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Inducible and Reversible NR1 Knockout Reveals Crucial Role of the NMDA Receptor in Preserving Remote Memories in the Brain

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Summary

Long-term storage of information is a hallmark feature of the brain, yet routine turnover of synaptic receptors appears to be intrinsically paradoxical to this capability. To investigate how the brain preserves its delicate synaptic efficacies, we generated inducible and reversible knockout mice in which the NMDA receptor can be temporarily switched off in the forebrain specifically during the storage stage. Retention of 9-month contextual and cued fear memories is severely disrupted by prolonged, but not transient, loss of the NMDA receptor that occurs 6 months after initial training and at least 2 months prior to memory retrieval. Normal learning and memory function in subsequent tasks following the 9-month retention tests suggest that the observed retention deficits did not result from recall or performance impairment. Thus, our study reveals a hitherto unrecognized role of the NMDA receptor in dynamically maintaining the long-term synaptic stability of memory storage circuits in the brain.

Introduction

The long-term memory process can be divided into four distinct stages: learning, consolidation, storage, and retrieval. Over the past century, substantial progress has been made toward the molecular and systems understanding of the first two stages of the memory process, namely, learning and consolidation (Muller, 1900; Dudai, 1996; Lechner et al., 1999; Tsien, 2000b; Sara, 2000; McGaugh, 2000; Frey, 2001; Wittenberg et al., 2002). It is known that the hippocampus plays an important role in consolidating new memories into long-lasting ones (Scoville and Milner, 2000; Zola-Morgan et al., 1986; Rempel-Clower et al., 1996). Upon completion of hippocampal consolidation, those memories are believed to be transferred and stored somewhere in the cortex as "remote memories" (Zola-Morgan and Squire, 1990; Bontempi et al., 1999; Frankland et al., 2001; Debiec et al., 2002). However, the molecular and neural processes through which the stored remote memories are stably maintained have not been investigated.

At the molecular level, structural changes in synaptic connection have been postulated as the basis for long-term storage of memory traces. However, synaptic structures in the brain are not static, but rather subject to the routine metabolic turnovers of synaptic receptors and proteins (Shimizu et al., 2000). If not appropriately regulated, such dynamic turnovers would inevitably cause accumulative drift in synaptic efficacy, thus undermining the long-term storage of information in the brain. Therefore, it is important to investigate how the brain overcomes those destabilizing turnover effects in order to achieve the stable storage of memory over months, years, and decades.

The NMDA receptor serves as a cellular coincidence detector for synaptic plasticity and memory formation (Wigstrom and Gustafsson, 1985; Tsien, 2000a, 2000b). For example, genetic knockout of the NMDA receptor in the hippocampal CA1 region causes severe deficits in both spatial and nonspatial learning in mice (Tsien et al., 1996b; Rampon et al., 2000; Shimizu et al., 2000). On the other hand, genetic enhancement of NMDA receptor function results in enhanced synaptic coincidence detection and superior learning and memory (Tang et al., 1999, 2001; Wong et al., 2002; for review, see Tsien, 2000b). The NMDA receptors are heteromeric complexes consisting of NR1 and various NR2 (NR2A, NR2B, NR2C, and NR2D) subunits (Nakanishi, 1992; Hollmann and Heinemann, 1994). The NR1 subunit serves as a key subunit essential for ion selectivity and agonist binding of the NMDA channels, whereas the NR2 subunit is mainly responsible for regulating channel gating and Mg^{2+} dependency. The combination of NR1 with different NR2 subunits shows functional diversity and uniqueness in electrophysiological and pharmacological properties (Monyer et al., 1992).

Despite evidence for the essential function of the NMDA receptor in learning (Tsien, 2000b) and memory consolidation (Wittenberg and Tsien, 2002), its temporal role in the storage of remote memories has not been examined. Systematic analysis of the molecular mechanism underlying memory storage requires experimental tools that can offer both molecular and temporal specificity. Recent development of a series of inducible and region-specific gene knockout (Shimizu et al., 2000; Mack, et al., 2001) or more recently, inducible protein knockout (Wang et al., 2003) as well as transgenic dominant-negative approaches (Mayford et al., 1996; Kida et al., 2002; Hedou and Mansuy, 2003) have permitted a more precise investigation of behavioral learning.

In the present study, we applied an inducible and forebrain-specific knockout technique to assess the involvement of the NMDA receptor in the storage of fear memories. It is well known that fear memories can persist for an extended period of time after brief training. Moreover, the time course for the hippocampal involvement in the fear memory consolidation has been well characterized in rodents. For example, lesion of the hippocampus during the first 2 posttraining weeks produces severe impairment in the retention of 1-month contextual fear memory, whereas lesions made during or after the fourth posttraining week have no significant effect (Kim and Fanselow, 1992; Anagnostaras et al., 1999). Similarly, inducible knockout of the NMDA receptor in the mouse CA1 region during the first 2 weeks after training causes profound impairment of 1-month contextual memory, whereas the knockout of the NMDA

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receptor in CA1 during the fourth posttraining week does not (Shimizu et al., 2000). These findings are consistent with the notion that the fear memory completes the hippocampal consolidation and may enter the cortical storage stage 4 weeks after initial learning has occurred.

To explore the temporal role of the NMDA receptor in memory process, we have previously built a computational model to examine the effects of synaptic receptor turnovers on the long-term stability of stored memory traces (Wittenberg et al., 2002). Our computational analysis suggests that over time, accumulative drift of synaptic efficacy caused by metabolic turnover would undermine the long-term storage of information in the brain; however, periodic reactivations of the NMDA receptor could theoretically reinforce synaptic connections in pre- and postsynaptic neuron paired-wise fashion and thereby provide an effective cellular means for preserving the synaptic stability of long-term memory (Wittenberg et al., 2002; Wittenberg and Tsien, 2002).

Here, we set out to test this prediction through a series of genetic and behavioral experiments by applying inducible and reversible knockout of the NMDA receptor's NR1 subunit in the excitatory neurons of the mouse forebrain. Specifically, we manipulated NR1 expression specifically during the storage stage of the memory process. Six months after fear conditioning and at least 2 months prior to recall, we used controlled feeding of doxycycline to temporarily switch off NMDA receptor expression for variable periods of time. Our integrated analysis reveals that the prolonged absence of NMDA receptor activity indeed leads to synaptic drifts in the brain with devastating consequences on the stability of stored remote fear memories.

Results

Generation and Basic Characterization of Inducible and Forebrain-Specific NR1 Knockout Mice

To investigate whether the NMDA receptor is required for the maintenance of remote memories during the storage phase, we have established inducible, reversible, and forebrain-specific NR1 knockout mice (iFB-KO) by combining the bacteriophage P1-derived Cre/loxP recombination system with the tTA/tetO transactivator system. Our overall strategy involved forebrain-specific tetracycline-regulated expression of the NR1-GFP transgene to restore NMDA receptor function in iFB-KO mice (Figure 1). Feeding the iFB-KO mice with drinking water or food pellets containing doxycycline (dox), a tetracycline analog with higher permeability through the blood-brain barrier, enabled us to switch off NR1-GFP transgene expression in the forebrain region and thus to induce a regional and temporally specific NR1 knockout state.

We used one of the forebrain excitatory neuron-specific CaMKII promoter-driven Tg-Cre lines for making forebrain-specific mutant mice (Tsien et al., 1996a). Large breeding colonies and extended breeding cycles were employed to generate iFB-KO mice carrying the necessary allelic combinations (*Cre/+, tTA/+, NR1-GFP/+, fNR1/fNR1*). The numbers of inducible knockout mice in the offspring were according to the Mendelian inheritance, at the ratio of either 1/16 or 1/32, depending on whether the parent mice carried either heterozygous or homozygous *fNR1*. The genotypes of progeny mice were determined by both PCR and Southern blot analyses of tail DNA (representative data are shown in Figures 1B and 1C). In stark contrast to the conventional NR1 knockout mice that die within hours after birth (Li et al., 1994; Forrest et al., 1994), the iFB-KO mice grow and mate normally, and their overall behaviors are indistinguishable from those of control littermate mice (*tTA/+, NR1-GFP/+, fNR1/fNR1*; or *tTA/+, NR1-GFP/+, fNR1/+*).

As an initial means of assessing the forebrain specificity of Cre/loxP recombination of the Tg-Cre line, we also crossed the forebrain excitatory neuron-specific Cre mice with a *LacZ* reporter mouse so that the recombination could be easily detected by the X-gal staining method (Tsien et al., 1996b). The spatial pattern of X-gal staining (depicted by blue cells) shows that recombination in the adult mouse brain was completely restricted to the cortex, hippocampus, and striatum (Figures 1D–1G). We further assessed the proportion of neurons that had undergone the Cre/loxP recombination by comparing the numbers of X-gal-stained cells and Nissl-stained cells in the brain sections. Our analysis revealed that the Cre/loxP recombination occurred reliably but differentially in distinct forebrain structures, reflecting the selective nature of CaMKII promoter activity in different types of neuronal populations. For example, it occurred in about 98% of CA1 pyramidal cells (Figures 1E and 1H) and the averaged 62% of cortical neurons (Figures 1F–1H), but was not in the olfactory bulb.

In order to directly determine and confirm the forebrain-specific knockout of the endogenous NR1 gene in iFB-KO mice, we have performed in situ hybridization to probe for the NR1 mRNA sequence that corresponds to a loxP flanked exon of the NR1 gene, which should be deleted once recombination occurs. Our in situ hybridization showed broad and robust expression of NR1 mRNA in the brain of the control littermate (Figure 2A). In contrast, the dox-treated iFB-KO mice exhibited drastically reduced NR1 mRNA levels in the cortex, striatum, and hippocampus (Figure 2B). As expected from the pattern of CaMKII promoter-driven Cre expression, NR1 mRNA expression in other brain regions such as the olfactory bulb, thalamus, brainstem, and cerebellum appeared to be normal in iFB-KO mice.

We next measured the protein level of NR1 in different brain regions of these mice using an antibody specific to the C terminus of NR1. As shown by the Western blot, there were significant reductions of NR1 protein in both the cortex and hippocampus, but not in the cerebellum of dox-treated iFB-KO mice (Figure 2C). For instance, the level of NR1 in the iFB-KO cortex was reduced significantly in comparison to the normal level (see CTX lanes in Figure 2C). The remaining signals in the cortex and hippocampus (Figure 2C) may reflect the intact NR1 expression in interneurons and other types of neurons in which the CaMKII promoter was not active.

To further define the anatomical location of NR1-GFP protein expression, we performed immunohistochemistry using an antibody directed against GFP. This antibody is highly specific to the GFP-tagged NR1 protein since the brains of control littermates do not show any immunoreactivity (Figure 2D). Our staining revealed that the NR1-GFP protein in iFB-KO mice localized to the cortex, hippocampus, and striatum (Figure 2E), thus

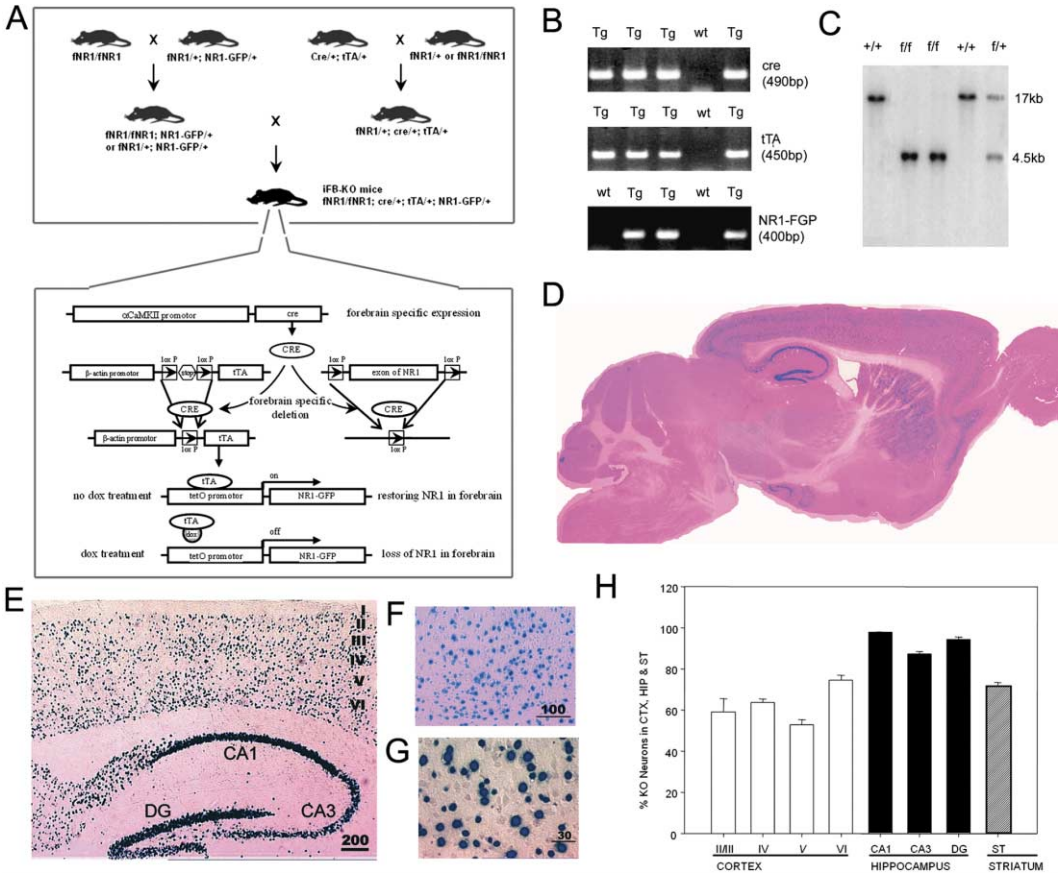

Figure 1. Production of Inducible, Reversible, and Forebrain-Specific NR1 Knockout Mice

(A) A general strategy for making inducible NR1 knockout mice. In the final progeny, only small numbers of the mice are the iFB-KO mice, which carry triple transgenes (*Cre*, *tTA*, and *NR-GFP1* transgenes) and homozygous or heterozygous floxed *NR1*. In these iFB-KO mice, expression of the tTA transgene in the forebrain excitatory neurons will be achieved by the Cre/loxP-mediated deletion of the "stop" sequence, which then allows the expression of the *NR1-GFP* transgene in these neurons, thereby rescuing the forebrain-specific knockout of floxed endogenous *NR1* gene. Feeding the iFB-KO mice with dox, a compound pulling tTA off the tetO promoter, will switch off *NR1-GFP* transgene expression and return the forebrain to the NR1 knockout state. On the other hand, the withdrawal of dox from their food will restore NR1 expression in the forebrain region.

(B) PCR genotyping of tail DNA from representative mice for detecting the *Cre*, *tTA*, and *NR1-GFP* transgenes, respectively. The sizes of the perspective bands were noted on the right.

(C) Southern blot shows the detection of the floxed *NR1* gene (4.5 kb band) and wild-type *NR1* alleles (17 kb band) in wild-type (+/+), heterozygous (*f*/+), and homozygous (*f*/*f*) mice.

(D) Sagittal sections show that the cre/loxP recombination (indicated by blue X-gal signals) is restricted to neurons of cortex, hippocampus, and striatum. For better visualization, eosin staining was used as background staining.

(E) LacZ and Nissl double staining shows that the cre/LoxP recombination occurred in neurons of the hippocampus and different layers of cortex.

(F) High magnification of cortical layer IV. Cre/loxP recombination (blue) occurred only a portion of the cortical cells.

(G) Higher magnification of layer V. Numbers above the bar in (E), (F), and (G) indicate the scale in micrometer (μm) under respective magnifications.

(H) Estimated percentages of the cre/loxP recombinant neurons in different layers (II–VI) of cortex, the CA1, CA3, and dentate gyrus regions of hippocampus as well as the striatum.

compensating for the knockout of the endogenous NR1 gene.

To assess how rapidly NR1-GFP expression can be switched off by dox, which can sequester tTA and terminate the tetO promoter activity, we subjected the mice to various periods of dox feeding (in food pellets) and prepared the brain tissues for GFP staining. The 3-day dox treatment significantly reduced the level of NR1-GFP protein in the forebrain of iFB-KO mice (data not shown), in comparison to that of the untreated iFB-KO mice (Figure 3A). Meanwhile, the 5-day dox treatment resulted in a complete loss of NR1-GFP in the cortex, striatum, and hippocampus (Figure 3B). This demonstrates not only that NR1-GFP protein is turned over in 5 days in vivo, but also that inducible knockout of NR1 can be achieved by a minimal 5-day dox treatment. Furthermore, the NR1-GFP expression can be reversed within 2 months even after the prolonged dox treatment (30 days) (Figure 3C), a useful feature for temporal analysis of the memory process.

Normal Physiological and Behavioral Function in Untreated iFB-KO Mice

To investigate the physiological effects of dox-regulated inducible NR1 knockout, we conducted field recordings of excitatory postsynaptic potentials (EPSPs) in the

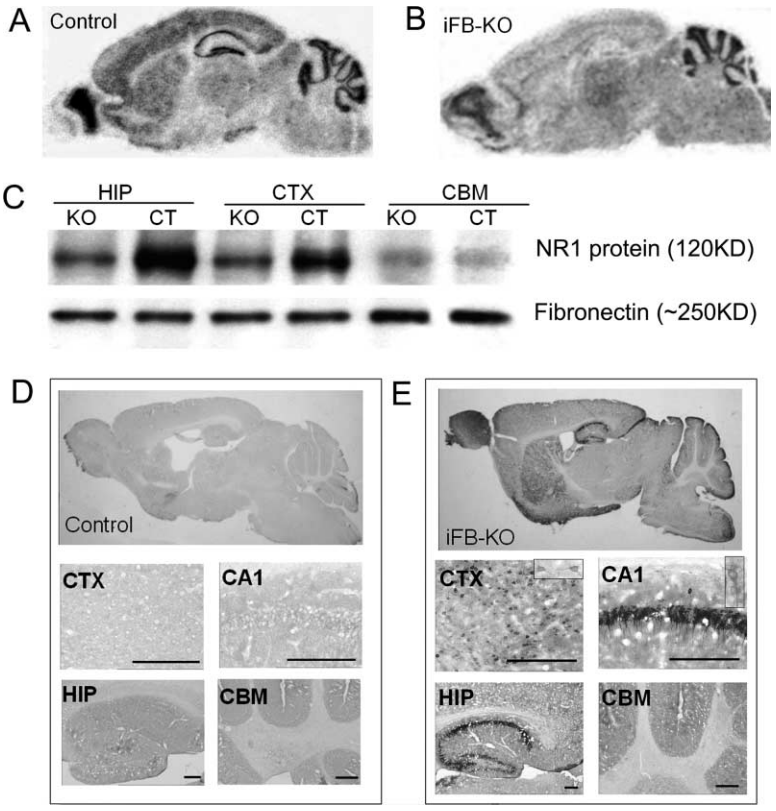

Figure 2. Histological and Biochemical Characterization of iFB-KO Mice and Control Mice

(A) In situ hybridization reveals the normal expression of NR1 mRNA in the control littermate.

(B) The 5-day dox-treated iFB-KO mice show the lack or great reduction of NR1 mRNA expression in the cortex, striatum, and hippocampus.

(C) Reduction of NR1 protein in dox-treated iFB-KO demonstrated by Western blots (antibody: Upstate, cat# 06-311). Dox-treated iFB-KO mice had reduced NR1 expression in the hippocampus to about 14% and in the cortex to about 27.5% of control mice (top) as assessed by OD measurement. The reduction in the NR1 protein level is largely consistent with knockout efficiency as assessed by X-gal method, and likely reflects the cell-type specific CaMKII promoter activity used to drive Cre expression. The same amount of proteins was loaded in each lane as shown by fibronectin signal (lower panel).

(D) GFP antibody does not exhibit nonspecific binding to the control mice. Sagittal section of control brain (top) shows no GFP-staining signal. Cortex (CTX), hippocampus (HIP), CA1, and cerebellum (CBM) sections at higher magnification are shown.

(E) GFP immunostaining shows the forebrain-specific expression of the NR1-GFP protein in untreated iFB-KO mice. Top panel is the whole view of a sagittal brain section of untreated iFB-KO mice. Bottom panels show the staining in the cortex, whole hippocampus, hippocampal CA1, and cerebellum sections at higher magnification. NR1-GFP expression is clearly visible in cortical and hippocampal neurons, but no signals were observed in cerebellum, thalamus, and brainstem regions. The small insets on the left corner of CTX and CA1 show the stained individual neurons in these regions. The scale bars represent 200 μm.

brain slices prepared from the entorhinal cortex of both iFB-KO and control mice upon the completion of the 5-day dox treatment. Although the NR1 knockout occurred in the excitatory neurons of the various cortical areas, we chose the entorhinal cortex as the site for measurement because of its implicated function in the long-term memory storage (Squire, 1987; Rosen et al., 1992; Corodimas and LeDoux, 1995; Suzuki, 1996; Bucci et al., 2000). The stimulating electrode was placed at layer II, and the recording electrode was positioned at layer III (Figure 3D). Paired-pulse protocol produced statistically indistinguishable responses between dox-treated iFB-KO slices (101.67 $\pm$ 5.68) and control slices (97.41 $\pm$ 5.65) or untreated iFB-KO (96.23 $\pm$ 9.59) (Figures 3E and 3F). The lack of pair-pulse facilitation in wild-type slices may reflect either the insensitivity of the field-recording technique in detecting complex changes within diverse cortical connections or the greatly reduced responses in slices prepared from relatively old (8–11 month) animals (so our results can provide better assessment for the inducible NR1 knockout in our long-term memory studies). We next examined the synaptic plasticity between layers II and III by application of tetanic stimulation (100 Hz, for 1 s, repeated twice with 5 s interval) (Figures 3G–3I). This stimulation protocol evoked significant long-term potentiation (LTP) in the control slices (130.25 $\pm$ 3.38) or dox-untreated iFB-KO mice (127.37 $\pm$ 4.99). However, the same protocol was incapable of eliciting LTP in the cortex of the dox-treated iFB-KO mice (104.63 $\pm$ 5.38) (Figures 3G–3I, $p < 0.01$). Thus, our data show that 5-day dox treatment sufficiently and specifically disables NMDA receptor-dependent synaptic plasticity in the cortex of iFB-KO mice.

Finally, we used the fear conditioning paradigm to assess learning and memory capabilities in untreated iFB-KO mice, especially to determine whether the mice could form and retain the remote contextual and cued fear memories. The initial training of these mice involved three paired presentations (separated by 5 min intervals) of a pure tone conditioned stimulus (CS, 2800 Hz at 85 dB) and a mild foot shock unconditioned stimulus (US). A measurement of immediate freezing responses indicated that the untreated iFB-KO mice exhibited normal learning in comparison to the control littermates (Figure 4A).

Nine months after the initial training, both untreated iFB-KO and control mice were tested for the retention of remote fear memories. Upon returning to the original training chamber, the untreated iFB-KO mice exhibited significant freezing responses in comparison to those of control mice, indicating the intact contextual fear memories (Figure 4A). Student's t test revealed no statistical difference in contextual freezing responses between untreated iFB-KO mice and control littermates. We further measured the cued fear memories in these mice by placing them in a contextually and visually dis-

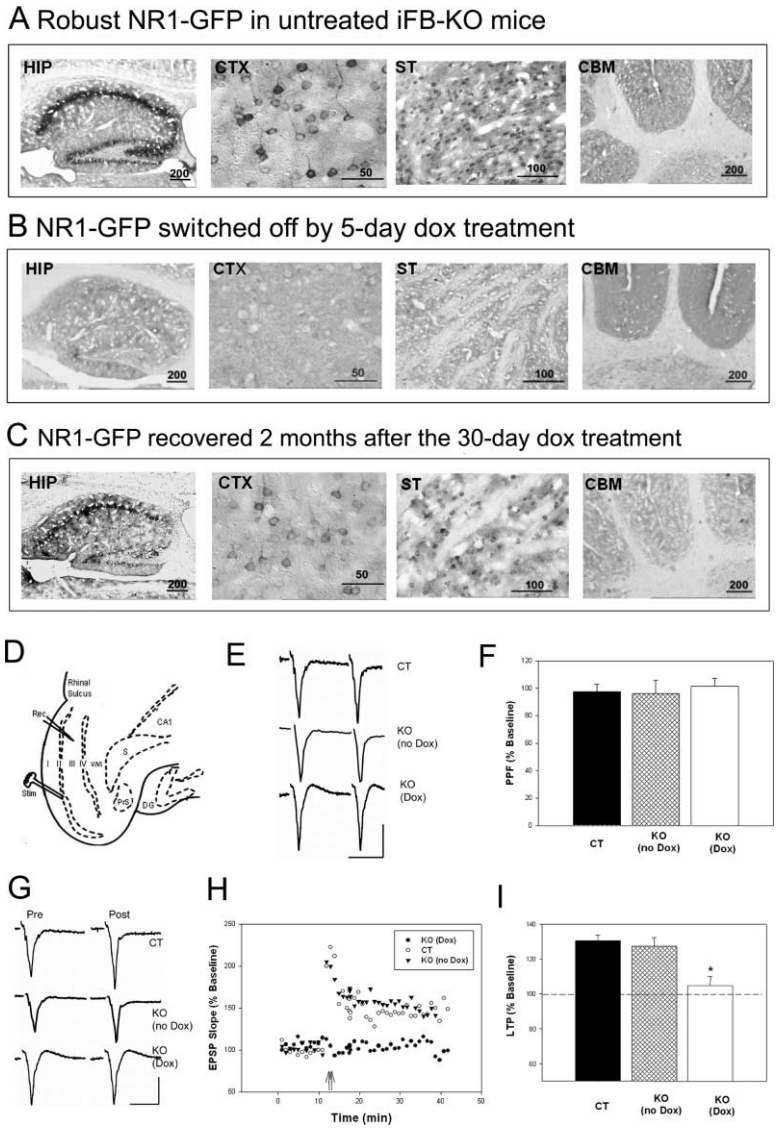

Figure 3. Inducible and Reversible Switch Off of NR1 in the Forebrain of iFB-KO Mice by Dox Treatment

(A) The forebrain neurons in hippocampus (HIP), cortex (CTX), and striatum (ST) of iFB-KO mice before dox treatment had robust NR1-GFP signal, but no NR1-GFP signal was observed in cerebellum (CBM) and brainstem (date not show).

(B) 5-day dox treatment leads to complete loss of NR1-GFP protein in the cortex, hippocampus, and striatum of iFB-KO mice.

(C) NR1-GFP signals in these forebrain regions of iFB-KO mice are completely recovered by 2 months after the completion of 30-day dox treatment. The scale bars are in μm.

(D) Schematic drawing of the entorhinal cortical slice and positions where the stimulating (Stim) and recording (Rec) electrodes were placed in layer II and III, respectively. S, subicullum; PrS, presubicullum; DG, dental gyrus.

(E) Representative traces for paired pulse responses obtained from controls (CT), dox-untreated iFB-KO (KO, no dox), and dox-treated iFB-KO (KO, dox) mice.

(F) Pooled results show indistinguishable paired-pulse responses of 5-day dox-treated iFB-KO slices in comparison to that of untreated iFB-KO or controls.

(G) Representative traces of LTP from a single slice prepared from controls (CT), dox-untreated iFB-KO (KO, no dox), and dox-treated iFB-KO (KO, dox) animal.

(H) Representative EPSP slope measurement of LTP response for 30 min after LTP induction from a single slice of control (CT), dox-untreated iFB-KO (KO, no dox), and dox-treated iFB-KO (KO, dox) animal. Two trains (arrows) of 100 Hz, each presented for a duration of 1 s and separated by a 5 s interval, produces robust LTP, as measured by increased EPSPs, in both untreated iFB-KO and control slices. No LTP was observed in the iFB-KO slice.

(I) Pooled results showing LTP, as measured in changes of EPSP slopes, was abolished in 5-day dox-treated iFB-KO slices (KO with dox: 104.63 $\pm$ 5.38, n = 10 slices, 3 mice), whereas normal LTP was reliably evoked in dox-treated control mice (CT: 130.25 $\pm$ 3.38, n = 6 slices, 3 mice) and untreated iFB-KO mice (KO with no dox: 127.37 $\pm$ 4.99, n = 6 slices, 2 mice). We used two-pulse protocol (40 ms interpulse interval) as test stimulus at a frequency of 0.017 Hz. 0.05–0.20 nA and 50 μs duration were used so that half of the maximal EPSP was elicited as by first pulse (the baseline response). The scale bars in (E) and (G) equal 20 ms for the *x* axis and 0.4 mV for the *y* axis. *$p < 0.01$.

tinct chamber. When presented with 3 min of continuous tone (original CS), these untreated iFB-KO mice showed the same amount of freezing responses in comparison to that of control mice (Figure 4B), suggesting the normal retention of 9-month cued fear memory. Taken together, the above results confirm that the untreated iFB-KO mice and control littermates had indistinguishable physiology and learning behaviors.

Temporal Involvement of the NMDA Receptor in the Storage of Remote Fear Memories

We used the same 9-month fear conditioning paradigm as described above to investigate the molecular mechanism underlying the storage of remote fear memories. After training, both iFB-KO and control mice were returned to their home cages. Regular food pellets were replaced with dox-containing food pellets during either the first 7 or all 30 days of the seventh month (thereby switching off NR1 in iFB-KO mice), but these mice were otherwise left undisturbed for 9 months. After the dox treatment, feeding of regular food pellets was resumed, thus allowing at least 8 or 11 weeks for NR1 expression to recover in iFB-KO mice before retention tests were conducted. As we have shown biochemically (Figure 3C), this feeding schedule permits the full restoration of NR1 expression in the forebrain of iFB-KO mice, thereby enabling temporally controlled analysis of the memory storage process.

At the end of 9 months, we measured the retention of fear memories in all mice. First, we asked whether transient knockout of the NMDA receptor function by 7-day dox treatment was sufficient to affect the mainte-

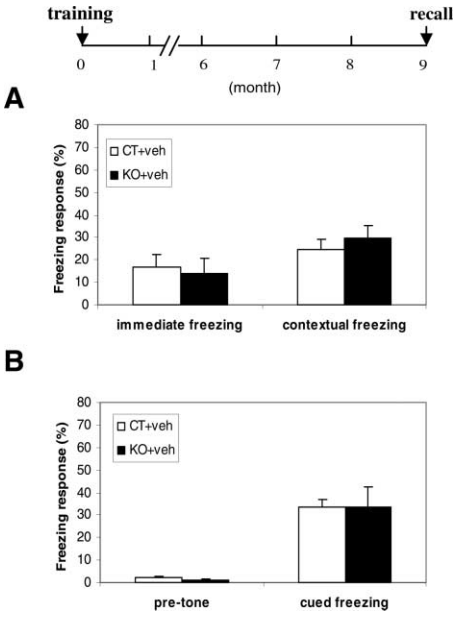

Figure 4. Both Control and iFB-KO Mice without Dox Treatment Exhibited Significant Retention of 9-Month Fear Memories

Three CS/US pairings were used during training. Upon the completion of training, the mice were returned to their home cages for 9 months before recall.

(A) Normal learning and retention of 9-month contextual fear memory in untreated iFB-KO mice (KO, n = 12) mice, in comparison to that of untreated control (CT, n = 11). No significant difference in immediate freezing during learning and contextual freezing during recall was found between the two groups.

(B) Indistinguishable retention of cued fear memory in untreated control (CT, n = 11) and KO (KO, n = 12) mice. No significant difference was found between two groups. The time line indicates the duration of 9-month retention. Regular food pellets without dox were used as vehicle (veh).

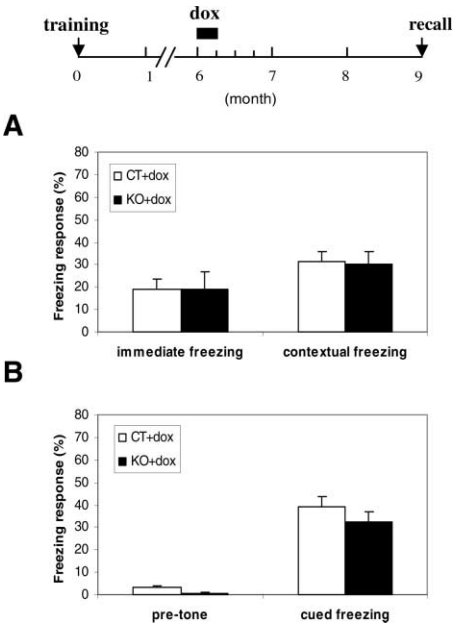

Figure 5. 7-Day Dox Treatment during the First Week of the 7th Month Had no Effect on the Maintenance of 9-Month Fear Memories

(A) No significant difference was found in the retention of contextual fear memory between dox-treated control (n = 8) and dox-treated KO (n = 8) mice.

(B) No significant difference was found in cued freezing response between dox-treated control (n = 8) and dox-treated KO (n = 8) mice. The black bar above the time line indicates the duration of dox treatment (7 days), which occurred 6 months after initial training.

nance of remote fear memories. We found that both dox-treated iFB-KO and control mice exhibited significant retention of fear memories (Figure 5). Student's t test reveals no statistical difference between iFB-KO and controls in the retention of 9-month-old contextual memory (Figure 5A). Similarly, these mice showed indistinguishable freezing responses in the retention of the 9-month-old cued memories (Figure 5B). Thus, the transient loss of the NMDA receptor in the forebrain of iFB-KO mice during the storage phase did not produce detectable disruption of the stored fear memories.

In our previous computation modeling analysis, we show that the synaptic drift caused by turnovers of synaptic receptors is a time-dependent and accumulative process (Wittenberg and Tsien, 2002). We thus reasoned that the temporal duration of NMDA receptor knockout by 1-week dox treatment could be too short for synaptic drifts to detrimentally alter the stored memories. Thus, we decided to have another set of mice undergo the same behavioral paradigm, except this time, the duration of dox treatment would be for 30 days beginning at the end of 6 months after training. At the end of the 30 days of dox feeding, normal food was then provided for the remaining 2 months so that the NMDA receptor in the iFB-KO is functional by the time of recall.

We conducted the 9-month contextual retention test in these iFB-KO mice, and indeed found that these 30-day dox-treated mice exhibited severe deficits in the retention of remote contextual fear memory in comparison to that of control mice that had undergone the same dox treatment (Figure 6A). To measure the effects of NMDA receptor knockout on the storage of cued fear memories, we placed these mice individually in the cue test chamber. We observed that while the amount of freezing before the onset of the tone (pretone) was the same among the groups, the dox-treated iFB-KO mice exhibited profound deficits in the retention of the 9-month-old cued fear memories (Figure 6B). Furthermore, significant difference exists between dox-treated iFB-KO mice and dox-untreated iFB-KO as well as dox-untreated control mice (see Figure 4) in both contextual and cued memory retention. Therefore, these experiments show that in contrast to the ineffectiveness of transient NMDA knockout by the 7-day dox treatment, the prolonged disabling of NMDA receptor function in the forebrain of iFB-KO mice by the 30-day dox treatment during the storage phase was sufficient to disrupt the retention of the remote contextual and cued fear memories.

Previously 30-Day Dox-Treated iFB-KO Mice Were Fully Capable of Learning and Retaining Subsequent New Memories

To evaluate the molecular and temporal specificity of the observed storage deficits in the 30-day dox-treated iFB-KO mice, we conducted a series of "post remote

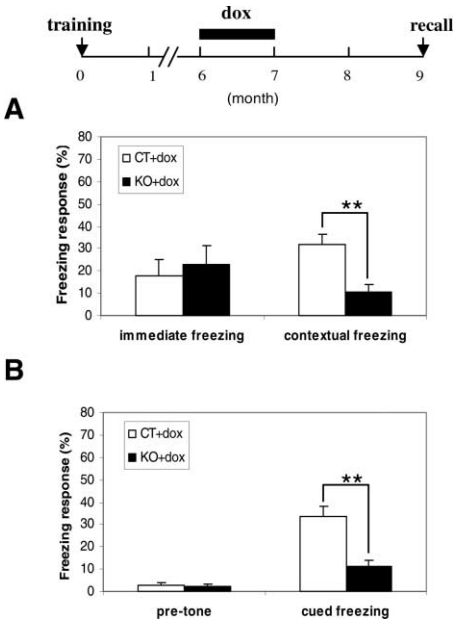

Figure 6. 30-Day Dox Treatment during the 7th Month Disrupts the 9-Month-Old Remote Fear Memories

Mice received dox-containing food pellets for 30 days as indicated by black bar above the time line. Retrieval tests were conducted at the end of 9 months after initial training.

(A) Contextual learning in these two groups of mice during initial training are the same as shown by similar amount of immediately freezing. However, the retention of 9-month-old contextual fear memory was significantly impaired in dox-treated iFB-KO mice ($n = 11$) in comparison to that of dox-treated control ($n = 12$) ($p < 0.001$). Further Student's t test reveals significant difference in the responses of dox-treated iFB-KO mice, compared with untreated control ($p < 0.05$) and untreated KO ($p < 0.01$) in the 9-month contextual fear retention tests (see Figure 4). It should be noted that the behavioral experiments using untreated mice was run together with the treated mice for ensuring proper statistical comparisons.

(B) Although no significant difference was found in pretone freezing response in these mice, a significant difference in tone-elicited freezing responses was found between 30-day dox-treated KO mice and dox-treated control ($p < 0.001$). Significant differences were also found when dox-treated FB-KO mice were compared to untreated control ($p < 0.01$) and untreated KO ($p < 0.01$) (see Figure 4). **$p < 0.001$.

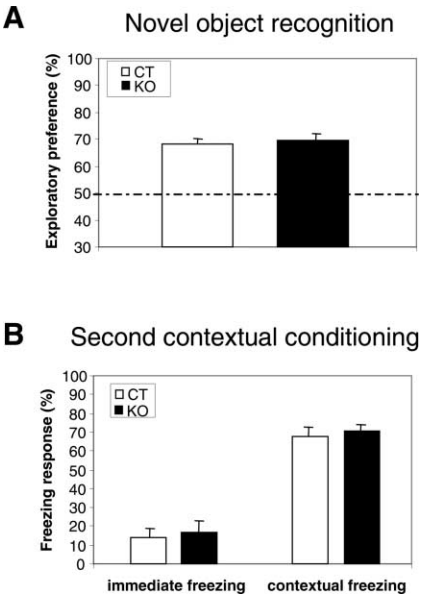

Figure 7. Normal learning and Memory Function in Previously Dox-Treated iFB-KO Mice

These mice received 30-day dox treatment during the storage of remote fear memories experiments and were subject to a set of new learning and memory tests.

(A) In the novel object recognition test, previously dox-treated iFB-KO mice exhibited good recognition memories as indicated by strong preference toward novel objects. No differences were found among control ($n = 12$) and iFB-KO ($n = 11$) mice. The dotted line indicates the chance performance (50%).

(B) Previously dox-treated iFB-KO mice also showed robust retention in a second contextual fear conditioning (with a totally new context and different floor grids and in a new room). Experimenters also wore different colored lab coats and gloves during this experiment. No significant differences were found between groups. The retention of contextual fear memory was measured 24 hr after training. All the above follow-up tests were concluded within 4–6 days of completion of the 9-month retention tests.

memory test" behavioral assessments immediately after their completion of the 9-month retention tests. Specifically, we aimed to determine whether the observed retention deficits in 30-day dox-treated iFB-KO mice were due to any kind of leftover effects of NR1 knockout or nonspecific interferences in the animals' general behaviors at the time of recall.

Thus, we subjected the same groups of mice (used in Figure 6 experiments) to the novel object recognition test (Rampon et al., 2000). This test measures visual recognition memory and is known to require both the NMDA receptor function (Rampon et al., 2000; Tang et al., 1999) and the structural integrity of the hippocampus and cortex (Mumby et al., 1996; Zola-Morgan and Squire, 2001; Manns et al., 2003). Consistent with our histological evidence that showed the full recovery of NR1-GFP protein in iFB-KO mice 2 months after the completion of the 30-day dox treatment (Figure 3C), we found that these iFB-KO mice performed normally in this visual memory test. There was no obvious difference in the amount of time the mice spent exploring the two novel objects, indicating that both groups of the mice had the same curiosity and motivation to explore objects. Moreover, during the subsequent 24 hr retention test, iFB-KO mice and control littermates exhibited indistinguishable preference for the novel object (Figure 7A). Thus, this experiment demonstrates that these iFB-KO mice, which previously received 30-day dox treatment and exhibited memory storage deficits in the 9-month retention tests, possessed normal ability to acquire, retain, and retrieve the object recognition memory.

Given that the normal performances of these iFB-KO mice in novel object recognition may not give us the best assessment of the circuitry function involved in fear memory and behaviors, we designed a new, second contextual memory test to further examine the memory function in these dox-treated mice. We used an entirely different shock chamber (different shape and wall color) equipped with distinct shock-floor grids featuring different textural patterns and rod size. Furthermore, experimenters wore different colored lab coats and gloves and conducted the new fear conditioning in a different

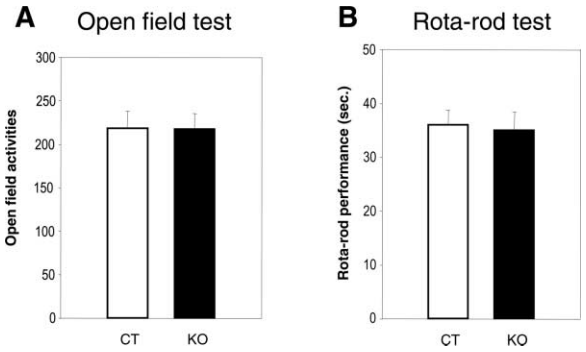

Figure 8. Dox Treatment Had no Effect on Locomotor Activity and Cerebellar Coordination in Mice

(A) No significant difference was found between control group and iFB-KO group in the open field test. The tests were conducted 2 months after these mice had completed a 30-day dox treatment (CT, n = 24, open field activities 218.60 ± 19.77, KO, n = 24, 217.69 ± 17.89, open field activity: the number of squares the mice passed within the 3 min period, the data is expressed as mean ± SEM).

(B) Normal motor coordination, as measured by the rotarod test, was also observed in both iFB-KO and control littermates (CT, n = 24, rotarod performance 36.03 ± 2.78, KO, n = 24, 34.99 ± 3.45). Rotarod performance was measured by the amount of time (in seconds) that mice can manage to stay on the accelerating rotating rod; the rod is 1 inch in diameter and the rotating rate starts at 4 rpm/min.

room, thereby minimizing any similarity between the original fear conditioning and this second fear conditioning. Notably, the previously 30-day dox-treated iFB-KO mice showed the full capability in acquiring, retaining, and retrieving new contextual fear memory (Figure 7B). They exhibited indistinguishable freezing responses in the 1-day retention test. Student's t test further indicated no statistical difference in the retentions of 1-day contextual memory between these iFB-KO and control groups. Taken together, the above two new memory tests conducted immediately after the completion of the 9-month retention tests have allowed us to exclude the possibility that the observed storage deficits of the 9-month remote fear memories in the 30-day dox-treated iFB-KO mice were due to performance or memory impairment at the time of recall.

Finally, we conducted an additional set of behavioral measurement on these mice in the open-field test and the rotarod test. Both previously dox-treated iFB-KO mice and control littermates showed comparable locomotor activity as measured by the open field behavior (Figure 8A). In addition, they also exhibited indistinguishable motor coordination as revealed by the rotarod test (Figure 8B). Therefore, we conclude that these mice had normal locomotor and cerebellar function.

Discussion

The NMDA Receptor and the Long-Term Storage of Remote Memories

Although long-term memory is known to consist of several distinct temporal stages, the vast majority of experiments to date have been focused on the analysis of learning and consolidation. Memory consolidation, an intermediate process required for converting a new memory into long-lasting one, seems to operate at the time scale of weeks in rodents and monkeys. For example, it is known that lesion of the hippocampus in rats 1 or 7 days after training produces significant impairment in long-term fear memories, but the same lesion 28 days afterwards has no effect (Kim and Fanselow, 1992; Maren et al., 1997; Anagnostaras et al., 1999). Similarly, posttraining lesion of the hippocampus in monkeys within 2 to 4 weeks, but not at 8–16 weeks, produced severe deficits in object discrimination tests (Zola-Morgan and Squire, 1990). Furthermore, inducible knockout of the NMDA receptor in the mouse CA1 region during the first 2 weeks after contextual fear conditioning causes severe memory impairment in 1-month retention tests, whereas the knockout of the NMDA receptor in CA1 during the fourth posttraining week does not impair long-term memory (Shimizu et al., 2000). The postlearning processing of memory consolidation by the hippocampus is also indicated by a recent experiment showing that posttetanic pharmacological prevention of LTP decay, possibly via suppressing NMDA receptor-mediated new interference from the nearby neurons within the activated pathways, is associated with the stabilization of initial hippocampal spatial memory traces (Villarreal et al., 2001). Thus, those studies point to the notion that hippocampal-mediated systems-level consolidation of memories occurs for a limited time period (weeks or month), after which the memories enter a more interference-resistant state and are perhaps stored somewhere in the cortex (Zola-Morgan and Squire, 1990; Kim and Fanselow, 1992; Bontempi et al., 1999; Frankland et al., 2001). Memories that have completed such a temporal and mechanistic transition to a hippocampal-independent state are referred in the literature as remote memories (Debiec et al., 2002).

Recent research on memory reactivation and reconsolidation has generated increasing interest in the study of remote memory (Sara, 2000; Nader, 2003). However, little attention has been directed toward understanding the molecular process underlying the storage of remote memories. It has been postulated that long-lasting memory is stored in the form of structural synaptic modifications triggered by original learning. Such structural changes, once laid down, have been assumed to confer the long-term stability of stored memories. Recent observation that molecular and structural machineries at the synapse undergo routine metabolic turnover (Shimizu et al., 2000), an intrinsic process likely independent of whether memory is in the dormant or active form, raises the fundamental question as to how the memory remains stable over time.

As a first step toward the genetic analysis of the memory storage mechanism, we focused on the role of the NMDA receptor in the mouse forebrain. We combined the Cre/loxP-recombination system with tTA transactivator systems to generate inducible, reversible, and forebrain-specific NR1 knockout mice (iFB-KO). In contrast to the neonatal lethality of conventional NR1 knockout, iFB-KO mice are viable and their physiological and behavioral phenotypes are indistinguishable from control mice. By taking advantage of dox-mediated inducible and reversible switch off and on of the NR1 expression in the forebrain excitatory neurons of iFB-KO mice,

we analyzed the involvement of the NMDA receptor in the storage of remote fear memories.

To increase the certainty that our molecular manipulation occurred during the storage stage, we designed a series of experiments in which the NMDA receptor was temporarily disabled 6 months after the original training. Since this 6-month duration corresponds to approximately one-fourth of a mouse's life expectancy, we reasoned that memory consolidation should be completed in 6 months after learning occurs. Thus, our "inducible knockout 6 months after learning" paradigm should allow our temporal analysis of NMDA receptor function restricted to the storage, not consolidation, phase of remote memories.

Specifically, we have demonstrated that the 9-month retention of both contextual and cued fear memories in iFB-KO mice is profoundly impaired by administering dox for 30 days, beginning 6 months after fear conditioning but ending 2 months before memory retrieval. In a subsequent set of memory tests including novel object recognition and a new (second) contextual fear conditioning following the completion of the 9-month retention tests, these mice exhibited normal capacity to learn, retain, and retrieve new memories. Therefore, the observed retention deficits are likely to reflect the disruption of the storage process rather than the disturbance of recall or performance capability.

Furthermore, untreated iFB-KO mice learned and retained 9-month-old fear memories as effectively as untreated control mice, suggesting that observed deficits in the dox-treated iFB-KO mice were not simply caused by genotypic alterations. Equally important, the control mice receiving the same 30-day dox treatment showed significant retention of 9-month contextual and cued fear memories, indicating that dox feeding alone did not produce detectable side effects on learning behavior. In addition, long-term dox treatment (1 month) does not alter nociceptive responses as measured by the amount of current necessary to elicit flinching/running, jumping, and vocalizing in mice (Shimizu et al., 2000). Finally, as shown in Figure 8, the 30-day dox treatment in iFB-KO mice did not alter their performance in the open field test and rotarod test, indicating normal cerebellar coordination and locomotor function.

The use of both contextual and cued fear conditioning has allowed us to simultaneously investigate the storage mechanisms underlying neurologically different types of memories: namely, the hippocampal-dependent (contextual) and hippocampal-independent (cued) fear memories (Phillips and LeDoux, 1992; Davis et al., 1987; Maren, 2001). At the molecular level, the formation of both contextual and cued fear memories requires the NMDA receptor during learning. For example, pharmacological blockade of the NMDA receptor within the amygdala before training impairs cued fear conditioning (Rodrigues et al., 2001; Falls et al., 1992; Lee et al., 2001), whereas knockout of NMDA receptor in the CA1 region impairs contextual fear memory (Rampon et al., 2000; Shimizu et al., 2000). Furthermore, enhancement of NMDA receptor function by overexpression of NR2B in the forebrain enhances retention and accelerates extinction of both contextual and cued fear memories (Tang et al., 1999). Similar to the disruption of fear learning by disabling NMDA receptor function, the present study shows that the prolonged absence of the NMDA receptor during the storage stage (as shown in Figure 6) results in profound retention deficits for both contextual and cued fear memories in iFB-KO mice. Our finding suggests that the continued presence of the NMDA receptor may be a general requirement for the enduring storage of remote memories in the brain.

Where Are the Remote Fear Memories Stored in the Forebrain?

Since dox treatment of iFB-KO mice induces the knockout of NMDA receptor function in the excitatory neurons of forebrain regions including the cortex, hippocampus, and striatum, we currently do not know the precise anatomical site(s) involved in storing remote fear memories. Given the lack of strong evidence for striatal contribution to fear memories, it is unlikely that our genetic manipulation of the NMDA receptor in the striatum plays a significant role in the observed phenotype. In addition, the commonality of storage deficits in both contextual and cued memories excludes the hippocampus as a crucial site of action since cued fear memory is known to be independent of the hippocampus. This view is further supported by the fact that our inducible knockout of NR1 did not occur until 6 months after initial training, a time course far exceeding the temporally limited role of the hippocampus in processing contextual memory (less than 1 month in rodents) (Kim and Fanselow, 1992; Anagnostaras et al., 1999; Shimizu et al., 2000).

Based on previously conducted lesion studies, the neocortical regions may serve as the primary storage site(s) for fear memories. While the exact storage site within the cortex has not been clearly defined, the entorhinal and/or perirhinal cortical regions may play a particularly important role in the storage of fear memories. Notably, posttraining lesions of these sites produce severe retention deficits, whereas removal of the visual cortex, medial prefrontal cortex, or insular cortex has no obvious impact on fear memory (Rosen et al., 1992; Corodimas and LeDoux, 1995; Suzuki, 1996; Bucci et al., 2000; but see Romanski and LeDoux, 1992a, 1992b). However, due to the relatively short intervals between training and lesion in those lesion experiments (typically within 2–3 days after training), memory deficits observed following lesion of the perirhinal/entorhinal cortex could have arisen from the disruption of either consolidation or storage in those areas. In the future, similar experiments using longer intervals between training and lesion (e.g., at least 1 month after training) will be valuable for discriminating the storage effect from the consolidation process. Alternatively, development of cortical subregion-specific knockout systems (e.g., perirhinal or entorhinal cortex-specific knockout) will be required to address this issue.

Although our knockout did not appear to affect the amygadala region, it is nonetheless important to consider the possibility that the amygdala may participate in the storage of both contextual and cued fear memories. For example, lesions of the basal lateral amygdala 1, 14, or 28 days after training impair both contextual and acoustic fear memory (Maren et al., 1996). Since the amygdala is known to control fear responses, it is not clear whether the memory deficits produced by these

posttraining lesions reflect the blockade of the behavioral freezing responses or the destruction of stored fear memories.

Possible Molecular and Cellular Mechanism for the Storage of Remote Memories

How might the NMDA receptor maintain and stabilize the stored remote memories? One explanation might be that it is the physical presence, not the activity, of the NMDA receptor at synapses that is responsible for memory retention. However, the transient deletion of the NMDA receptor in the forebrain of iFB-KO mice by 1-week dox treatment had no effect on the storage of remote fear memories, thus nullifying this scenario.

Alternatively, since the activation of the NMDA receptor is crucial for its physiological function, periodic reactivations of the NMDA receptor at the synapse may constitute an indispensable synaptic reentry reinforcement (SRR) mechanism for the dynamic maintenance of remote memory traces. Under this SRR scenario, the strongly connected neurons will reactivate together to stay strongly connected, whereas the weakly connected neurons tend not to reactivate together and thereby remains weakly connected. By serving as a cellular means for monitoring and ensuring accurate replacement of synaptic molecular machinery, these reactivations could exert the quality-control mechanism to prevent turnover-caused deleterious drift in synaptic efficacy over time.

This view is further elaborated by our recent computational analysis of the synaptic reinforcement process in a neural network that stores memory traces (Wittenberg et al., 2002). Our simulation using a network consisting of 2500 neurons shows that periodic NMDA receptor reactivations can indeed provide an effective way for the circuit to overcome the synaptic drift. Without such reactivation-mediated SRR, synaptic efficacies cannot be stably preserved in face of receptor turnover. Consequently, the stored memory traces gradually become unreliable, thereby undermining long-term storage of information in the brain.

Our experimental data here is not only consistent with this view, but also reveals the temporal duration required for detrimental manifestation of such synaptic drift in the absence of the NMDA receptor. Since the 1-month, but not 1-week, dox feeding was sufficient to disrupt the storage of remote fear memories, these results allow us to estimate that multiple rounds of receptor turnovers would be required before synaptic errors accumulate to the critical level that would compromise the integrity of stored memories.

Currently, little information is available regarding what triggers the reactivation of the NMDA receptor during the memory storage. One triggering mechanism could be spontaneous recall; however, achieving stable memory storage by such a mechanism would require the systematic recall of all past remote experiences. A more likely triggering mechanism may depend on sleep, a process that would not necessitate sequential or orderly reactivation of the memory traces (Maquet, 2001; Siegel, 2001; Stickgold et al., 2001). In fact, our computation simulation suggests that pair-wise reactivation of coupled neurons would be sufficient to reactivate the NMDA receptors, thereby achieving the long-term stabilization of synaptic connections (Wittenberg et al., 2002; Wittenberg and Tsien, 2002).

In conclusion, using an inducible, reversible, and region-specific gene knockout technique, we have revealed a hitherto unrecognized role of the NMDA receptor in the ongoing preservation of the stored remote memory traces in the brain. We propose that periodic NMDA receptor reactivation is the key synaptic reentry reinforcement (SRR) mechanism for dynamically and stably preserving long-term stability of the brain.

Experiments Procedure

Production and Genotyping of Inducible Knockout Mice

Construction of conditional knockout mice was the same as described previously for inducible and CA1-specific knockout mice (Shimizu et al., 2000). All our transgenic mice were produced on BCF hybrid background (B6 × CBAF1) (Shimizu et al., 2000). The original floxed NR1 heterozygous mice produced on the hybrid background 129 and B6 (Tsien et al., 1996a) have also been backcrossed to the BCF hybrid strain for at least 20 generations. The iFB-KO mice are homozygous for the floxed-*NR1* gene and heterozygous for the CaMKII-*Cre* transgene, the *NR1-GFP* transgene under control of the tet-O promoter, and the tetracycline transactivator (*tTA*) transgene, which is driven by the β-actin promoter and contains a floxed stop sequence (*fNR1/fNR1*, *Cre/+*, *tTA/+*, and *NR1-GFP/+*). In our experiments, the inducible switch off and on event occurred at the age of 8 months or older when we fed the mice with food pellets containing dox at 6 mg/g. Mice showed no preference or dislike for the dox-containing food and consumed approximately the same amount in comparison to the regular food pellets. The littermates lacking the Cre gene (*fNR1/fNR1*, *tTA/+*, *NR1-GFP/+*; or *fNR1/+*, *tTA/+*, *NR1-GFP/+*) were used as control mice. For our experiments, both male and female mice were equally used at the ratio of about 50:50.

For genotyping, Southern blot method was used to detect the floxed *NR1* gene and the protocol is the same as described (Rampon et al., 2000; Tang et al., 1999). About 10 μg purified tail DNA were digested by EcoR I, fractionated by electrophoresis on 0.7% agarose gels, and transferred onto Zeta-probe GT membranes (BioRad). A 1.2 kb DNA fragment of 3′ NR1 gene probe was labeled by [α-^{32}P]dCTP and hybridized to the GT membranes. For PCR detection of the *Cre, tTA*, and *NR1-GFP* transgenes, approximately 0.5 to 1 μg of mouse tail DNA was amplified in PT100 thermal cycler using the programs as follows: 1 min, 94°C; 45 s, 55°C; and 1 min, 55°C for 35 cycles. The primers for *Cre* detection are 5′-AGA TGT TCG CGA TTA TC and 5′-AGC TAC ACC AGA GAC GG; for *tTA* detection are 5′-CAA TTA CGG GTC TAC CAT and 5′-GGT TCC TTC ACA AAG ATC CTC; and for *NR1-GFP* detection are 5′-GGT AGA GCA GAG CCC GAC CCT and 5′-GTA TCT GGA AAA GCA CTG, respectively. The size of specific PCR products is 490 bp for *Cre*, 450 bp for *tTA*, and 400 bp for *NR1-GFP*.

LacZ Staining to Detect the Cre Expression Pattern

Mice carrying Tg-*Cre* were crossed with β-actin promoter loxp-stop-loxp-*LacZ* transgenic mice (Tsien et al., 1996b). To closely match the pattern of Cre/loxP recombination in iFB-KO mice, we used the iFB-KO mice to cross with *LacZ* reporter. The double transgenic mice (carrying Tg-*Cre* and Tg-*LacZ*) were examined at the age of 8 months. Brain sections were stained by X-gal solution (in 10 ml of PBS, add 200 μl 0.2 mol $K_4Fe(CN)_6 \cdot 3H_2O$ and 0.2 mol $K_3Fe(CN)_6$; 20 μl of 1 mol $MgCl_2$; 80 μl of 125 mg/ml x-gal (in Dimethylformamide) at 37°C for 3 hr. The sections were washed twice by PBS and water, respectively, and stained in 1% eosin for 3 min. The sections were dipped successively in 50%, 70%, 80%, 90%, and 100% ethanol for 30 s each, followed by xylene 1 min. For storing the slides, 1 drop of Permount was put on the section and covered with cover glass.

In Situ Hybridization

The protocol for in situ hybridization is similar to the one described previously (Rampon et al., 2000). The antisense 42-mer oligonucleo-

tide probe (5′-TCT ACC ACT CTT TCT ATC CTG CAG GTT CTT CCT CCA CAC GTT), which recognizes the exon 20 of NR1 gene, was end-labeled with [α-^{35}S]dATP (50 μCi, Amsham) by terminal transferase (Roche). Fresh brain tissue was frozen by fine dry ice powder and sectioned to 20 μm by using cryostat. Prior to hybridization, the sections were fixed with 4% paraformaldehyde (PFA) in 0.1 M PB (pH 7.2) for 20 min, washed twice for 5 min each in 0.1 M PB (pH 7.2), and then subjected to acetylation for 10 min followed by wash in deionized water for 5 min before dehydration by successively incubating for 1 min in 80%, 90%, and 100% ethanol. The brain sections were hybridized with the oligonucleotide probe (1.0×10^5 cpm/slide) at 48°C for 12 hr. Sections were then washed in 0.2× SSC at 65°C for 1 hr and then in 0.1× SSC at room temperature. All washing solutions contained 10 mM DTT. KODAK MR films were used to expose for 2–3 weeks for autoradiography. The image was obtained by Epson scanner.

Immunostaining for the Detection of NR1-GFP Protein Expression

Tribromoethanol (avertin) were used for anesthesia, the animals were transcardially perfused with 4% PFA in PBS buffer, and the brain was removed and stored overnight at 4°C in the 4% PFA solution and then transferred to a 30% sucrose (in PBS) solution, where it was stored at 4°C for at least 48 hr. The sagittal sections were sliced at 25 μm using a cryostat and floated in PBST solution. The brain slices were successively incubated in three solutions: (1) the primary antibody, rabbit antibody against GFP (1/2500, Clontech 3867-1) overnight, (2) the secondary antibody, biotinylated goat anti-body to rabbit (1/2000, Jackson Immunoresearch Lab, 111-065-003) for 90 min, and (3) avidin-conjugated horseradish peroxidase (HRP) (ABC reagents PK6100) complex (1/1000, Vector Elite Kit, Vectastain) for 90 min in PBST. Prior to each of these incubations, the slices were washed three times for 15 min each in PBST. The GFP immunostaining was revealed by immersing the sections in a solution of 3-3′ diaminobenzidine (DAB substrate kit for peroxydase, Vectastain) containing H_2O_2 (0.003%) and nickel (0.6%) (DD water was used to make DAB solution instead of PBST) for 15 min followed by three times wash in PBST. Neurons containing GFP appeared black in both their soma and their processes.

Entorhinal Cortex Slice Recording

Adult mice were sacrificed by cervical dislocation and the brain was quickly removed, and horizontal slices (400 mM thick) were prepared on a vibratome (OTS 3000, EMS, Inc.) in ice-cold modified Ringer's solution (in mM: sucrose 212.5; KCl 3.5; KH_2PO_4 1.2; $MgSO_4$ 1.3; $CaCl_2$ 2.4; $NaHCO_3$ 26, glucose 10) saturated with 95% O_2 and 5% CO_2. Slices were then incubated in oxygenated 95% O_2 and 5% CO_2 normal Ringer (125 mM NaCl instead of 212.5 mM sucrose) for 1–8 hr at 34°C before recording. After the recovery period, slices were transferred one at a time to a submersion chamber for recording.

Field excitatory postsynaptic potentials (fEPSPs) were recorded in the layer III of entorhinal cortex in response to layer II stimulation by a concentric bipolar stimulating electrode (FHC). Extracellular recording electrodes were pulled from thin-wall glass capillaries (OD 1.2 mm, ID 0.9 mm, FHC) and filled with normal Ringer's solution (with tip impedance less than 2 MΩ). Paired pulses with an interpulse interval of 40 ms interval were delivered as test stimulus via a stimulus isolator (ISO-Flex, A.M.P.I), driven by a Master-8 (A.M.P.I.) at a frequency of 0.017 Hz. The pulse was 0.05–0.20 nA, 50 μs duration, adjusted so that half of the maximal EPSP was elicited as by first pulse (the baseline response). This two-pulse protocol (40 ms interval) is also used for paired pulse facilitation measurement. LTP was induced by two trains of 100 Hz with 1 s duration and 5 s interval. Data were collected by Axon Clamp 8.2 (Axon, Inc.). Paired pulse facilitation (PPF) was measured as a ratio of EPSP slope evoked by the second pulse over the EPSP slope elicited by the first pulse. LTP was measured as a ratio of slopes of EPSP at the last 10 min (30–40 min) after tetanic stimulation over the slopes of EPSP during the 10 min before tetanic stimulation.

Fear Conditioning Task

We used a fear conditioning shock chamber ($10 \times 10 \times 15$ high inches) and TruScan multiparameter activity monitors (Coulbourn Instrument), and the procedure were similar to the published ones (Rampon et al., 2000; Tang et al., 1999). The young adult mice (2- to 4-months-old) were handled for 3 days and then habituated to the training chamber for 5 min 1 day before the training began. The CS used was an 85 dB sound at 2800 Hz, and the US was a continuous scrambled foot shock at 0.75 mA for 2 s for 3 times (with 1 min interval). After the third CS/US paring, the animal was allowed to stay in the chamber for another 30 s for the measurement of immediate freezing. Freezing was judged as complete immobile of the body except for the respiratory movements. The animals were returned to their home for 9 months. During retention tests, each mouse was placed back into the same training chamber and the freezing responses were recorded and scored at every 5 s for 5 min (contextual freezing). Subsequently, the mice were put into a novel chamber and monitored for 3 min before the onset of the tone (pre-CS). Immediately after that, a tone identical to that in the training session was delivered for 3 min and freezing responses were recorded (cued conditioning). Freezing responses were time sampled at every 5 s for duration of 3 min. Student's t test was used to determine genotype effects on the freezing responses.

Novel Object Recognition Task

The experimental protocol was the same as described previously (Rampon et al., 2000; Tang et al., 1999). Briefly, mice were individually habituated to an open-field box ($20 \times 20 \times 10$ high inches) for 3 days. During the training sessions, two novel objects were placed in the open field, and the animal was allowed to explore for 15 min. The time spent exploring each object was recorded and found to be the same (data not shown). During the retention tests, the animal was placed back into the same box, in which one of the familiar objects during training was replaced by a novel object, and allowed to explore freely for 15 min. A preference index, a ratio of the time spent exploring any one of the two objects (training session) or the novel one (retention session) over the total time spent exploring both objects, was used to measure recognition memory. Student's t test was used to determine genotype effects on the behavioral responses.

Open Field and Rotarod Tests

For the measurement of the open field activity, mice were placed in an open field, made of a 14×14 inches black box. The box was marked by 2×2 inches small square grid (7 squares by 7 squares with 49 squares in total). The open field activity of animals was measured by the number of crosses that the mice have passed within the 3 min period. For the measurement of rotarod test, the mice were placed on an accelerating rotating wood-rod. The rod is 12 inches long and 1 inch in diameter. The initial rotation speed was 4 rpm and then steadily accelerated to 40 rpm. The performance was measured by the amount of time (in seconds) that mice managed to remain on the rotating rod. Student's t test was used to determine the significance of those behavioral measurements.

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