated deletion of the "stop" sequence (simultaneous recombination process used in the deletion of the floxed endogenous NR1 gene). Production of tTA transactivator subsequently switched on the expression of the NR1 transgene in the CA1 region. Therefore, expression of the NR1 transgene rescued the CA1-specific knockout of endogenous NR1 gene. However, feeding the mice with the drinking water containing doxycycline (doxy), a tetracycline analog with higher permeability through the blood-brain barrier, caused tTA to disassociate from the tetO promoter, thus switching off NR1 transgene expression and returning the mice to the NR1 knockout state in the CA1 region. Furthermore, removal of tetracycline from the drinking water restored NR1 expression in the CA1 region. To detect NR1 transgene expression, we used a previously reported NR1-GFP fusion transgene that retains both normal NMDA channel properties and GFP activity (28). Thus, the patterns and kinetics of NR1 transgene expression in the transgenic mice can also be assessed through the detection of GFP.

23. The transgenic founder mice were produced by pronuclear injection of the linearized DNA into B6/CBF1 inbred zygotes and then intercrossed with B6/CBF1 for various analyses. The tetracycline-controlled transactivator (tTA) construct was generated with the Eco RI-Bam HI tTA fragment from pUHD15-1 and the fragment after removing LacZ from pcAct-XstopX-LacZ plasmid. The tTA target construct containing NR1-GFP was obtained by cloning a 4-kilobase pair promoterless fragment downstream from the tTA responsive promoter (tetO) at the Xba I site of the pUHD10-1 vector. The NR1-GFP chimeric protein coding cDNA in pcDNA3 vector was generously provided by J. Marshall (28). We produced a total of seven independent tTA transgenic mouse lines and five NR1-GFP

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Fig. 4. Memory consolidation and retrieval in the fear-conditioning task. **(A)** Impaired long-term contextual fear memory in iCA1-KO mice if the CA1-NMDA receptor was suppressed during early consolidation. Regular water was administered to both control ($n = 13$) and iCA1-KO ($n = 11$) mice before training. Immediately after training, both groups received doxy treatment that continued for 2 weeks. On day 15, the doxy was stopped and switched to water for another 2 weeks. A retention test was carried out on day 29. A significant difference was observed between control and doxy-treated iCA1-KO mice (** $P < 0.01$, Student's t test). **(B)** Normal cued conditioning in the same group of both iCA1-KO and control mice [as described in (A)]. No significant difference was found in the long-term cued-fear memory between these two groups. **(C)** Retrieval and the late-stage consolidation of contextual fear memory are independent of CA1 NMDA receptor. Both control ($n = 12$) and iCA1-KO ($n = 13$) mice received regular water before training and after training until posttraining day 21; both



groups were then treated with doxy for 8 days. A retention test was carried out on day 29. No significant difference was found in either immediately freezing (imm freezing) or contextual conditioning. **(D)** Normal cued freezing response in the same group of mice [as described in (C)]. No significant difference was found in cued-fear memory between these two groups.

transgenic lines. After crossing each tTA line with an individual tetO-NR1/GFP line in a systematic and combinatorial manner [as illustrated in Web fig. 1 (21)], we obtained double-transgenic mice. These double-transgenic mice were then mated with our previously engineered CA1-KO mice that contained a heterozygous Cre transgene and the homozygous floxed NR1 genes (14, 16). After three generations of carefully planned crossings and subsequent histological analysis of each respective inducible knockout line, we obtained an iCA1-KO line (carrying tTA/+, NR1-GFP/+, Cre/+, fNR1/fNR1) with the expected expression pattern and properties. Littermates carrying fNR1/fNR1, tTA/+ or fNR1/fNR, NR1-GFP/+ were used as controls. For suppression of transgene expression, doxycycline (1 mg/ml) was added to the drinking water with 5% sucrose to mask the bitter taste.

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29. Animals were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Coronal brain sections were successively incubated in solutions of (i) rabbit antibody to GFP (1/2500, Clontech, and Chemicon) overnight, (ii) biotinylated goat antibody to rabbit (1/2000, Jackson ImmunoResearch Lab) for 90 min, and (iii) avidin-biotin-horseradish peroxidase complex (1/1000, Vector Elite Kit, Vectastain) for 90 min in PBST (0.1 M PB containing 0.9% NaCl and 0.3% Triton X-100). The GFP immunostaining was then revealed by immersing the sections in a solution of 3-3'-diaminobenzidine

(DAB substrate kit for peroxidase, Vectastain) containing H₂O₂ (0.003%) and nickel (0.6%). As a result, GFP-containing neurons were identified by the black coloration of their cell body and processes.

30. The recording procedures are the same as previously described (18), with the exception for the doxy-treatment schedule. Briefly, transverse slices of the hippocampus from iCA1-KO and control mice (3 to 4 months old) that were treated with doxy (1 mg/ml) or vehicle were rapidly prepared and maintained in an interface chamber. The slices prepared from doxy-treated mice were exposed to doxy (1 ng/ml) in the perfusate. A bipolar tungsten stimulating electrode was placed in the stratum radiatum in the CA1 region, and extracellular field potentials were recorded with a glass microelectrode (3 to 12 megaohms filled with artificial cerebral-spinal fluid) also in the stratum radiatum. Test responses were elicited at 0.02 Hz.
31. All the behavioral data presented in this report were collected in adult animals and collected at the ages of 7.5 to 14.5 weeks old in blind fashion. Analysis of variance (ANOVA) and Student's t test were used to determine genotype effects on the behavioral responses. The experimental protocol for the water maze was the same as described previously (18). The training procedures consisted of a total of seven sessions in 7 days; each session contained four trials per day.
32. The experimental protocol for fear conditioning was the same as that described previously (16, 18). The unconditional stimulus was a single foot shock 0.8 mA for 2 s, paired with an 86-dB sound at 2800 Hz in the shock chamber (context).
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35. We excluded the possibility that doxy has any side effect on these learning behaviors by measuring long-term treatment of doxy (1 mg/ml). After 1-month treatment, no obvious side effect on contextual or cued freezing responses in wild-type mice was found. Furthermore, we excluded the possibility that any changes in nociceptive responses might contribute to the difference observed in freezing responses because the amount of current to elicit flinching/running, jumping, and vocalizing was indistinguishable among all groups.
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