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Cellular and systems mechanisms of memory strength as a constraint on auditory fear reconsolidation

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Memory reconsolidation has been demonstrated in various tasks and species, suggesting it is a fundamental process. However, there are experimental parameters that can inhibit reconsolidation from occurring (boundary conditions). These conditions and their mechanisms remain poorly defined. Here, we characterize the ability of strong training to inhibit reconsolidation at the behavioral, systems and molecular levels. We demonstrate that strong memories in rats initially are resistant to reconsolidation, but after sufficient time will undergo reconsolidation, suggesting that boundary conditions can be transient. At the systems level, we show that the hippocampus is necessary for inhibiting reconsolidation in the amygdala. At the molecular level, we demonstrate that NR2B NMDA-receptor subunits which are critical for the induction of reconsolidation of auditory memories in the amygdala, are downregulated only under conditions when strong memories do not undergo reconsolidation. This suggests that one molecular mechanism for mediating boundary conditions is through downregulation of reconsolidation induction mechanisms.

Memories not only undergo a time-dependent process of stabilization after the initial learning, which is called consolidation[1]; they can also undergo another restabilization process after reactivation that typically entails presentation of a training-related stimulus to call up the memory[2,3], which is now called reconsolidation[4–7]. The memory process induced by reactivation of consolidated memories meets the standards of being a consolidation process[8]. There have been many demonstrations of reconsolidation across species, tasks and amnesic agents, suggesting that it is a fundamental process. However, reconsolidation is not ubiquitous. There are experimental conditions under which reconsolidation does not seem to occur. These conditions, which we define as boundary conditions on reconsolidation, have recently drawn research attention.

A number of boundary conditions have been suggested, such as trace dominance between cues that no longer predict the occurrence of a reinforcement (extinction) and reconsolidation[9–11], memory age[12,13], directly versus indirectly activated memories[14] and training strength[12]. However, comprehensive descriptions of the behavioral conditions and the mechanisms for inhibiting reconsolidation under specific parameters are limited. Further, for each condition—extinction[15,16], strength of training[17,18] and age[19]—there are contradictory findings (for review see ref. 8).

One source probably contributing to the observed inconsistencies is that the typical logic used to conclude that a boundary condition exists is through challenging a memory's sensitivity to post-reactivation amnesic agents under one set of experimental parameters. If memory disruption is not observed, then it is concluded that the memory does not undergo reconsolidation under those conditions. Several reports, however, have demonstrated that a memory may undergo reconsolidation only under specific reactivation conditions[12,20,21]. The implication of these findings is that it is extremely difficult to conclude on the basis of behavioral studies that a memory never undergoes reconsolidation. Do the negative effects upon which the boundary conditions are based imply that a given memory never undergoes reconsolidation, or is the memory still capable of undergoing reconsolidation with another reactivation protocol? Given that the parameter space of possible reactivation procedures is essentially infinite, a real boundary condition is very difficult to prove at the behavioral level. This is likely to be part of why there is so much inconsistency in the field of boundary conditions[8].

Here we have taken a complementary approach to identify some of the molecular mechanisms by which boundary conditions inhibit reconsolidation from occurring. If a molecular or conceptual definition of how they are manifested in the brain could be identified, then we could make strong predictions concerning when we should see these molecular mechanisms expressed. For example, if strong memories represent real boundary conditions, then the putative mechanisms used to inhibit reconsolidation from occurring should only be expressed after strong but not weak training. This strategy would significantly complement the behavioral studies described above in their search for true boundary conditions and help resolve some of the conflicting findings in the field.

An understanding of how boundary conditions are mediated across levels of analysis is critical because targeting reconsolidation of traumatic memories has been proposed to be a potential treatment for many psychopathologies, including post-traumatic stress disorder (PTSD)[19,22]. For PTSD, blocking the reconsolidation of traumatic memories might weaken the long-term maintenance of these traumatic memories, in turn reducing PTSD pathology. However, if strong aversive experiences act as a boundary condition on reconsolidation[12],

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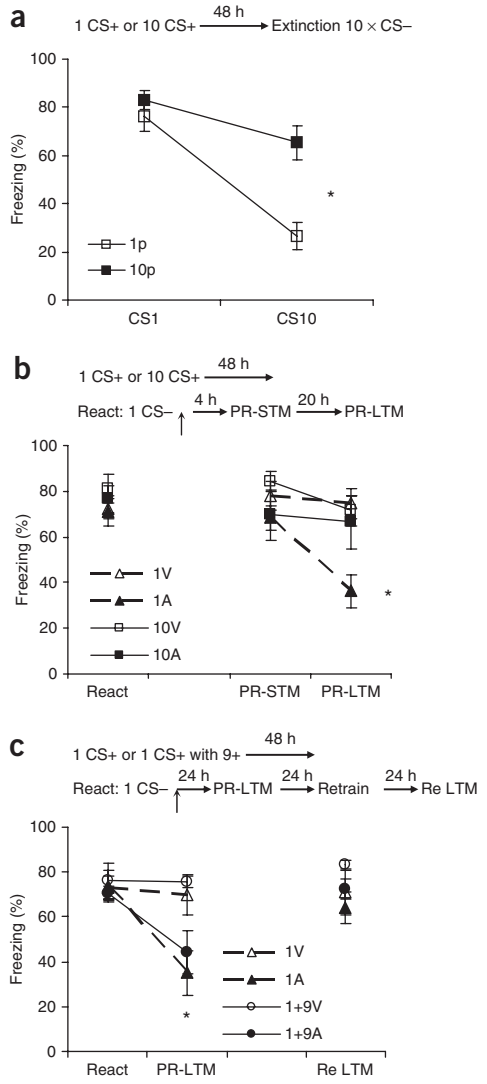

Figure 1 Strong auditory fear memories are insensitive to anisomycin 2 d after training. (**a**) Top: behavioral protocol. Separate groups of rats received one or ten tone–shock pairings (1P/1CS+ or 10P/10CS+). Two days after training they received extinction sessions (CS–). At the end of the extinction session, rats trained with 10P had significantly more freezing. Freezing percentage was defined as percentage of time during tests that the animal stayed immobile except for breathing. (**b**) Top: behavioral protocol. LBA-cannulated rats received either 1P or 10P. Two days after training, the memory was reactivated (React) with one unreinforced tone (1CS–) immediately followed by intra-LBA infusions (vertical arrow) of vehicle (V) or anisomycin (A). PR-STM and PR-LTM tests were done at 4 h and 24 h after reactivation, respectively. All groups (1V, 1A, 10V, $N = 7$ per group and 10A, $N = 8$) froze similarly during reactivation and PR-STM. Although anisomycin blocked PR-LTM in the 1P group (1V > 1A), it, however, did not impair PR-LTM in the 10P group (10V ≈ 10A). (**c**) Top: behavioral protocol. Rats received either 1P or 1P followed by nine unsignaled footshocks (1 CS+ with 9+). Post-reactivation anisomycin infusion blocked both groups' PR-LTM compared to vehicle controls (1V and 1+9V > 1A and 1+9A; $n = 6, 7, 6, 8$, respectively). When rats were retrained, they were capable of maintaining the retrained long-term memory (Re LTM). *$P < 0.05$. Means ± s.e.m.

then this would suggest that the traumatic memories in PTSD patients may be resistant to undergoing reconsolidation, negating reconsolidation as a therapeutic target. Therefore, it is critical to determine what the optimal conditions are to allow an extremely strong fear memory to undergo reconsolidation.

To this end, we show that strong auditory fear memories initially did not undergo reconsolidation but did over time, suggesting that the boundary condition induced by strong training is transient. The time course resembled the time course over which contextual fear memories are thought to be transformed from a hippocampus-dependent to hippocampus-independent memory[23]. We hypothesized and found that the hippocampus inhibited the auditory fear memory from undergoing reconsolidation in the lateral and basal amygdala (LBA). On the basis of our previous findings[24], we hypothesized that one principle that could mediate boundary conditions is downregulation of the mechanisms that allow memories to undergo reconsolidation. Using two complementary methods, we demonstrated that NR2B expression in the LBA, which is critical for the induction of fear reconsolidation but not the expression of fear[24], was reduced under conditions when memories did not undergo reconsolidation and was normal when memories underwent reconsolidation.

RESULTS

Recent strong memories do not undergo reconsolidation

We first determined whether the strength of auditory fear memories acquired with ten tone–shock pairings (10P) was stronger than with one pairing (1P). Separate groups of rats were conditioned with either 1P or 10P and then received multiple extinction trials in a single session (see Online Methods). Extinction reduced freezing significantly more in the 1P group than in the 10P group (group by trial interaction $F_{1,6} = 9.28$, $P < 0.05$; **Fig. 1a**). This indicates that the 10P memory was stronger than the 1P memory.

To test whether these stronger memories underwent reconsolidation, 2 d after conditioning the auditory fear memory was reactivated in a context different from the context used for training and followed by intra-LBA infusion of anisomycin or its vehicle. Post-reactivation short-term memory (PR-STM) and post-reactivation long-term memory (PR-LTM) tests, assayed by conditioned freezing[25], were given 4 and 24 h later, respectively. The results showed that the strong memory was not sensitive to anisomycin challenge (**Fig. 1b**). A two-way analysis of variance (ANOVA) on reactivation performance showed no significant training effect (1P versus 10P), drug effect (vehicle versus anisomycin), or training by drug interaction (all $F_{1,25} < 1.5$, $P > 0.25$). A three-way, one-repeated ANOVA comparing training, drug and test (PR-STM versus PR-LTM, repeated measure) showed a significant three-way interaction ($F_{1,25} = 7.68$, $P = 0.01$). Further analyses revealed that all groups had comparable PR-STM scores (all $F_{1,25} < 2.5$, $P > 0.1$). At PR-LTM, however, only the 1P-anisomycin group showed significantly impaired performance compared to the other groups (*post hoc* tests, all $P < 0.02$) which did not differ from each other (all $P > 0.4$). These data are consistent with the possibility that strong training either inhibited the memory from undergoing reconsolidation or made it more difficult for auditory fear memories to undergo reconsolidation 2 d after training[12]. The negative finding in the 10P group demonstrates that anisomycin infusion did not induce damage that was sufficient to compromise behavioral functions[26].

The boundary condition is due to increased associative strength

We asked whether the inability of 10P memories to undergo reconsolidation was due to the learning or to some non-associative factor caused by multiple footshocks. We trained two groups of rats with a single tone–footshock pairing followed by nine un-signaled, unpaired footshocks (that is, 1+9UP). Two more groups were trained with 1P

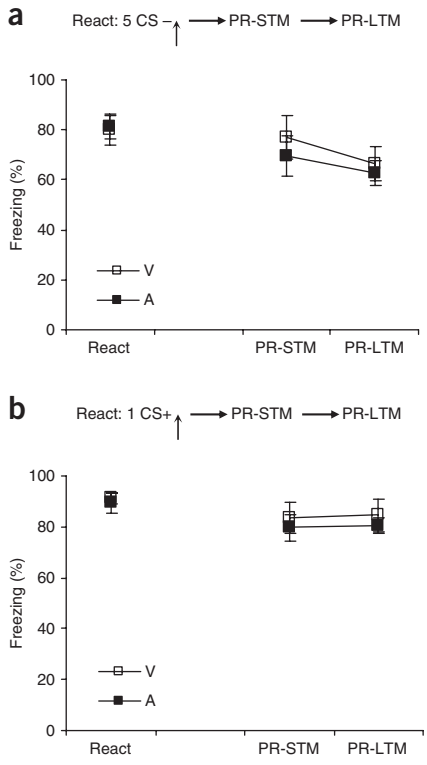

Figure 2 Alternative reactivation protocols are not sufficient to make the strong auditory fear memories sensitive to anisomycin. Top subpanel of each panel represents the behavioral protocol, which is similar to **Figure 1b** except for the reactivation procedure. All LBA-cannulated rats received ten tone–footshock pairings. The memory was reactivated 2 d later with five unreinforced tone presentations (5CS–, $n = 7$ per group) (**a**) or 1 tone–footshock pairing (1CS+, $n = 6$ per group) (**b**). In both cases, post-reactivation anisomycin (A), compared to vehicle (V), infusion did not block post-reactivation short-term and long-term memory in the strongly trained rats. Means ± s.e.m.

and served as positive controls. If greater associative strength inhibits memories from undergoing reconsolidation, then the 1+9UP memory, sharing a similar associative strength with the 1P memory, should undergo reconsolidation. Results showed that the 1+9UP memory underwent reconsolidation (**Fig. 1c**). A three-way, one-repeated ANOVA comparing training, drug and test (reactivation versus PR-LTM, repeated measure) showed a significant drug by test interaction ($F_{1,24} = 11.98$, $P = 0.0002$). Further analyses showed all groups had similar performance at reactivation (that is, no training effect, drug effect or training by drug interaction, all $F < 1$). An analysis of PR-LTM showed that both anisomycin groups were impaired compared to both vehicle groups ($F_{1,24} < 15.93$, $P < 0.001$). There was neither main effect of training nor an interaction between training and drug (both $F < 1$).

All rats were retrained with 1P. The long-term memory for this relearning was tested on the next day. All four groups showed similar long-term memory of relearning (that is, no effect of training history, drug history, or training by drug interaction, all $F_{1,24} < 2.9$, $P > 0.1$). This again demonstrates that memory impairment caused by anisomycin is unlikely due to LBA damage[26].

These results demonstrate that auditory fear memories formed after 1P followed by nine unsignaled footshocks is as labile as memories formed after 1P alone. We conclude that the resistance of 10P memories to undergoing reconsolidation is due to the stronger association (**Fig. 1c** versus **Fig. 1b**).

Other reactivation protocols do not induce reconsolidation

The above findings suggest that either strong memories do not undergo reconsolidation or it is harder to induce reconsolidation of strong memories. To partially address this, we asked whether reconsolidation of strong memories could be induced with other reactivation protocols. One reactivation protocol was to extend the tone presentation by giving five tone presentations without footshock[12]. The second protocol used

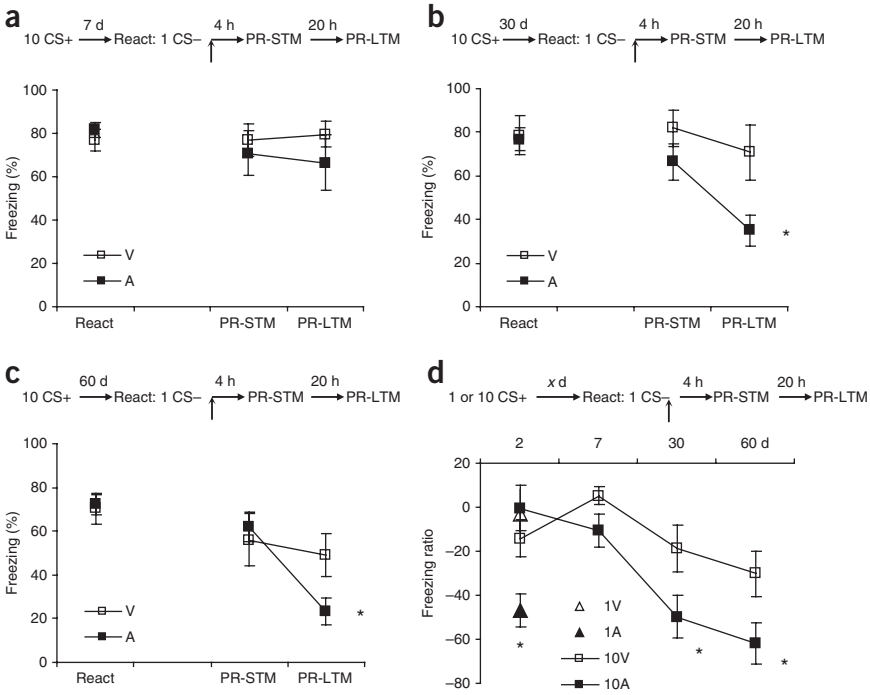

Figure 3 Strong memories undergo reconsolidation at 30 and 60 d, but not 7 d, after training. Top subpanel of each panel represents the behavioral protocol. (**a**–**c**) Separate groups of rats were LBA-cannulated and trained with ten tone–footshock pairings. The memory was reactivated at 7 (**a**), 30 (**b**) or 60 (**c**) days after training. Intra-LBA anisomycin infusion (vertical arrow) after memory reactivation with 1 tone (1CS–) impaired the PR-LTM only when the memory was reactivated at 30 and 60 d but not 7 d after training. In all cases, the reactivation was similar and PR-STM was intact in the anisomycin (A) rats compared to the vehicle (V) rats. (**d**) Strong memories undergo reconsolidation over time. For the purposes of comparison, the data in **Figures 3a**–**c** and **1b** were converted to a freezing ratio, (PR-LTM – PR-STM)/PR-STM) × 100%. Intra-LBA anisomycin infusion impaired the PR-LTM only when the strong memory was reactivated at 30 and 60 d after training. Each data point represents separate groups of rats (data for 2 d were adapted from **Figure 1b**; $n = 7$ per group for 7 d; $n = 5$ (10V), 7 (10A) for 30 d; $n = 8$ per group for 60 d). *$P < 0.05$. Means ± s.e.m.

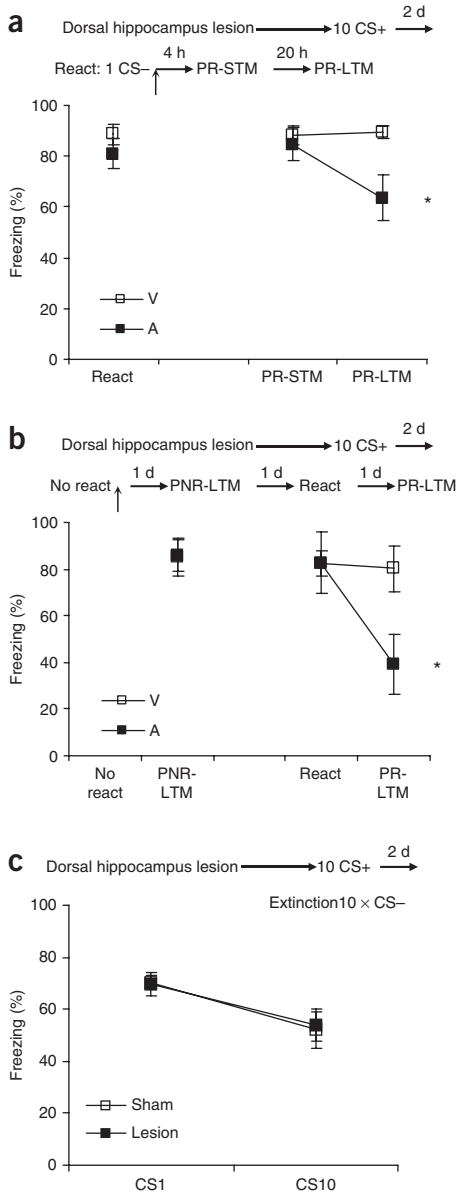

Figure 4 Pretraining dorsal hippocampus lesions cause strong fear memory to undergo reconsolidation in the LBA. Top: behavioral protocol. All rats received electrolytic dorsal hippocampus lesion when cannulae were implanted. After recovery from surgery, they received 10 tone–footshock pairings. (**a**) Post-reactivation anisomycin (A, $n = 7$) infusion in LBA did not block PR-STM but did impair PR-LTM compared to the vehicle infusion (V, $n = 7$). (**b**) When the memory reactivation was omitted before the drug infusion, the PNR-LTM was comparable in vehicle (V, $n = 6$) and anisomycin (A, $n = 4$) groups. The same rats further received memory reactivation and were divided in two subgroups in a counterbalanced manner ($n = 5$ per group for vehicle or anisomycin infusion). The group assignments yielded a comparable baseline (see the text). The PR-LTM then was impaired by intra-LBA anisomycin when the drug was contingent on the memory reactivation (React). (**c**) Rats received hippocampus or sham lesion ($n = 7$ per group) followed by 10 pairings and an extinction session (10CS–). The two groups showed comparable extinction rates. $^*P < 0.05$. Means ± s.e.m.

was a reinforced trial that has been shown to induce reconsolidation in the LBA[27]. This should be a very strong reactivation because the LBA neurons mediating the memory will be reactivated by sensory input from both tone and footshock afferents to the LBA.

Results showed that neither reactivation protocol was sufficient to detect an anisomycin impairment (**Fig. 2**). When the reactivation trial contained five tone presentations, the PR-LTM was still normal in the anisomycin group ($F < 1$, **Fig. 2a**) as it was when another pairing was used to reactivate the memory ($F < 1$, **Fig. 2b**).

Strong training boundary condition is transient

In clinical settings, PTSD patients have experienced extremely aversive past events. Often years will have passed between the trauma and the opportunity for intervention (R. Pitman, personal communication). Therefore, we asked whether the time between training and memory reactivation would interact with a strong memory's inability to undergo reconsolidation. To this end, we increased the time between strong training and reactivation. When the strong memory was reactivated 7 d after training, post-reactivation anisomycin infusions did not impair PR-LTM ($F < 1$, **Fig. 3a**). However, when the memory was reactivated 30 or 60 d after training, anisomycin infusions induced a behavioral impairment at PR-LTM (30 d: $F_{1,10} = 7$, $P < 0.02$, **Fig. 3b**; 60 d: $F_{1,14} = 5.19$, $P < 0.04$, **Fig. 3c**) but not PR-STM. To summarize the relationship of the interval between training and reactivation and whether the strong memory undergoes reconsolidation, we standardized the behavioral results as a freezing ratio (**Fig. 3d**). Anisomycin did not induce any detectable impairment 2 or 7 d after training but did 30 and 60 d after training (a significant group by day interaction, $F_{3,49} = 2.83$, $P < 0.05$). *Post hoc* tests showed that, compared to day 2, the significant group differences only emerged at days 30 and 60, not day 7 ($P < 0.03$, $P < 0.01$ and $P > 0.5$, respectively).

The freezing percentage during reactivation at 2, 7, 30 or 60 d after strong training did not change significantly and did not differ between vehicle and anisomycin groups (both $F < 1$). Moreover, the freezing elicited by the test context before the onset of the tone is also consistent across days and between groups (both $F < 1$, **Supplementary Fig. 1** online). This suggests that the age of a memory at the time of reactivation interacts with its strength and that this interaction determines the memory's susceptibility to reconsolidation.

Dorsal hippocampus is necessary for the boundary condition

If strong training indeed transiently inhibits a fear memory from undergoing reconsolidation, why would this time course resemble the time course of systems consolidation, in which the hippocampus has been proposed to play a time-limited role[23,28–30]? Specifically, a lesion of the dorsal hippocampus 1 d after training impairs contextual, but not auditory, fear conditioning. However, the same lesion has no effect on memory retention if made 28 d after training[23]. Auditory fear conditioning usually also leads to contextual fear[31]. Therefore, it is possible that the strong contextual fear, acquired with strong training, would inhibit the ability of memories in the LBA to undergo reconsolidation for as long as the contextual memory is hippocampus dependent. Once the contextual memory is putatively hippocampus independent, this could allow the strong fear memory in the LBA to undergo reconsolidation. This inhibition could be mediated through the LBA's connections with the hippocampus[32,33]. The ability of the hippocampus to modulate the amygdala's plasticity has been proposed[34].

To test whether the systems consolidation of contextual fear memory imposes the strong training boundary condition, we applied electrolytic dorsal hippocampus lesions to the rats before strong training. We chose this lesion method on the basis of its effect on causing temporally-graded amnesia of contextual, but not auditory, freezing[17,23]. We predicted that if the dorsal hippocampus is critical for inhibiting new strong fear memories from undergoing

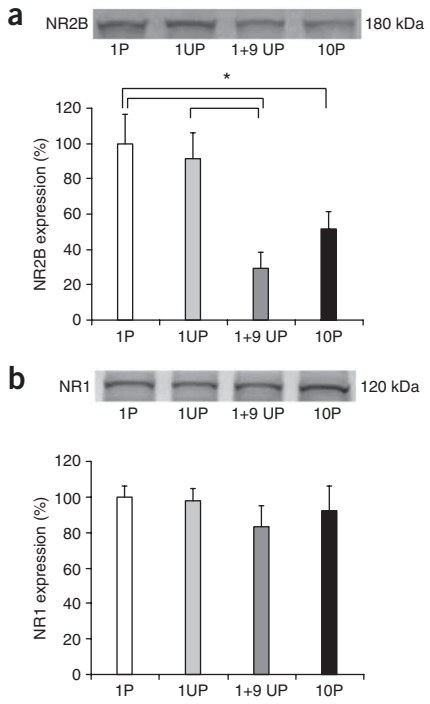

Figure 5 NR2B-subunit abundance is inversely related to the ability of strong memories to undergo reconsolidation over time. (**a**) Western blot and quantification of NR2B subunits in the LBA 2 d after training. Rats received one pairing (1P, $n = 7$), one unpaired tone and shock presentation (1UP, $n = 4$), ten pairings (10P, $n = 7$) or one pairing followed by nine (1+9UP, $n = 4$) unsignaled foot shocks. Rats receiving 10P or 1+9UP showed less NR2B than rats receiving 1P or 1UP. (**b**) Western blot and quantification of NR1 subunits in the LBA 2 d after training. Rats received one pairing (1P, $n = 4$), one unpaired tone and shock presentation (1UP, $n = 4$), ten pairings (10P, $n = 4$) or one pairing followed by nine unsignaled foot shocks (1+9UP, $n = 4$). No differences were found. $^*P < 0.05$. Means ± s.e.m.

reconsolidation, then strong memories should undergo reconsolidation 2 d after training in dorsal hippocampus–lesioned rats, a time when the memory does not undergo reconsolidation in intact rats. Dorsal hippocampus lesions did not impair auditory fear memory given comparable freezing at reactivation in unlesioned rats that received strong training (**Fig. 4a** versus **Fig. 1b**, $F_{1,25} < 1.1$, $P > 0.3$; **Supplementary Figs. 2** and **3** online).

We found that strong memories in lesioned rats were sensitive to post-reactivation anisomycin infusions 2 d after training (**Fig. 4a**). Specifically, both anisomycin and vehicle groups had similar freezing percentages at reactivation and PR-STM test (both $F < 1$), whereas the anisomycin group froze significantly less during PR-LTM test than the vehicle group ($F_{1,12} = 6.95$, $P < 0.03$). This is in contrast to results in intact rats, in which the strong memory remained insensitive to anisomycin when it was reactivated 2 d after training.

We then performed the identical experiment in different rats but omitting memory reactivation. Anisomycin had no effect on the post-non-reactivation long-term memory (PNR-LTM) test (**Fig. 4b**, $F < 1$). These data demonstrate that reconsolidation of a strong memory 2 d after training occurs in rats with dorsal hippocampus lesions and is dependent on memory reactivation. We then used these rats, which did not receive a reactivation session, to replicate the reactivation-dependent reconsolidation. One day after the PNR-LTM test, these rats received a reactivation session and were infused with either vehicle or anisomycin in a counterbalanced manner (that is, the vehicle group consisted of equal numbers of animals that had received vehicle and anisomycin in the previous experiment, and vice versa). The group assignment showed comparable performance between groups (rats that were to receive vehicle versus those that were to receive anisomycin) in the previous PNR-LTM test ($F < 1$). The reactivation result showed that both groups had similar freezing to the conditioned tone ($F < 1$). However, a significant impairment of PR-LTM was observed in the anisomycin group ($F_{1,8} = 6.48$, $P < 0.04$). This replicates the previous experiment.

These findings suggest that the dorsal hippocampus actively inhibits strong memories from undergoing reconsolidation 2 d after training. However, if the absence of an anisomycin effect in normal rats is due to a ceiling effect 2 d after training, perhaps the anisomycin sensitivity after lesioning might be due to the hippocampus lesions decreasing freezing to a range in which a putative anisomycin impairment could be behaviorally detected. To directly test this possibility, two groups of rats received either sham or electrolytic lesions of the dorsal hippocampus, followed by 10P and extinction. The lesion and the sham groups showed comparable extinction rates 2 d after strong training (**Fig. 4c**, group by trial interaction $F < 1$). This rules out a ceiling-effect interpretation of our findings.

The molecular mechanism for the boundary condition

What could be the molecular mechanism in the LBA that inhibits reconsolidation of strong memories for up to 30 d after training? Our group recently demonstrated that NMDA receptors containing NR2B subunits are necessary in transforming stable, consolidated memories into labile ones during reactivation[24]. New strong memories show similar properties: normal expression of freezing during reactivation but insensitivity to post-reactivation anisomycin. We reasoned that strong training may downregulate NR2B expression in the LBA, thereby making the memory insensitive to post-reactivation anisomycin infusions but capable of being expressed normally.

The ability of robust fear conditioning to strongly affect NMDA receptor subtypes has already been described[35]. An earlier finding used electrophysiological recordings to show that there is a postsynaptic decrease of NMDA receptors during the maintenance phase of strong fear conditioning. Furthermore, this study used western blots to show that protein expression in the amygdala of NR2B, but not total NR1 NMDA-receptor subunit, is reduced after strong fear conditioning[35]. We measured NR2B in the LBA using quantitative western blots and immunohistochemistry (IHC) under conditions that prevent (2 d) or allow (60 d) reconsolidation to occur after training.

Four groups (1P, 1UP, 1+9UP and 10P) were killed 2 d after training, a time when strong memories do not undergo reconsolidation. Western blot results demonstrated that strong training reduced NR2B expression (**Fig. 5a**, **Supplementary Fig. 4a** online). A one-way ANOVA showed a significant group effect ($F_{3,17} = 5.92$, $P < 0.01$). *Post hoc* tests showed that NR2B expression decreased after both 10P and 1+9UP compared with the 1P control group ($P < 0.05$). We further found NR1 expression comparable for all groups (**Fig. 5b**, **Supplementary Fig. 4b**, $F_{3,12} = 0.501$, $P > 0.6$). This result shows that the decrease of NR2B was selective in the LBA, replicating the previous report[35].

Using IHC, we counted NR2B-containing cells within the lateral and basal amygdala separately (**Supplementary Fig. 5a** online). A two-way ANOVA showed significant group effect ($F_{2,9} = 19.86$, $P < 0.001$), area effect (lateral > basal amygdala, $F_{1,9} = 82.02$, $P < 0.001$) and group by area interaction ($F_{2,9} = 7.76$, $P = 0.01$). *Post hoc* tests revealed that the 1P group had similar NR2B-positive cells compared to the 1UP group ($P > 0.5$). However, strong training significantly reduced NR2B-positive cells compared to either the 1P or 1UP group (both $P < 0.01$).

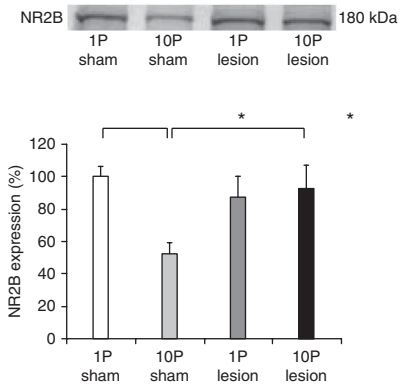

Figure 6 Pretraining dorsal hippocampus lesions prevent the downregulation of NR2B in strongly trained rats. Rats received a pretraining electrolytic dorsal hippocampus lesion or sham lesion. After recovered from the surgery, they were trained with 1 or 10 tone–footshock pairings (1P and 10P, respectively). Two days after training, tissue was extracted from the amygdala for NR2B quantification. The 1P sham ($n = 6$), 1P lesion ($n = 7$) and 10P lesion ($n = 7$) all had comparable NR2B subunit expression. The 10P sham ($n = 7$) group showed less expression of NR2B in the LBA, an effect that was not present in 10P lesion group. *$P < 0.05$. Means ± s.e.m.

We next asked whether this decrease was reversed 60 d after strong training, a time when strong memories undergo reconsolidation. Western blot results showed that NR2B downregulation disappeared 60 d after training ($t_7 = 1.98$, $P > 0.05$, **Supplementary Fig. 6** online), at a time when the strong memory can undergo reconsolidation (**Fig. 5** and **Supplementary Figs. 5** and **6**). IHC results confirmed this (**Supplementary Fig. 5b**). A two-way ANOVA (1P versus 10P in lateral versus basal amygdala) detected insignificant group difference or group by area interaction (both $F < 1$). Overall results demonstrated an inverse relationship between NR2B abundance and the ability of a memory to undergo reconsolidation.

NR2B levels functionally relate to reconsolidation

If the NR2B abundance in the LBA had a functional relationship with whether fear memories undergo reconsolidation, then we predicted that manipulations that allow reconsolidation of strong memories 2 d after training should also prevent NR2B downregulation. We applied pretraining dorsal hippocampus lesions, which allows new strong memories to undergo reconsolidation (**Fig. 4**). Four groups of rats received sham or electrolytic lesions and 1P or 10P.

Western blot results confirmed the prediction (**Fig. 6** and **Supplementary Fig. 7** online): a one-way ANOVA showed significant difference among groups ($F_{3,23} = 4.001$, $P = 0.019$). *Post hoc* tests showed that only 10P-sham group had less NR2B expression than the 1P-sham and 10P-lesion groups ($P < 0.05$). Using IHC (**Supplementary Fig. 8** online), a two-way ANOVA showed significant group ($F_{2,9} = 6.57$, $P < 0.05$) and area effects (lateral > basal amygdala, $F_{1,9} = 75.21$, $P < 0.001$). *Post hoc* tests showed that the 10P-sham group had fewer NR2B-positive cells ($P < 0.05$) than the 1P-sham group. This again replicated the downregulation (**Fig. 5a** and **Supplementary Fig. 5a**). However, the downregulation was absent in the 10P-lesion group.

DISCUSSION

Previous work has demonstrated that reconsolidation is a fundamental phenomenon, but it is not ubiquitous. There are reports of boundary conditions[9,12,13,36]. Here we show that strong training–induced boundary conditions can (i) be transient, (ii) require a separate brain system and (iii) be manifested by downregulation of a mechanism mediating the induction of reconsolidation, which in the case of fear conditioning in the LBA is the NR2B NMDA receptor subunit (**Supplementary Figs. 9** and **10** online). The reduction in mechanisms necessary for reconsolidation is likely to be a graded phenomenon and would be maximally reduced under conditions when the memory is resistant to undergoing reconsolidation using a variety of reactivation protocols. Under moderate conditions—for example, after 5 tone–shock pairings—the NR2B reduction could be more modest, which might leave enough NR2B receptors for the memory to undergo reconsolidation with alternative reactivation protocols.

One possible alternative interpretation of our conclusion that strong training boundary conditions are transient would posit that strong auditory fear memories are initially amygdala independent and then become amygdala dependent over time. However, the amygdala is thought to be always critical for acquisition and consolidation of pavlovian fear memories[37,38]. Even strong memories acquired with 75 shocks are dependent on the LBA[39]. In addition, this alternative interpretation cannot explain why lesions of the dorsal hippocampus make the auditory fear memories sensitive to anisomycin challenge in the LBA.

A second alternative interpretation of the transient boundary condition would suggest that strong memories undergo reconsolidation; however, the freezing level might reach a ceiling and this prevents us from detecting a positive effect of anisomycin at 2 and 7 d after strong training. However, to explain the positive findings at 30 and 60 d after training, this interpretation would have to posit that the levels of freezing decline over 30 d to allow an anisomycin-induced deficit to be detectable. This interpretation has more difficulty explaining the findings that strong memories in rats with dorsal hippocampus lesion are sensitive to post-reactivation anisomycin. This is because lesions of the dorsal hippocampus are not thought to affect the level of auditory freezing[23]. To explain the anisomycin impairment in the dorsal hippocampus lesioned rats, the ceiling effect interpretation would have to posit that the lesions substantially decreased auditory fear to levels at which an anisomycin impairment could be detected. We directly tested this and found no change in the rate of extinction (**Fig. 4c**), suggesting that the lesions did not affect the strength of the memory. This last finding rules out the ceiling effect interpretation of our data.

Lastly, the pattern of findings cannot be explained by nonspecific effects, such as state-dependent learning, or by toxicity, such as apoptosis, due to anisomycin infusions[26]. This is because PR-STM was always intact, and the identical infusions have both negative and positive effects on the long-term memory, depending on the training protocol, the reactivation time after training and the presence or absence of hippocampus lesions. Thus, the most parsimonious interpretation of the data is that the strong fear memory remains consolidated in the LBA and over time can again begin to undergo reconsolidation.

We found that the strong training–induced boundary condition was due to associative effects of the shocks. Memories for a single pairing followed by nine unsignaled footshocks (1+9UP) underwent reconsolidation. This suggests that at the behavioral level, it is the change in associative strength acquired with the ten pairings that induces the boundary condition. At the molecular level, both of these groups showed decreases in NR2B subunit abundance. It could be argued that the ability of the auditory fear memory to undergo reconsolidation in the 1+9UP group, while it also reduces NR2B subunit abundance in the LBA, dissociates amounts of the NR2B subunit from a memory's ability to undergo reconsolidation. In turn, it could be argued that on

the basis of this finding the decrease in NR2B subunit is nonspecific. However, if this interpretation is correct, then there is no reason why a nonspecific effect would reverse over time (60 d after strong straining), be subunit specific (decreased NR2B but not NR1) and be reversed by lesions of the dorsal hippocampus.

An alternative specific interpretation of the decreased NR2B expression in the 1+9UP group is that the protocol induces strong contextual fear conditioning. The decrease in NR2B subunit would serve to inhibit reconsolidation of the contextual memory. Projections of auditory and contextual information are thought to be acquired by different populations of neurons within the LBA[40]. The tissue analyzed in our experiments included both regions. We assume that the reduction in NR2B subunit after 10P occurs predominantly at LBA afferents relaying the 5-kHz frequency of the conditioned stimulus and adjoining frequencies. Similarly, the decreased NR2B expression in the 1+9UP group would predominantly be reduced at the afferents mediating the contextual memory within the LBA. In this case, the 1+9UP should decrease the total NR2B subunits (induced at the afferents mediating the contextual memory), but the abundance of NR2B on the afferents mediating auditory fear conditioning would be sufficient to permit the auditory memory to undergo reconsolidation.

The dorsal hippocampus has been previously shown to be mostly dispensable for the acquisition and expression of auditory fear conditioning[23,41,42]. For this reason, it is unexpected that lesions of the dorsal hippocampus allowed strong memories to undergo reconsolidation in the amygdala 2 d after training. This is not due to nonspecific effects of the lesions, as the rats showed freezing levels comparable to those in unlesioned rats during reactivation, with intact PR-STM scores, and the impairment was only seen when the memory was reactivated. Any nonspecific effects of the lesion, such as increased locomotion, that could compete with freezing would have led to a decrease in freezing during reactivation in both the reactivated and non-reactivated groups. Further evidence for the specificity of dorsal hippocampus lesions on the mechanisms associated with the boundary condition was that the abundance of NR2B subunits in these lesioned rats was comparable to that in sham-lesioned rats with weak 1P training. This cross-region regulation of reconsolidation needs future studies to identify which stage of the training experience the hippocampus is critical for. It is possible that the hippocampus is only involved in the initial training in order to inhibit reconsolidation. Alternatively, the dorsal hippocampus may only be involved in the maintenance of the boundary condition. Attempts to answer these questions are under way.

Because of the novelty of the ability of the hippocampus to affect reconsolidation in the amygdala, we can only speculate as to the nature of the information mediated by the hippocampus that is inhibiting strong new memories from undergoing reconsolidation in the amygdala. Current models of hippocampus functions suggest the involvement of the dorsal hippocampus in the time-dependent reorganization of contextual memories[28]; but see ref. 43). It is possible that during the reorganization of the contextual memory into a remote memory, the strong auditory fear memories consolidated in the LBA are inhibited from undergoing reconsolidation. Over time, however, the memory is thought to become independent of the hippocampus and dependent on the anterior cingulate cortex[29]. Once the memory has become hippocampus independent, it would cease to inhibit reconsolidation within the LBA.

Reconsolidation experiments entail two processes. First, reactivation induces the consolidated memory to return to a labile state. Second, the memory must be reconsolidated from this labile state[8,24]. Recent findings suggest that NR2B subunits must be activated in the LBA during reactivation for the consolidated auditory fear memory to return to a labile state[24]. We found a clear relationship between NR2B expression and the ability of a strong auditory fear memory to undergo reconsolidation in the LBA. NR2B downregulation coincides with time points at which strong memories do not undergo reconsolidation and returns to normal (either passively by the passage of time or by dorsal hippocampus lesion) at times when the strong memories can undergo reconsolidation. It is unlikely that the initial decrease in NR2B subunits was due to increased cellular stress from strong training because (i) NR1 expression was normal in rats that received strong training and (ii) NR2B expression was normal in hippocampus-lesioned rats. Last, the finding that dorsal hippocampus lesion did prevent both the strong training boundary condition and the decrease in NR2B expression demonstrates a functional relationship between these two factors.

The reduced NR2B but normal NR1 expression is congruent with an earlier report using physiological recording and western blot[35]. The authors suggested that one protective effect on the strong memory that results from the downregulation of NMDA receptors would be that it would prevent the acquisition of new fear memories that could interfere with the original strong memory. This downregulation may be a homeostatic response to overstimulation[44]. Another effect on a recently acquired strong fear memory of decreasing NR2B subunits in the LBA is that it would prevent the strong fear memory from returning to a labile state during which it could be changed or weakened. Thus, the very strong memory is protected for some time from interference. Substantial downregulation of NMDA receptors is also seen during development, often at the end of a critical period[45,46]. Therefore, decreasing NMDA receptor abundance could be a general mechanism by the brain to preserve the learned experience and reduce the potential interference from future events. This reduction would, theoretically, compromise any computations, memory-related or not, performed by afferents with a very low abundance of NR2B subunits.

The insensitivity of the strong memory to anisomycin 2 d after training could be interpreted as a memory that does not undergo reconsolidation or as one that is harder to induce to undergo reconsolidation. The finding that three different reactivation procedures (**Figs. 1b** and **2**) did not induce any amnesia suggests that strong memories initially do not undergo reconsolidation. It is always possible that some other protocol would be effective. However, 2 d after training, the receptor mechanisms critical for inducing reconsolidation are downregulated. Because the mechanisms that are necessary for inducing a consolidated memory to enter a labile state are extremely reduced, we could consider strong memories as a real boundary condition in the LBA.

Our suggested role of the NR2B subunits in regulating when fear memory in the LBA will undergo reconsolidation may not generalize to all memory systems or types of memory. Thus far, there are four studies that have examined the mechanisms involved in transforming a consolidated memory into one in a labile state. While we have demonstrated that NR2B subunit is critical for memories to return to a labile state within the LBA for fear conditioning[24], NMDA receptors in the hippocampus for fear memories and within the amygdala for appetitive memories are thought to play a role in restabilization process[47,48]. In the hippocampus, voltage-gated calcium channels (VGCC)[47] and protein degradation[49] are critical for return of a memory to a labile state. Thus, boundary conditions within the hippocampus may work by decreasing VGCC abundance or by preventing protein degradation or any molecular mechanism initiated by VGCC activation that will putatively lead to protein degradation. For each system, the specific molecules mediating boundary conditions are likely to change, but the conceptual mechanisms should remain the same: boundary conditions inhibit reconsolidation by downregulating a mechanism that is critical for transformation of a memory from a stable to a labile state.

In summary, these results begin to describe the training-strength boundary conditions on reconsolidation from the perspectives of behavioral variables, brain system dynamics and molecular mechanisms. These data provide new insights into the nature of the mechanisms that constrain reconsolidation: (i) they can be transient, (ii) different brain areas can be necessary for the boundary conditions on other brain areas and (iii) one conceptual mechanism mediating boundary conditions is the downregulation of the mechanisms mediating the induction of reconsolidation. These findings contain important clinical implications: treating PTSD too soon after the memory has consolidated may be fruitless as the memory is less likely to undergo reconsolidation.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

K.N. and S.-H.W. designed and developed this study. S.-H.W. conducted the behavioral, pharmacological, lesion and immunohistochemical experiments, performed the statistical and histological analyses and wrote the paper with K.N. L.d.O.A. conducted the western blot and lesion with extinction experiments and performed related data analyses. K.N. supervised this project.

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1. Müller, G.E. & Pilzecker, A. Experimentelle Beitrage zur Lehre vom Gedachtnis [Experimental contributions to the theory of memory]. *Z. Psychol. Erganzungsband (Z. Psychol. suppl.)* **1** 1:300 (1900).
2. Misanin, J.R., Miller, R.R. & Lewis, D.J. Retrograde amnesia produced by electroconvulsive shock after reactivation of a consolidated memory trace. *Science* **160**, 554–555 (1968).
3. Schneider, A.M. & Sherman, W. Amnesia: a function of the temporal relation of footshock to electroconvulsive shock. *Science* **159**, 219–221 (1968).
4. Alberini, C.M. Mechanisms of memory stabilization: are consolidation and reconsolidation similar or distinct processes? *Trends Neurosci.* **28**, 51–56 (2005).
5. Nader, K., Schafe, G.E. & LeDoux, J.E. The labile nature of consolidation theory. *Nat. Rev. Neurosci.* **1**, 216–219 (2000).
6. Dudai, Y. Reconsolidation: the advantage of being refocused. *Curr. Opin. Neurobiol.* **16**, 174–178 (2006).
7. Sara, S.J. Retrieval and reconsolidation: toward a neurobiology of remembering. *Learn. Mem.* **7**, 73–84 (2000).
8. Nader, K. & Hardt, O. A single standard for memory: the case for reconsolidation. *Nat. Rev. Neurosci.* **10**, 224–234 (2009).
9. Eisenberg, M., Kobilo, T., Berman, D.E. & Dudai, Y. Stability of retrieved memory: inverse correlation with trace dominance. *Science* **301**, 1102–1104 (2003).
10. Nader, K. Memory traces unbound. *Trends Neurosci.* **26**, 65–72 (2003).
11. Pedreira, M.E. & Maldonado, H. Protein synthesis subserves reconsolidation or extinction depending on reminder duration. *Neuron* **38**, 863–869 (2003).
12. Suzuki, A. *et al.* Memory reconsolidation and extinction have distinct temporal and biochemical signatures. *J. Neurosci.* **24**, 4787–4795 (2004).
13. Milekic, M.H. & Alberini, C.M. Temporally graded requirement for protein synthesis following memory reactivation. *Neuron* **36**, 521–525 (2002).
14. Debiec, J., Doyere, V., Nader, K. & Ledoux, J.E. Directly reactivated, but not indirectly reactivated, memories undergo reconsolidation in the amygdala. *Proc. Natl. Acad. Sci. USA* **103**, 3428–3433 (2006).
15. Stollhoff, N., Menzel, R. & Eisenhardt, D. Spontaneous recovery from extinction depends on the reconsolidation of the acquisition memory in an appetitive learning paradigm in the honeybee (*Apis mellifera*). *J. Neurosci.* **25**, 4485–4492 (2005).
16. Duvarci, S., Mamou, C.B. & Nader, K. Extinction is not a sufficient condition to prevent fear memories from undergoing reconsolidation in the basolateral amygdala. *Eur. J. Neurosci.* **24**, 249–260 (2006).
17. Debiec, J., LeDoux, J.E. & Nader, K. Cellular and systems reconsolidation in the hippocampus. *Neuron* **36**, 527–538 (2002).
18. Lee, J.L., Di Ciano, P., Thomas, K.L. & Everitt, B.J. Disrupting reconsolidation of drug memories reduces cocaine-seeking behavior. *Neuron* **47**, 795–801 (2005).
19. Debiec, J. & Ledoux, J.E. Disruption of reconsolidation but not consolidation of auditory fear conditioning by noradrenergic blockade in the amygdala. *Neuroscience* **129**, 267–272 (2004).
20. DeVietti, T.L. & Holliday, J.H. Retrograde amnesia produced by electroconvulsive shock after reactivation of a consolidated memory trace: a replication. *Psychon. Sci.* **29**, 137–138 (1972).
21. Bozon, B., Davis, S. & Laroche, S. A requirement for the immediate early gene zif268 in reconsolidation of recognition memory after retrieval. *Neuron* **40**, 695–701 (2003).
22. Przybyslawski, J., Roullet, P. & Sara, S.J. Attenuation of emotional and nonemotional memories after their reactivation: role of beta adrenergic receptors. *J. Neurosci.* **19**, 6623–6628 (1999).
23. Kim, J.J. & Fanselow, M.S. Modality-specific retrograde amnesia of fear. *Science* **256**, 675–677 (1992).
24. Ben Mamou, C., Gamache, K. & Nader, K. NMDA receptors are critical for unleashing consolidated auditory fear memories. *Nat. Neurosci.* **9**, 1237–1239 (2006).
25. Blanchard, R.J. & Blanchard, D.C. Crouching as an index of fear. *J. Comp. Physiol. Psychol.* **67**, 370–375 (1969).
26. Rudy, J.W., Biedenkapp, J.C., Moineau, J. & Bolding, K. Anisomycin and the reconsolidation hypothesis. *Learn. Mem.* **13**, 1–3 (2006).
27. Duvarci, S. & Nader, K. Characterization of fear memory reconsolidation. *J. Neurosci.* **24**, 9269–9275 (2004).
28. Frankland, P.W. & Bontempi, B. The organization of recent and remote memories. *Nat. Rev. Neurosci.* **6**, 119–130 (2005).
29. Frankland, P.W., Bontempi, B., Talton, L.E., Kaczmarek, L. & Silva, A.J. The involvement of the anterior cingulate cortex in remote contextual fear memory. *Science* **304**, 881–883 (2004).
30. Zola-Morgan, S.M. & Squire, L.R. The primate hippocampal formation: evidence for a time-limited role in memory storage. *Science* **250**, 288–290 (1990).
31. Blanchard, D.C., Blanchard, R.J. & Fukunaga, K.K. Environmental control of defensive reactions to footshock. *Bull. Psychon. Soc.* **8**, 129–130 (1976).
32. McDonald, A.J. Cortical pathways to the mammalian amygdala. *Prog. Neurobiol.* **55**, 257–332 (1998).
33. Pitkanen, A., Pikkarainen, M., Nurminen, N. & Ylinen, A. Reciprocal connections between the amygdala and the hippocampal formation, perirhinal cortex, and postrhinal cortex in rat: a review. *Ann. NY Acad. Sci.* **911**, 369–391 (2000).
34. Richter-Levin, G. & Akirav, I. Amygdala-hippocampus dynamic interaction in relation to memory. *Mol. Neurobiol.* **22**, 11–20 (2000).
35. Zinebi, F. *et al.* NMDA currents and receptor protein are downregulated in the amygdala during maintenance of fear memory. *J. Neurosci.* **23**, 10283–10291 (2003).
36. Morris, R.G. *et al.* Memory reconsolidation: sensitivity of spatial memory to inhibition of protein synthesis in dorsal hippocampus during encoding and retrieval. *Neuron* **50**, 479–489 (2006).
37. Fanselow, M.S. & Gale, G.D. The amygdala, fear, and memory. *Ann. NY Acad. Sci.* **985**, 125–134 (2003).
38. Schafe, G.E., Nader, K., Blair, H.T. & LeDoux, J.E. Memory consolidation of Pavlovian fear conditioning: a cellular and molecular perspective. *Trends Neurosci.* **24**, 540–546 (2001).
39. Maren, S. Neurotoxic basolateral amygdala lesions impair learning and memory but not the performance of conditional fear in rats. *J. Neurosci.* **19**, 8696–8703 (1999).
40. Reijmers, L.G., Perkins, B.L., Matsuo, N. & Mayford, M. Localization of a stable neural correlate of associative memory. *Science* **317**, 1230–1233 (2007).
41. Phillips, R.G. & LeDoux, J.E. Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. *Behav. Neurosci.* **106**, 274–285 (1992).
42. Ambrogi Lorenzini, C.G., Baldi, E., Bucherelli, C., Sacchetti, B. & Tassoni, G. Neural topography and chronology of memory consolidation: a review of functional inactivation findings. *Neurobiol. Learn. Mem.* **71**, 1–18 (1999).
43. Moscovitch, M., Nadel, L., Winocur, G., Gilboa, A. & Rosenbaum, R.S. The cognitive neuroscience of remote episodic, semantic and spatial memory. *Curr. Opin. Neurobiol.* **16**, 179–190 (2006).
44. Turrigiano, G.G. & Nelson, S.B. Hebb and homeostasis in neuronal plasticity. *Curr. Opin. Neurobiol.* **10**, 358–364 (2000).
45. Franks, K.M. & Isaacson, J.S. Synapse-specific downregulation of NMDA receptors by early experience: a critical period for plasticity of sensory input to olfactory cortex. *Neuron* **47**, 101–114 (2005).
46. Joshi, I. & Wang, L.Y. Developmental profiles of glutamate receptors and synaptic transmission at a single synapse in the mouse auditory brainstem. *J. Physiol. (Lond.)* **540**, 861–873 (2002).
47. Suzuki, A., Mukawa, T., Tsukagoshi, A., Frankland, P.W. & Kida, S. Activation of LVGCCs and CB1 receptors required for destabilization of reactivated contextual fear memories. *Learn. Mem.* **15**, 426–433 (2008).
48. Milton, A.L., Lee, J.L., Butler, V.J., Gardner, R. & Everitt, B.J. Intra-amygdala and systemic antagonism of NMDA receptors prevents the reconsolidation of drug-associated memory and impairs subsequently both novel and previously acquired drug-seeking behaviors. *J. Neurosci.* **28**, 8230–8237 (2008).
49. Lee, S.H. *et al.* Synaptic protein degradation underlies destabilization of retrieved fear memory. *Science* **319**, 1253–1256 (2008).

ONLINE METHODS

Subjects. Adult male Sprague-Dawley rats bred at Charles River were used in this study. Both food and water were provided *ad libitum*. The 12-h light-dark cycle began at 7 am daily. All experiments were conducted at the light cycle and followed the protocols approved by McGill University Animal Care Center.

Surgery. Rats were anesthetized with sodium pentobarbital (60 mg ml^{-1}), injected with atropine to prevent the obstruction of the respiration and placed in stereotaxic frames. Guide cannula (22 gauge) were bilaterally implanted and aimed at lateral and basal nuclei of the amygdala (LBA). The coordinates were 3 mm posterior, 5.3 mm lateral, and 8 mm ventral from the bregma based on rat brain atlas. Three jewelry screws were implanted into the skull and acrylic cement was applied to stabilize the cannula. The rats were then allowed 7–10 d to recover from the surgery. During the recovery period, rats were handled daily.

For dorsal hippocampus lesions, rats were given electrolytic or sham lesions at the time of LBA cannula implantation. The screws were first anchored. The electrodes were then placed at two sites of dorsal hippocampus in each hemisphere. The coordinates were 2.8 mm posterior, 2 mm lateral and 4 mm ventral; 4.2 mm posterior, 3 mm lateral and 4 mm ventral from the bregma. Stainless steel microelectrodes (FHC, model KK1) with 500 μm of the tip insulation removed were lowered through an incision in the dura into the target area. Lesions were made by passing a positive current (1.0 mA, 20 s) through a lesion-making device (Ugo Basile). Sham lesioned rats underwent a similar surgery procedure except for the electrolytic current being omitted.

Drugs and micro-infusions. Anisomycin (125 μg μl^{-1}, Sigma-Aldrich) was dissolved in 1 M HCl and normal physiological saline. The pH was adjusted to 7.4 with 1 M NaOH. Normal physiological saline was used as vehicle solution. For intra-LBA microinfusion, injectors (28 gauge, extending 1.5 mm below the guide cannula) were connected to microsyringe (5 μl, Hamilton) with polyethylene tubes. The solution (0.5 μl per side) was infused with a pump (Harvard) over the course of 2 min. The injector was left for an additional minute to allow the complete diffusion.

Histology. After completing all behavioral procedures, rats were transcardially perfused with physiological saline followed by 10% formalin-saline. The brains were then cryosectioned at 50-μm thickness and stained with formal-thionin to identify cannula placement.

Behavioral procedures. *Habituation.* After recovery from the surgery, rats were given 2 d of habituation in the training and testing chambers, which had different olfactory, tactile and visual properties from each other. On day 1, half of the rats were habituated to the training contexts for 30 min and 5 h later, they were habituated to the testing contexts for 30 min. On the next day, the same rats received a reversed order of habituation (that is, testing context first and then training context). The remaining half rats received the reverse sequence of habituation.

Training. The day after habituation rats were conditioned. After 3 min of acclimation, one tone (5 kHz, 75 dB) was presented for 30 s and it coterminated with a scrambled footshock (1.5 mA, 1 s). In the strong training paradigm, 10 tone–footshock pairings were given. The interpairing interval was variable with an average of 4 min. One minute after the final pairing, rats were returned to their home cages.

Reactivation. Reactivation entailed one 30-s tone presentation in the testing box. One minute after the offset of the tone, rats were removed from the testing chamber. Half of the rats were immediately infused with anisomycin and the remaining were infused with vehicle. They were then returned to the home cage. Four hours later, they were given a post-reactivation short-term memory (PR-STM) test. The test session was 8 min long and composed of three presentations of the 30-s tone. Twenty-four hours after reactivation, rats were given post-reactivation long-term (PR-LTM) memory test which was 8 min long and composed of three presentations of the 30-s tone.

Extinction. Rats were habituated and trained with 1 pairing or 10 pairings as described above. Two days later, rats received 10 presentations of the tones (30 s each) without any footshocks in the testing context. The intertone interval was varied between 2 and 5 min (average 3 min).

Western blots and antibodies. The rats were deeply anesthetized with urethane (50 mg ml^{-1}) and put to death and their brains were rapidly removed and frozen. Amygdala punches were obtained with a neuro punch (1 mm; Fine Science Tools) from frozen brains. The punches included the lateral amygdala and the basal nucleus and possibly portions of the lateral central nucleus. The samples were homogenized in cold lysis buffer with protease inhibitors. Equal amounts of protein (15 μg) were resolved using 7.5% SDS-PAGE and transferred to nitrocellulose membranes as previously described[50]. The protein blots were incubated with primary antibodies (NR2B, 1:300 (Zymed) or NR1, 1:1,000 (Chemicon)), followed by incubation with horseradish peroxidase–conjugated antibody to goat IgG. For quantification of immunoblots, they were scanned and analyzed using ImageQuant software (Amersham).

Immunohistochemistry. Two days after training, the rats were deeply anesthetized with urethane (50 mg ml^{-1}). They were then transcardially perfused with cold PBS followed by 4% paraformaldehyde in phosphate buffer. Brains were removed and postfixed in the same fixative overnight. Brains were then sliced with vibratome (Leica) at 50-μm thickness. Sections were collected from the region around 2.8 to 3.5 mm posterior to the bregma, where it contains amygdalar structures. IHC was done using a free-flotation method. Selected sections were then incubated in 0.3% H_2O_2 to quench endogenous peroxidase activity, blocked in PBS containing 1% bovine serum with 0.2% Triton X-100 and incubated in antibodies to NR2B (rabbit polyclonal antibody, 1:500; Upstate) in the same blocking buffer at room temperature (23–26 °C) overnight. After washing with PBS, slices were then incubated in biotinylated goat anti-rabbit antibodies (1:1,000, Vector) for 1 h at room temperature, washed with PBS and incubated in ABC (Elite kit, Vector). The color development was done with 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) for 2 min. After a series of dehydration procedures, sections were mounted on coated slides and coverslipped.

Quantification of NR2B-labeled cells. In each rat, cell counts were taken from two to three sections, separated by 200 μm, from 2.8 to 3.2 mm posterior to the bregma. The boundary of lateral amygdala and basal amygdala was defined under the microscope (Olympus, IX81) using a ×20 objective. Cell counting was done under a ×40 objective. We used ImagePro software (Media Cybernetics) to identify circular, stained objects that were substantially darker than the background. We later verified that these objects were cell bodies. A region of interest (ROI; dimensions, a 210 μm × 150 μm rectangle) was randomly selected ~0.2 mm below the tip of the lateral amygdala or below the boundary of the lateral and basal amygdala. In total, eight similar ROIs were randomly collected within lateral and basal amygdala. The cell numbers within these ROIs were later averaged for statistic analysis.

Statistics. We used one-way independent, two-way independent, and two-way or three-way with one repeated measure ANOVA for behavioral data analysis. *Post hoc* tests were further used to identify the critical differences that contributed to significant interaction. Type-one error rate was set at 0.05. Behavioral data entered statistical analysis only when the cannula correctly targeted LBA bilaterally.

50. Lengyel, I. *et al.* Autonomous activity of CaMKII is only transiently increased following the induction of long-term potentiation in the rat hippocampus. *Eur J. Neurosci.* **20**, 3063–3072 (2004).