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β-catenin is required for memory consolidation

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β-catenin has been implicated in neuronal synapse regulation and remodeling. Here we have examined β-catenin expression in the adult mouse brain and its role in amygdala-dependent learning and memory. We found alterations in β-catenin mRNA and protein phosphorylation during fear-memory consolidation. Such alterations correlated with a change in the association of β-catenin with cadherin. Pharmacologically, this consolidation was enhanced by lithium-mediated facilitation of β-catenin. Genetically, the role of β-catenin was confirmed with site-specific deletions of *loxP*-flanked *Ctnnb1* (encoding β-catenin) in the amygdala. Baseline locomotion, anxiety-related behaviors and acquisition or expression of conditioned fear were normal. However, amygdala-specific deletion of *Ctnnb1* prevented the normal transfer of newly formed fear learning into long-term memory. Thus, β-catenin may be required in the amygdala for the normal consolidation, but not acquisition, of fear memory. This suggests a general role for β-catenin in the synaptic remodeling and stabilization underlying long-term memory in adults.

Structural changes at synapses are thought to underpin long-term memory formation. Dendritic spines, where most excitatory synapses terminate[1,2], show alterations in motility and morphology after a learning event[3–5]. The processes governing dendritic morphogenesis are many and varied, but recent work has focused on the role of cell adhesion molecules in mediating activity-dependent changes at synapses.

β-catenin is a candidate molecule that may function in mediating the structural changes associated with long-term memory formation. It associates with the cytoplasmic domain of cadherin and directly links to the actin cytoskeleton through α-catenin[6]. This cadherin-catenin complex is localized in synaptic junctions, and alterations in this complex are thought to influence synaptic size and strength[7]. Recent work has suggested that the cadherin-catenin complex is involved not only in synapse development but also in modulation of synaptic connectivity and activity[8,9].

In addition to its role in cadherin-mediated cell-cell adhesion, β-catenin has an important role in the Wnt signal transduction pathway. In the resting state, β-catenin is phosphorylated by glycogen synthase kinase-3β (GSK-3β) and rapidly degraded by the proteasome pathway. Upon activation of Wnt signaling, β-catenin is stabilized through the inhibition of GSK-3β and translocates to the nucleus, where it binds the TCF/LEF family of transcription factors to regulate the expression of Wnt target genes[10,11]. This signaling pathway has recently been shown to be involved in the regulation of synaptic plasticity in a hippocampal slice preparation[12].

Thus, β-catenin seems to be an important 'hub' protein in synaptic plasticity, with involvement in regulating both activity-dependent synaptic remodeling and gene transcription. Taken together, there is tremendous face validity to the hypothesis that β-catenin is directly involved in crucial events that mediate learning and memory. However, because knockouts of β-catenin are embryonic lethal[13], it has not been possible to examine the potentially crucial role of this protein in learning and memory assays in animals. Also, no specific pharmacological agents that target β-catenin have yet been identified, so no pharmacological studies have directly examined learning and memory modulation by β-catenin.

These experiments described here outline a role for the regulation of β-catenin and its interaction with cadherin during the consolidation phase of fear-memory formation. We showed that memory formation is enhanced by acute administration of lithium, which acts in part by stabilizing β-catenin through the inhibition of GSK-3β. We used an inducible genetic approach to examine whether β-catenin is required for the consolidation of fear memories *in vivo*. When examining the effects of temporal- and region-specific deletion, we found that β-catenin within the amygdala is required for the consolidation, but not the acquisition or expression, of fear memory.

RESULTS

β-catenin mRNA expression changes with learning

The heavy emphasis on the role of β-catenin in development has resulted in a scarcity of data on the expression of β-catenin in adult animals. We therefore examined β-catenin expression in the brains of wild-type adult (8- to 10-week-old) C57Bl/6J mice. *In situ* hybridization analyses using an antisense probe spanning exons 2 through 6 of *Ctnnb1* revealed very dense expression of this gene throughout the adult brain, particularly in regions associated with synaptic plasticity (**Fig. 1**). A sense probe spanning the same region was used as a negative control, resulting in no significant labeling above background (data not shown). These data indicate that β-catenin is present in the adult brain and may be required for normal neuronal functioning in adults.

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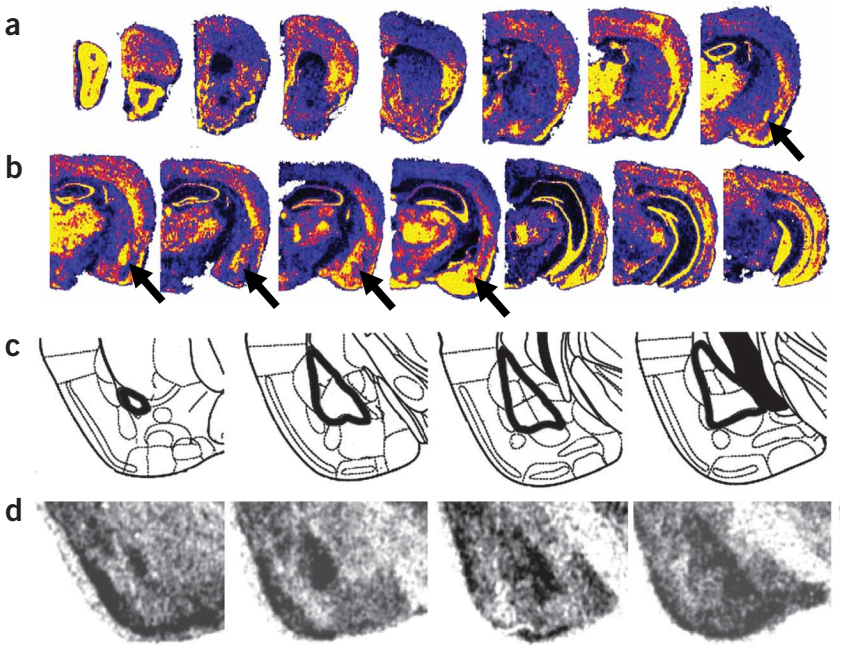

Figure 1 β-catenin expression in the adult mouse brain. (**a**,**b**) Pseudocolored *in situ* hybridization photomicrographs showing high β-catenin mRNA throughout the brain, particularly within the amygdala, some cortical regions, thalamus and hippocampus. Arrows, basolateral amygdala (BLA). Yellow, highest expression; blue-black, lowest expression. (**c**) Schematic diagram from Paxinos and Watson[36] showing the location of the amygdala and its subdivisions in the temporal lobe (BLA is outlined). (**d**) β-catenin mRNA is present at high levels spanning the basolateral nuclei of the amygdala, as outlined in **c**.

We next examined the hypothesis that β-catenin is involved in the synaptic plasticity underlying learning and memory in adults, specifically fear conditioning in the amygdala. After 3 d of habituation to the conditioning chambers, mice received five tone–shock pairings. A context control group was placed in the conditioning chambers for the same amount of time, but no stimuli were presented. We collected brains from the control mice 2 h after context exposure; brains from the trained mice were collected immediately, 0.5 or 2 h after conditioning. Mice that had received the five tone–shock pairings were able to acquire and express fear, as shown by increased freezing throughout the training (**Fig. 2a**). We then measured β-catenin mRNA in various brain regions at various time points after fear conditioning. β-catenin mRNA in the basolateral amygdala (BLA) was altered with fear conditioning, with a significant increase at 2 h after training (control, 1.00 ± 0.06 versus 2 h of fear conditioning, 1.27 ± 0.08; $t_{14} = 2.764$, $P < 0.05$; **Fig. 2b**,**c**). We did not find any significant differences in β-catenin mRNA in the somatosensory cortex or striatum ($P > 0.05$; **Fig. 2c**).

β-catenin is post-translationally modified with learning

We next examined whether the increase in β-catenin mRNA with fear learning is the result of altered expression or altered post-translational modification. We used western blot analyses to examine β-catenin expression in mice exposed to the context alone, mice exposed to unpaired tone and shock presentations, and mice trained and killed 0, 0.5, 2, 4, 12 or 24 h after fear conditioning with five tone–shock trials (**Fig. 3a**). In contrast to the observed increase in β-catenin mRNA, total β-catenin protein in the amygdala did not change with training (analysis of variance (ANOVA), $P > 0.05$; **Fig. 3b**), suggesting that protein modification or degradation occurs in concert with the increases in gene transcription.

Thus, we wanted to determine whether post-translational modifications of β-catenin occur with learning. We first measured changes in the stabilization of β-catenin after GSK-3β inactivation. GSK-3β destabilizes β-catenin by phosphorylating it at Ser33, Ser37 and Thr41. However, when GSK-3β is phosphorylated, it becomes unable to destabilize β-catenin. We measured phosphorylated GSK-3β after learning and found a significant main effect for time ($F_{7,132} = 3.943$, $P \leq 0.001$ by ANOVA). *Post hoc* least-squares difference analyses indicated that phosphorylated GSK-3β was significantly higher in trained mice 2 h after fear conditioning (1.71 ± 0.18) than in unpaired control mice (1.03 ± 0.16; $P \leq 0.005$), context-exposed mice (1.00 ± 0.10; $P \leq 0.001$) and trained mice killed immediately after conditioning (0-h time point; 0.68 ± 0.11; $P \leq 0.001$; **Fig. 3c**). Notably, the amount at the 0-h time point was also significantly different from the amount at the 4-h time point (1.34 ± 0.28; $P \leq 0.001$). This significant increase in phosphorylated GSK-3β after fear conditioning is consistent with enhanced stabilization of β-catenin during fear consolidation.

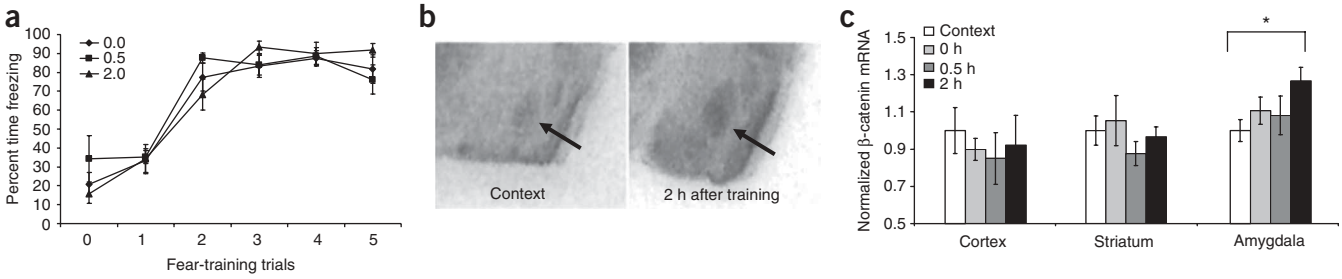

Figure 2 β-catenin gene expression in the amygdala is increased after fear conditioning. Mice were exposed to five tone–shock pairings and then killed 0, 0.5 or 2 h after training. (**a**) Acquisition curve showing the percentage of time spent freezing during each tone before the presentation of the footshock. Mice in all groups showed similar levels of freezing before the presentation of any tones (0) and then showed increased freezing during the tone trials throughout training (trials 1–5). Arrows, the presentation of footshock. (**b**) Qualitative *in situ* hybridization analysis of β-catenin mRNA in the amygdala in context-exposed mice (left) and mice killed 2 h after training (right). (**c**) Relative expression of β-catenin mRNA in the somatosensory cortex, striatum and amygdala, normalized to expression in context-exposed mice. Only β-catenin mRNA expression in the amygdala was significantly increased 2 h after fear conditioning. $n = 8$ for context, 0 h and 2 h; $n = 7$ for 0.5 h. Error bars, s.e.m. $^*P < 0.05$.

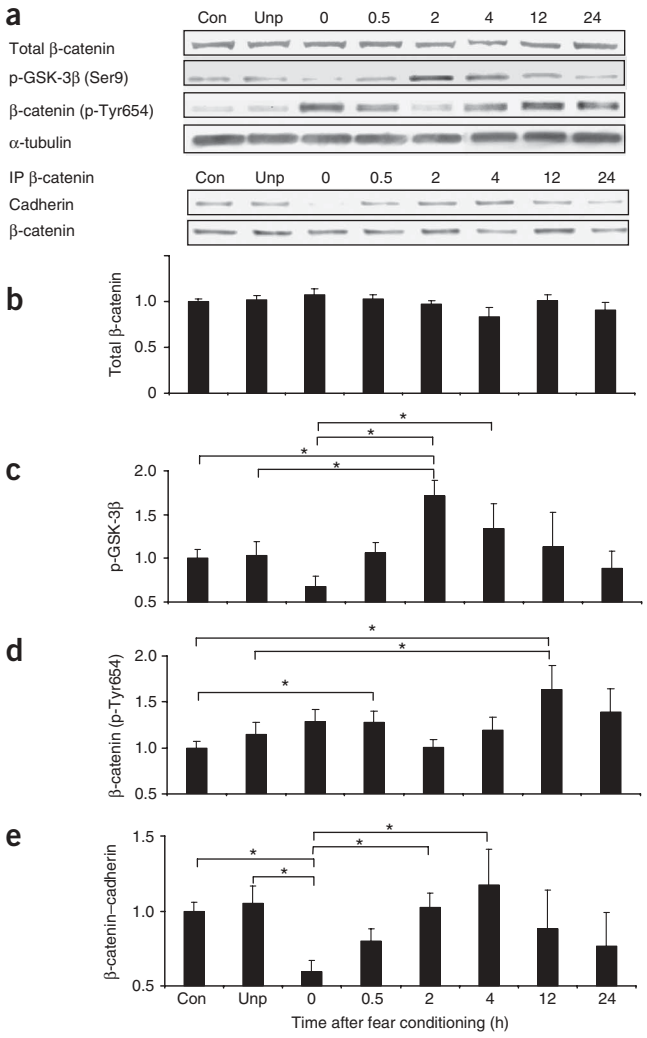

Figure 3 Phosphorylation states of β-catenin and GSK-3β are altered after fear conditioning. Mice were exposed to five tone–shock pairings and then killed 0, 0.5, 2, 4, 12 or 24 h after training. (**a**) Qualitative western blot data. In all bar graphs, protein amounts determined from these western blots are expressed relative to the α-tubulin loading control. (**b**) Total β-catenin did not change with fear conditioning. (**c**) Phosphorylated GSK-3β (p-GSK-3β) changed significantly over time. (**d**) Tyr654-phosphorylated β-catenin (p-Tyr654) changed significantly over time. (**e**) Coimmunoprecipitation of cadherin and β-catenin. Cadherin interaction with β-catenin changed significantly over time. $n = 31$ for context; $n = 13$ for unpaired; $n = 14$ for 0 h; $n = 27$ for 0.5 and 2 h; $n = 8$ for 4 h; $n = 6$ for 12 h; $n = 7$ for 24 h. Con, context control group; Unp, unpaired shock control group. Error bars, s.e.m. $^*P \leq 0.05$.

We then examined phosphorylation of β-catenin at Tyr654, which decreases its affinity for cadherin[14]. Overall, ANOVA indicated a significant main effect for time ($F_{7,132} = 2.107$, $P \leq 0.05$). *Post hoc* tests revealed that Tyr654-phosphorylated β-catenin was significantly higher in trained mice 12 h after conditioning (1.63 ± 0.26) than in unpaired control mice (1.14 ± 0.14; $P \leq 0.05$) and context-exposed mice (1.00 ± 0.08; $P \leq 0.01$; **Fig. 3d**). Notably, compared to the context-exposed group, Tyr654-phosphorylated β-catenin was also significantly increased 0.5 h after conditioning (1.28 ± 0.12; $P \leq 0.05$). Thus, the affinity of β-catenin for cadherin in the amygdala seems to be dynamically regulated during fear consolidation.

Given these results, we wanted to determine whether these changes in Tyr654-phosphorylated β-catenin abundance significantly affect the association of β-catenin with cadherin. We immunoprecipitated β-catenin from the amygdalas of the above mice and then probed with an antibody to pan-cadherin. ANOVA indicated a significant main effect for time ($F_{7,132} = 2.320$, $P \leq 0.05$; **Fig. 3e**). *Post hoc* analyses revealed that the amount of cadherin coimmunoprecipitated with β-catenin was significantly lower in trained mice immediately after conditioning (0.60 ± 0.08) than in unpaired mice (1.05 ± 0.12; $P \leq 0.01$) and context control mice (1.00 ± 0.06; $P \leq 0.01$). Notably, this immediate decrease was followed by a significant increase in binding at 2 h (1.02 ± 0.10; $P \leq 0.01$) and 4 h (1.17 ± 0.24; $P \leq 0.01$) after conditioning, returning cadherin binding to normal. We also found a significant negative correlation between the amount of cadherin coimmunoprecipitated with β-catenin and the amount of Tyr654-phosphorylated β-catenin ($r_{133} = -0.184$, $P \leq 0.05$), confirming a significant relationship between these measures.

None of the blot analyses showed significant differences between context-exposed mice and mice receiving unpaired tones and shocks ($P > 0.05$). Thus, the time-dependent differences we observed in β-catenin modulation are likely to result from associative learning and not from the stress of shock alone.

Increasing β-catenin stability enhances learning

Because β-catenin regulation in the BLA is correlated with fear conditioning, we examined whether manipulating β-catenin function would affect this learning process. No specific pharmacological agents that target β-catenin have yet been identified, making it difficult to directly examine the effect of β-catenin function on learning. However, lithium chloride (LiCl), though not as specific as we would like, is widely accepted as a modulator of β-catenin. LiCl inhibits GSK-3β, decreasing its ability to phosphorylate β-catenin at Ser33, Ser37 and Thr41. As a consequence, the unphosphorylated β-catenin is more stable and less prone to degradation[15–17]. The temporal changes we observed in phosphorylated GSK-3β suggested that is a good target for pharmacological manipulation of β-catenin with learning.

To examine the effects of acute LiCl administration on learning, we first confirmed that systemic administration of LiCl inhibits GSK-3β in the amygdala. We injected mice intraperitoneally with either saline or LiCl (100 mg kg^{-1}) and killed them 30 min later. As expected, acute administration of LiCl significantly increased phosphorylated GSK-3β in the amygdala (1.43 ± 0.30) compared to controls (0.64 ± 0.13; $t_{17} = 2.344$, $P < 0.05$; **Fig. 4a**). We then examined whether LiCl alters GSK-3β-dependent phosphorylation of β-catenin after fear conditioning. We injected mice with either saline or LiCl 30 min before training, and then killed them 0.5 or 2 h after fear conditioning. Total β-catenin in the amygdala was higher, although not significantly, in LiCl-treated mice compared to saline-treated mice (**Fig. 4b**). Notably, in agreement with the model of LiCl inhibiting GSK-3β, the ratio of GSK-3β-phosphorylated β-catenin to total β-catenin was significantly lower at the 0.5- and 2-h time points than the ratio in saline-treated mice ($F_{1,28} = 11.931$, $P < 0.01$; **Fig. 4c**). Together, these results suggest that acute LiCl administration inhibits GSK-3β-mediated phosphorylation of β-catenin, potentially enhancing its overall stability, during the consolidation period after fear conditioning.

We next determined whether decreasing GSK-3β-mediated phosphorylation of β-catenin through acute LiCl administration could affect learning. We injected mice with either saline or LiCl and then fear-conditioned them 30 min later. The intensity of the unconditioned stimulus was lowered to 0.6 mA to prevent ceiling effects on fear expression. Throughout this training paradigm, we measured freezing

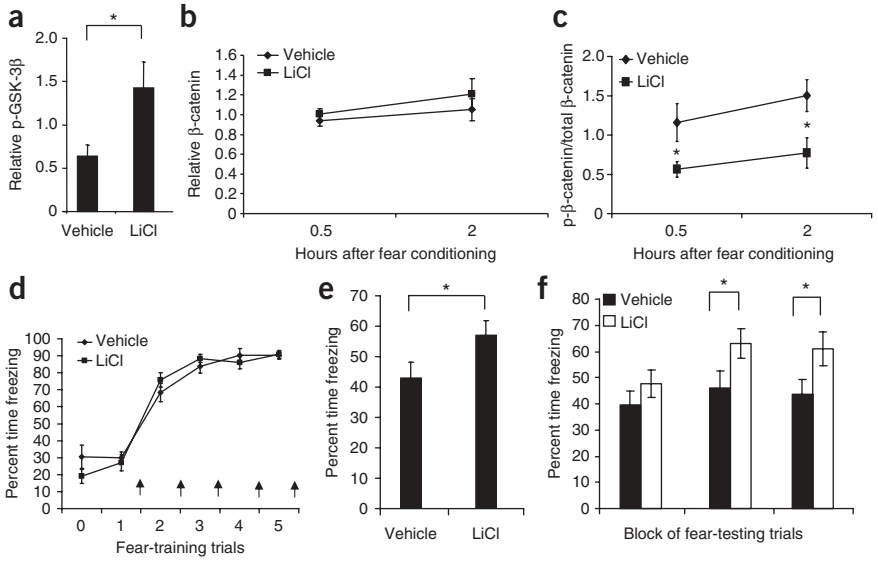

Figure 4 LiCl decreases GSK-3β-mediated β-catenin phosphorylation in the amygdala and enhances learning. (**a**) Mice in their home cages were killed 30 min after systemic injections of either vehicle or LiCl (100 mg kg^{-1}). There was significantly more phosphorylated GSK-3β in the amygdala in LiCl-treated mice than in vehicle-treated mice ($n = 9$ for vehicle group, $n = 10$ for vehicle group). (**b**,**c**) A separate group of animals was injected with either vehicle or LiCl 30 min before fear conditioning and then killed 0.5 or 2 h after training ($n = 8$ per group). (**b**) Total β-catenin in the amygdala was increased, although not significantly, at the 0.5- and 2-h time points in LiCl-treated mice. (**c**) The ratio of β-catenin phosphorylated at the GSK-3β-dependent sites (Ser33, Ser37 and Thr41) to total β-catenin was significantly decreased in LiCl-treated mice at the 0.5- and 2-h time points after fear conditioning. In **a**–**c**, protein amounts are expressed relative to α-tubulin loading control, as in **Figure 3**. (**d**–**f**) An additional group of mice was injected with either vehicle or LiCl 30 min before fear conditioning and then tested 48 h later ($n = 19$ per group). (**d**) Acquisition curve showing percentage time spent freezing during each tone before presentation of the footshock. All mice were able to acquire and express equal levels of fear. Arrows, the presentation of footshock. (**e**) Mice that received LiCl before fear training showed significantly more fear than did mice that received saline upon retesting 48 h later in the absence of drug. (**f**) Freezing data from **e** were grouped in blocks of five, revealing that this difference in fear retention is maintained across the testing session. Error bars, s.e.m. $^*P < 0.05$.

behavior during each tone presentation (conditioned stimulus) before the presentation of footshock (**Fig. 4d**). We found a significant main effect of time across all mice ($F_{5,180} = 111.495$, $P \leq 0.01$); however, there was no main effect of LiCl treatment ($F_{1,36} = 0.167$, $P = 0.685$).

Forty-eight hours after fear conditioning, in the absence of drug, mice were placed in a new chamber and presented with 15 conditioned-stimulus tones. The mean percent time spent freezing during these tones was recorded and used as a measure of conditioned fear (**Fig. 4e**). Mice that received LiCl before the fear training 2 d earlier now showed significantly more fear (57.00 ± 4.64) than did mice that had received saline (43.07 ± 4.99; $t_{38} = 2.044$, $P < 0.05$). This increase in fear retention was present throughout the testing session but was most notable toward the middle and end of the session (**Fig. 4f**). Percent time spent freezing was recorded in blocks of five trials each as follows (vehicle versus lithium): block 1, 39.5 ± 5.5 versus 47.6 ± 5.3 ($t_{38} = 1.06$, not significant); block 2, 46.2 ± 6.4 versus 63.0 ± 5.7 ($t_{38} = 1.95$, $P \leq 0.05$); block 3, 43.7 ± 5.6 versus 61.2 ± 6.5 ($t_{38} = 2.05$, $P \leq 0.05$). These data suggest that the difference in retention of fear memory was not caused by differences in extinction within testing. The enhancement of fear memory also did not seem to be caused by effects on locomotor behavior, as the mice did not show any significant differences across groups in activity level or freezing behavior before the first conditioned stimulus. Similarly, previous reports have shown that this specific dose of LiCl does not produce locomotor effects[18].

Together, these data suggest that a single, albeit nonspecific, pharmacological manipulation that increases functional β-catenin during or soon after fear conditioning leads to relatively specific increases in the expression of fear behavior 48 h later. This is consistent with the hypothesis that increasing functional β-catenin enhances consolidation of new memories.

Ctnnb1 deletion in the BLA does not alter baseline measures

As stated above, LiCl is somewhat nonspecific[19], so we sought to examine the effect of β-catenin on learning and memory through a more direct genetic mechanism: 'floxed' β-catenin mice[20], which possess *loxP* sites located in introns 1 and 6 of *Ctnnb1*. Injection of these mice with a Cre recombinase–expressing lentivirus (LV-Cre) resulted in region-specific deletion of the floxed *Ctnnb1* allele (**Fig. 5**). Ten days after unilateral infection with LV-Cre virus, we probed adjacent brain sections with radiolabeled *Ctnnb1* antisense mRNA or Cre recombinase. These experiments showed that relatively specific deletion of *Ctnnb1* in the amygdala can be achieved with LV-Cre injection. When mice were injected with a control lentivirus expressing green fluorescent protein (LV-GFP), the abundance of β-catenin remained similar to that in wild-type mice. Notably, there were no effects of Cre-mediated *Ctnnb1* deletion or LV-GFP injection on the cellular or anatomical structure of the amygdala, as shown with a Nissl stain (**Supplementary Fig. 1** online).

Having confirmed our ability to locally delete *Ctnnb1* in a temporally specific, inducible manner, we examined whether amygdala-specific deletion affects baseline anxiety or activity measures. We injected the mice with either LV-GFP or LV-Cre bilaterally in the amygdala at 6–8 weeks of age. Ten days later, we examined the mice in a series of basic behavioral tasks. In these baseline measures, we found no difference ($P > 0.1$) in anxiety as measured by baseline startle, elevated plus-maze (time in open or closed arms) and open-field maze (distance traveled in center, time at rest and average velocity; **Fig. 6a**–**e**). These data suggest that amygdala-specific deletions of *Ctnnb1* do not alter motor activity or anxiety levels.

We then wanted to quantitatively confirm that injections of LV-Cre into the amygdala decrease β-catenin mRNA. We processed brains for *in situ* hybridization and measured β-catenin mRNA. Mice injected with LV-Cre had significantly less β-catenin mRNA (26.73 ± 7.20) than did mice injected with LV-GFP (54.60 ± 2.96; $t_{6.88} = 3.580$, $P \leq 0.01$; **Fig. 6f**).

β-catenin is required for fear-memory consolidation

We then examined whether amygdala-specific deletion of *Ctnnb1* affects amygdala-dependent learning, as outlined in **Figure 7a**. We fear-conditioned the mice and obtained freezing measures during fear acquisition (**Fig. 7b**). As with acute LiCl administration, there was a significant main effect of trial ($F_{5,125} = 104.698$, $P \leq 0.01$) but no effect

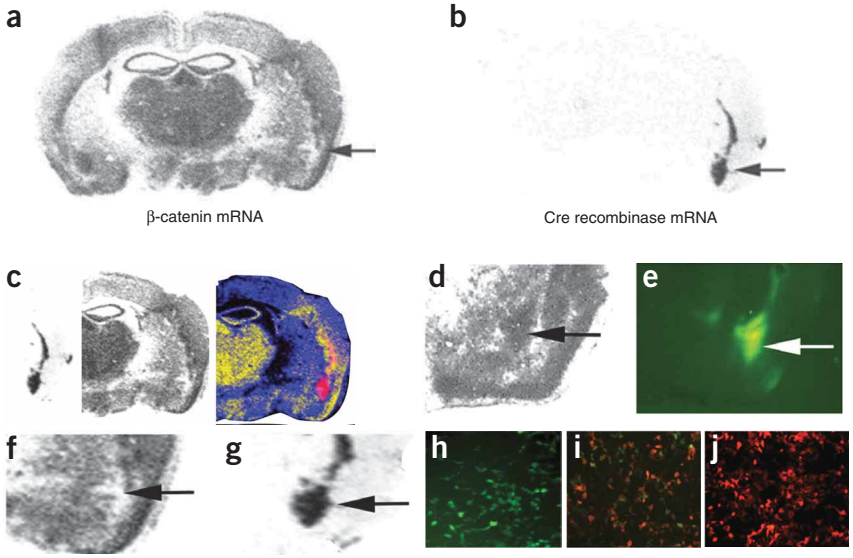

Figure 5 Region-specific deletion of β-catenin in the adult brain. (**a**,**b**) β-catenin–floxed mice were injected with either LV-Cre or LV-GFP and killed 10 d later. Unilateral injection of LV-Cre resulted in site-specific loss of expression. Adjacent sections were probed with radiolabeled antisense β-catenin mRNA (**a**) or Cre recombinase (**b**). A pseudocolor overlay of these two adjacent sections (**c**) shows the regional specificity. (**d**–**f**) Mice that that received LV-GFP injections into the amygdala had normal β-catenin abundance (**d**) where the LV-GFP was injected (**e**). This is in contrast to β-catenin mRNA expression (**f**) in the amygdala of a mouse injected with LV-Cre (**g**). (**h**–**j**) *In vitro* functional assay of lentivirus-expressed Cre recombinase. HEK293T cells were transiently transfected with a vector containing a floxed GFP reporter, pLoxpGFP-DsRed, in the absence (**h**) or presence (**i**) of LV-Cre and visualized using a green filter. (**j**) HEK293T cells transfected with pLoxpGFP-DsRed in the presence of LV-Cre, visualized using a red filter.

of virus ($F_{1,25} = 1.964$, $P = 0.173$). The similar acquisition curves for LV-GFP and LV-Cre mice suggest that mice with *Ctnnb1* deletions are initially able to encode and express fear memories normally.

Forty-eight hours after the first five trials, we tested mice for cue fear conditioning in a new context (**Fig. 7c**). In contrast to the acquisition data above, mice infected with LV-Cre—and thus, those with *Ctnnb1* deletions—showed over 40% less freezing averaged across all sessions (35.00 ± 7.26) compared to mice infected with LV-GFP (60.00 ± 4.94; $t_{25} = 2.935$, $P < 0.01$). Notably, even in the first freezing trial of the test, LV-Cre–infected mice froze less than LV-GFP–infected mice, which froze at rates near those seen during acquisition. The data for within-session freezing across the full testing session (**Fig. 7d**) were in grouped blocks of five trials each as follows (LV-GFP versus LV-Cre): block 1, 66.33 ± 6.24 versus 38.33 ± 7.67 ($t_{25} = 2.863$, $P < 0.01$); block 2, 58.67 ± 6.35 versus 35.00 ± 8.77 ($t_{25} = 2.239$, $P < 0.05$); block 3, 55.00 ± 6.45 versus 31.67 ± 7.11 ($t_{25} = 2.426$, $P < 0.05$). These data confirm that the decrease in fear is most likely to be a function of decreased consolidation at, or soon after, the initial learning event, as mice were able to acquire and express fear normally (**Fig. 7b**) and did not show decreased average fear resulting from increased within-session extinction (**Fig. 7d**). In addition, there was a positive correlation between β-catenin mRNA and freezing as a measure of fear ($r_{13} = 0.752$, $P < 0.01$; **Fig. 7e**).

Together, these data suggest that β-catenin expression in the amygdala is not required for normal anxiety-related behaviors or for the acquisition of fear, an amygdala-dependent task. However, consistent with the dynamic regulation of β-catenin abundance during the consolidation period after fear acquisition, these data suggest that β-catenin is required for the normal consolidation of fear memory. In the absence of β-catenin, newly formed memories do not seem to be stabilized and thus cannot be expressed 48 h later.

β-catenin is not required for expression of fear memory

Thus far, our pharmacological and genetic manipulations of β-catenin have shown that β-catenin is not involved in the acquisition of fear but rather in the stabilization of fear memory. We therefore examined whether deletion of *Ctnnb1* after the consolidation of fear memory would affect further expression of conditioned fear (outlined in **Fig. 7a**). To determine the effect of *Ctnnb1* deletion on expression, we trained mice and then presented them 48 h later with a three-trial 'short test' for freezing to confirm that they had acquired and consolidated the fear memory (**Fig. 7f,g**). We did not administer the full 15 trials to reduce the likelihood of extinction processes. We then injected the mice with either LV-GFP or LV-Cre bilaterally into the amygdala and allowed the mice to recover for 14 d. The mice were then tested again for fear memory after this delay (2–3 weeks after training; **Fig. 7f,h**). Although the levels of freezing in this 21-d fear expression test were lower than those in the 48-h experiments, both groups of mice showed significantly more freezing during the tone than before the conditioned stimulus during this expression test ($F_{1,17} = 14.786$, $P \leq 0.01$). Nissl staining of infected amygdala showed that the

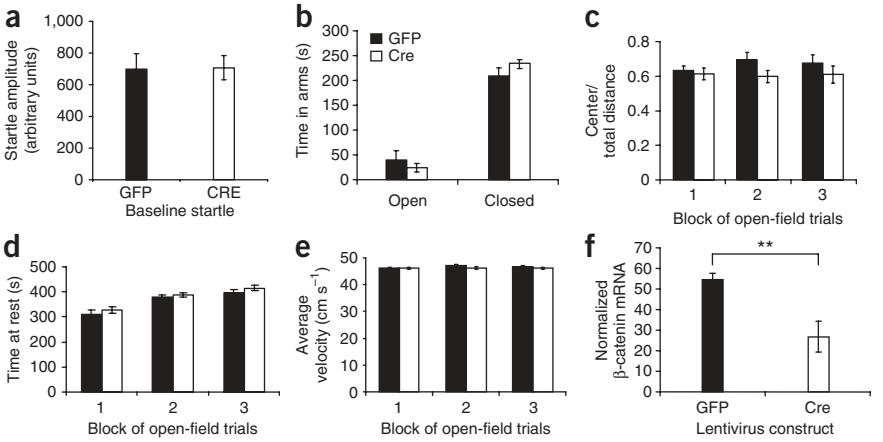

Figure 6 Amygdala-specific β-catenin deletions do not affect baseline anxiety or activity measures. Mice received bilateral injections of LV-GFP or LV-Cre into the amygdala and were allowed 10 d to recover. (**a**) Baseline startle for mice injected with LV-GFP or LV-Cre. (**b**) Time spent in the open and closed arms of the elevated plus-maze. (**c**–**e**) Activity measures, recorded in three blocks of 10 min, for mice placed in an open-field apparatus for 30 min. There were no differences between mice injected with LV-GFP or LV-Cre in terms of distance traveled in the center compared to total distance (**c**), time at rest (**d**) or average velocity (**e**). (**f**) β-catenin mRNA, normalized to local non-amygdala background. $n = 7$ for LV-GFP group; $n = 6$ for LV-Cre group. Error bars, s.e.m. $^{**}P \leq 0.01$.

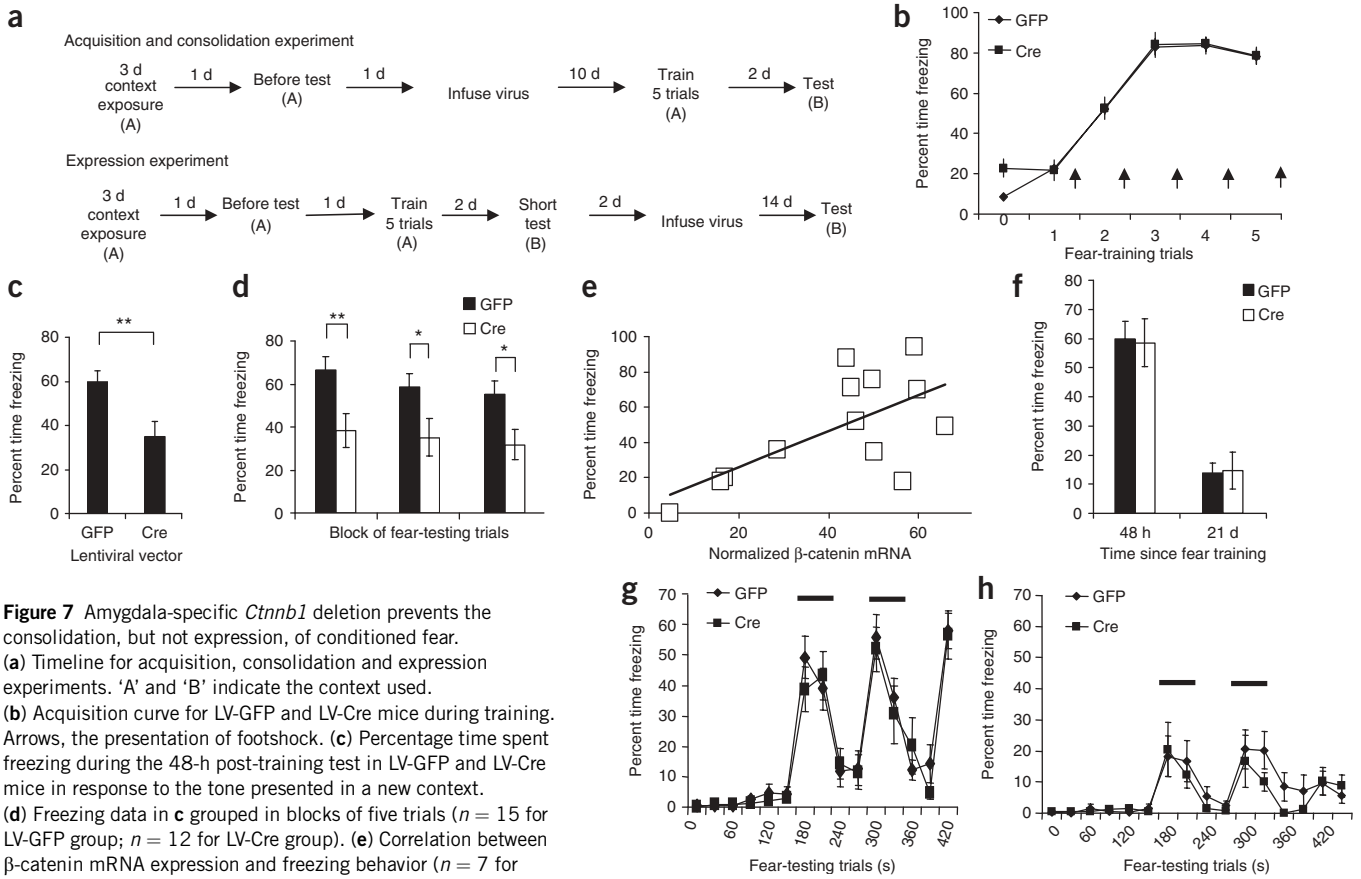

Figure 7 Amygdala-specific *Ctnnb1* deletion prevents the consolidation, but not expression, of conditioned fear. (**a**) Timeline for acquisition, consolidation and expression experiments. 'A' and 'B' indicate the context used. (**b**) Acquisition curve for LV-GFP and LV-Cre mice during training. Arrows, the presentation of footshock. (**c**) Percentage time spent freezing during the 48-h post-training test in LV-GFP and LV-Cre mice in response to the tone presented in a new context. (**d**) Freezing data in **c** grouped in blocks of five trials ($n = 15$ for LV-GFP group; $n = 12$ for LV-Cre group). (**e**) Correlation between β-catenin mRNA expression and freezing behavior ($n = 7$ for LV-GFP group; $n = 6$ for LV-Cre group). (**f**) Percentage time spent freezing by LV-GFP and LV-Cre mice at 48 h (before infusion) and 21 d (after infusion) after training. (**g**) Freezing behavior 48 h after training, shown in 30-s intervals. (**h**) Freezing behavior 21 d after training, shown in 30-s intervals. Horizontal bars in **f**–**h** indicate periods during which the conditioned stimulus was present ($n = 10$ for LV-GFP group; $n = 9$ for LV-Cre group). Error bars, s.e.m. $^*P < 0.05$; $^{**}P \leq 0.01$.

observed decrease in freezing in both groups was not caused by damage to the amygdala. Thus, it is more likely that the decrease in freezing resulted from the passage of time between training and testing. Notably, when testing the mice after this delay, we found that mice receiving LV-Cre showed similar levels of freezing (14.58 ± 6.34) to mice receiving LV-GFP (13.91 ± 3.45; $t_{17} = 0.095$, $P > 0.05$). Because both the LV-Cre and LV-GFP groups showed comparable, and statistically significant, levels of freezing when tested for fear expression while Cre recombinase and GFP protein were expressed in the amygdala, these data suggest that β-catenin in the amygdala is not required for fear expression after the memory has been consolidated.

DISCUSSION

Our data suggest that β-catenin has a role in long-term memory formation in adults. We showed that β-catenin is highly expressed in the adult mouse amygdala and is dynamically regulated at both the transcriptional and post-translational levels with fear learning. Pharmacological stabilization of β-catenin with LiCl resulted in enhanced learning, whereas genetic deletion of *Ctnnb1* in the amygdala resulted in deficient learning. By studying the effects of *Ctnnb1* deletion in adult mice, we have identified a role for β-catenin in learning and memory that is distinct from its role in development.

Our data also suggest that β-catenin is required for the consolidation, but not the acquisition, of fear memory. However, once the memory has been consolidated, we found that β-catenin is no longer required to express the memory. During this consolidation period, the interaction between β-catenin and cadherin is dynamically regulated, suggesting that β-catenin is involved in the structural conversion of short-term labile to long-term stable memory traces.

We found that β-catenin mRNA expression was increased in the BLA, but not the somatosensory cortex and striatum, after fear training. To our knowledge, this is the first study to examine β-catenin *in vivo* with learning, but this result is consistent with previous *in vitro* studies of hippocampal slices showing an increase in nuclear β-catenin with tetanic stimulation[12]. Wnt target genes have also been shown to be upregulated with long-term potentiation in hippocampal slices, anywhere from 15 to 120 min after stimulation[12].

Notably, when we measured total β-catenin protein, we did not see any alterations with training. It has been shown that depolarization of hippocampal neurons with KCl does not change the total amount of β-catenin at the synapse but instead causes a redistribution from dendritic shafts to spines[7]. It is possible that rapid dynamic changes in breakdown, redistribution and replacement do not result in apparent changes in total protein visualized with immunoblots.

We observed biochemical changes suggesting that the roles of β-catenin in both cell-cell adhesion and Wnt signaling are affected by fear conditioning. Phosphorylation of β-catenin on Tyr654 has been shown to decrease the affinity of β-catenin for cadherin[14,21]. In

addition, inhibiting the phosphorylation of Tyr654 with a point mutation redistributes β-catenin from dendritic shafts to spines, thereby increasing the β-catenin–cadherin interaction[7]. In our study, Tyr654-phosphorylated β-catenin were dynamically regulated after training. Our coimmunoprecipitation experiments show a very rapid period of β-catenin–cadherin destabilization, followed by a period of stabilization during consolidation. Overall, these findings suggest that the affinity of β-catenin for cadherin initially weakens to allow for modifications of the synapse and then strengthens to stabilize the synapse, which we hypothesize to be a molecular and cellular correlate of memory consolidation.

Such dynamic regulation of β-catenin phosphorylation on Tyr654 has previously been proposed. Treatment with brain-derived neurotrophic factor has been shown to induce synaptic vesicle dispersion in hippocampal cultures, which is associated with an increase in β-catenin tyrosine phosphorylation and a decrease in β-catenin–cadherin interactions. Soon after this dispersion, phosphorylation decreases, and the β-catenin–cadherin interaction is restored[22]. Notably, we previously showed that brain-derived neurotrophic factor activation of the TrkB receptor is required in the amygdala for consolidation of fear memories[23]. Thus, a similar mechanism may be taking place in this *in vivo* learning paradigm, such that when new memories are formed, pre-existing synapses must become destabilized transiently before the stabilization of synapses involved in memory formation.

We have provided both biochemical and behavioral evidence suggesting that increased stabilization of β-catenin, through the inhibition of GSK-3β, is important for learning and memory. Normally, GSK-3β phosphorylates β-catenin at Ser33, Ser37 and Thr41, marking the protein for degradation. However, when GSK-3β is inactivated by phosphorylation at Ser9, β-catenin becomes stabilized[10]. In our study, there was an increase in phosphorylated GSK-3β in the amygdala 2 h after fear conditioning. In addition, increasing the inhibition of GSK-3β with LiCl decreased β-catenin phosphorylation. Acute administration of LiCl 30 min before training resulted in an enhancement in learning measured 48 h later, without any effect on acquisition.

Although administration of LiCl has been shown to produce behaviors similar to those resulting from overexpression of β-catenin in the mouse brain[24], the actions of LiCl are not necessarily specific to β-catenin. To more definitively identify the role of β-catenin in long-term memory formation, we used genetic manipulation to delete *Ctnnb1* from the adult amygdala. We found that deletion of *Ctnnb1* before training does not affect the acquisition or immediate expression of fear but does produce deficits in learning when measured 48 h after training. In addition, deletion of *Ctnnb1* after consolidation has occurred does not affect the expression of learned fear. These findings provide further support that normal β-catenin expression is necessary to consolidate newly acquired memory.

One limitation of this study is our inability to specifically inhibit or delete *Ctnnb1* immediately after training. Previous work has elegantly shown, using consolidation of inhibitory avoidance, that post-training manipulations are the gold standard for demonstrating disruption of fear consolidation[25,26]. Although the data on consolidation of amygdala-dependent classical conditioning paradigms have been less clear, this is an important manipulation for interpretation of consolidation effects. Unfortunately, there are no drugs currently available that selectively inhibit β-catenin. Additionally, a minimum of 7–10 d is required for optimal lentiviral gene expression, so we are unable to delete *Ctnnb1* shortly after training. However, we feel that our current powerful method of genetic manipulation is an important approach to specifically examine the role of *Ctnnb1* in the amygdala during learning. Furthermore, we feel that the lack of an effect of *Ctnnb1* deletion on acquisition and expression of fear makes a strong case for its role during the consolidation period.

Given the results obtained thus far, we propose that synapses weaken during the acquisition of fear and immediately afterwards (as indicated by decreased β-catenin–cadherin association immediately and 0.5 h after training), thereby alleviating the requirement for β-catenin. Once the synapses have been modified during the consolidation process, β-catenin is required to convert that memory trace into long-term memory. These proposed changes in synaptic strength will need to be further explored. Additional studies are also needed to determine whether it is the role of β-catenin in cell-cell adhesion, Wnt signaling or both that contributes to its observed effects on learning and memory.

In summary, our results suggest that β-catenin, a 'hub' protein involved in both transcriptional regulation and stabilization of cell-cell contacts and synaptogenesis, is required for normal consolidation of new memories in adult mice. This finding adds to the body of knowledge describing the role of β-catenin in normal cell functioning, tumor regulation and development. Although β-catenin has been implicated with *in vitro* approaches in synaptogenesis and synaptic plasticity, our results provide definitive support for its function in learning and memory processes. Further understanding of its role may provide important insights into the nature of the molecular mechanisms underlying memory consolidation. In humans, the development of new small-molecule specific inhibitors of β-catenin function may eventually provide a powerful clinical approach to transiently inhibit the consolidation of newly formed trauma memories and thus prevent fear-related disorders, such as post-traumatic stress disorder. Similarly, enhancing β-catenin function may be helpful in treating disorders of memory such as Alzheimer's disease.

METHODS

Animals. Adult male C57BL/6J mice (Jackson Labs) were used for immunoblotting and drug treatment experiments. All other experiments were carried out with homozygous β-catenin floxed mice (B6.129-Ctnnb1tm2Kem/KnwJ[20]; Jackson Labs). Mice were housed four per cage in a temperature-controlled (24 °C) animal colony, with *ad libitum* access to food and water, on a 12-h light-dark cycle, with all behavioral procedures done during the light cycle.

Immunoblotting, immunoprecipitation and *in situ* hybridization. See **Supplementary Methods** online for full details. After behavioral procedures, brains were blocked rapidly and kept frozen at –80 °C. Bilateral amygdala punches were obtained and homogenized. Twenty micrograms of protein per mouse were electrophoretically separated on SDS-PAGE, transferred onto nitrocellulose, blocked for 1 h and incubated in primary antibody overnight at 4 °C. Antibodies to the following proteins were used: Tyr654-phosphorylated β-catenin (1:100, Abcam), β-catenin (1:500, BD Biosciences), phosphorylated β-catenin (1:1,000, Cell Signaling), phosphorylated GSK-3β (1:1,1000, Cell Signaling) and pan-cadherin (1:1,1000, Cell Signaling). Membranes were washed and incubated with a horseradish peroxidase–labeled secondary antibody, then detected by SuperSignal West Chemiluminescence (Pierce). Total blotted protein was normalized to α-tubulin, and relative values were expressed as the protein of interest divided by the loading control.

For immunoprecipitation, solubilized proteins were incubated with Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology) and centrifuged. Protein A/G PLUS-Agarose beads and antibody to β-catenin (Cell Signaling) were then added to the supernatant of each protein sample, incubated overnight at 4 °C, washed and subjected to western blot analyses.

In situ hybridization was carried out as previously described[20] (see **Supplementary Methods**). The full-length clone for β-catenin (GI accession no. 31419847) was used to amplify and subclone the regions between exons 2 and 6 of β-catenin, the area flanked by *loxP* sites in the mutant mouse. This *loxP*-flanked subclone was then linearized, and both antisense and sense 35S-riboprobes were generated using the appropriate RNA polymerase and ^{35}S-UTP in the reaction. After autoradiography, the density of β-catenin mRNA

in the BLA, somatosensory cortex and striatum was assessed using the mean luminosity function of Adobe Photoshop.

Lentiviral vectors and viral injections. See **Supplementary Methods** for full details. Viral vectors were produced and concentrated as previously described[27–33]. Briefly, a Cre recombinase–expressing vector (LV-Cre) or a GFP-expressing control vector (LV-GFP) with a final titer of 10^9 infectious units per ml was used for stereotaxic injections into the amygdala. Mice were anesthetized, and small holes were drilled into the skull above the injection site. BLA coordinates were as follows: anteroposterior, –1.8; dorsoventral, –4.9; mediolateral, ±3.2 relative to bregma. A 10-μl Hamilton microsyringe precoated with bovine serum albumin was used to deliver bilateral injections of lentiviral vectors into the amygdala (0.2 μl of virus per side, injected at a rate of 0.025 μl min^{-1}). The needle was left in place for 10 min after the injection, and mice were allowed to recover for 10–14 d before testing.

Behavioral studies. Elevated plus-maze and open-field maze were used according to standard protocol (behavioral techniques and materials described fully in **Supplementary Methods**). Mice were fear-conditioned in eight identical startle-response systems. After 3 d of exposure to the conditioning chambers, mice were given a pretraining test to examine baseline levels of startle in the presence of the tone (conditioned stimulus). Twenty-four hours after the pretest, mice were placed in the conditioning chamber and after 5 min presented with five tone–shock pairings at an intertrial interval of 5 min. Each pairing consisted of a 30-s tone (6 kHz, 85 db; conditioned stimulus) that terminated with a 0.5-s footshock (1.0 mA, except where noted; unconditioned stimulus). Freezing in startle-reflex chambers during fear acquisition was assessed as described previously[34,35]. Forty-eight hours after training, mice were tested for freezing in rodent modular test chambers with an inside volume of 30.5 cm × 24.1 cm × 21.0 cm. Three minutes later, 15 conditioned stimulus tones (6 kHz, 85 db) with an intertrial interval of 1.5 min were delivered through a high-frequency speaker attached to the side of each chamber. Percentage time spent freezing during the conditioned stimulus presentations was calculated for each mouse using FreezeFrame (ACT-100; Coulbourn Instruments).

Data analysis. Statistically significant differences were determined by Student's *t*-test or ANOVA, with *post hoc* least-squares difference tests for multiple comparisons. The results are presented as means ± s.e.m.

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

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AUTHOR CONTRIBUTIONS
K.A.M. initiated the project, conducted the experiments and data analysis and wrote the draft versions of the manuscript. K.J.R. supervised the project, assisted with data analysis and experimental planning, and contributed to draft and revised versions of the manuscript.

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1. Harris, K.M. & Kater, S.B. Dendritic spines: cellular specializations imparting both stability and flexibility to synaptic function. *Annu. Rev. Neurosci.* **17**, 341–371 (1994).
2. Nimchinsky, E.A., Sabatini, B.L. & Svoboda, K. Structure and function of dendritic spines. *Annu. Rev. Physiol.* **64**, 313–353 (2002).
3. Matsuzaki, M., Honkura, N., Ellis-Davies, G.C. & Kasai, H. Structural basis of long-term potentiation in single dendritic spines. *Nature* **429**, 761–766 (2004).
4. Nagerl, U.V., Eberhorn, N., Cambridge, S.B. & Bonhoeffer, T. Bidirectional activity-dependent morphological plasticity in hippocampal neurons. *Neuron* **44**, 759–767 (2004).
5. Yuste, R. & Bonhoeffer, T. Morphological changes in dendritic spines associated with long-term synaptic plasticity. *Annu. Rev. Neurosci.* **24**, 1071–1089 (2001).
6. Gumbiner, B.M. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* **84**, 345–357 (1996).
7. Murase, S., Mosser, E. & Schuman, E.M. Depolarization drives beta-catenin into neuronal spines promoting changes in synaptic structure and function. *Neuron* **35**, 91–105 (2002).
8. Salinas, P.C. & Price, S.R. Cadherins and catenins in synapse development. *Curr. Opin. Neurobiol.* **15**, 73–80 (2005).
9. Takeichi, M. & Abe, K. Synaptic contact dynamics controlled by cadherin and catenins. *Trends Cell Biol.* **15**, 216–221 (2005).
10. Logan, C.Y. & Nusse, R. The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.* **20**, 781–810 (2004).
11. Moon, R.T., Kohn, A.D., De Ferrari, G.V. & Kaykas, A. WNT and beta-catenin signalling: diseases and therapies. *Nat. Rev. Genet.* **5**, 691–701 (2004).
12. Chen, J., Park, C.S. & Tang, S.J. Activity-dependent synaptic Wnt release regulates hippocampal long term potentiation. *J. Biol. Chem.* **281**, 11910–11916 (2006).
13. Machon, O., van den Bout, C.J., Backman, M., Kemler, R. & Krauss, S. Role of beta-catenin in the developing cortical and hippocampal neuroepithelium. *Neuroscience* **122**, 129–143 (2003).
14. Roura, S., Miravet, S., Piedra, J., Garcia de Herreros, A. & Dunach, M. Regulation of E-cadherin/Catenin association by tyrosine phosphorylation. *J. Biol. Chem.* **274**, 36734–36740 (1999).
15. Behrens, J. *et al.* Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. *Science* **280**, 596–599 (1998).
16. Peifer, M., Pai, L.M. & Casey, M. Phosphorylation of the *Drosophila* adherens junction protein Armadillo: roles for wingless signal and zeste-white 3 kinase. *Dev. Biol.* **166**, 543–556 (1994).
17. Yost, C. *et al.* The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in *Xenopus* embryos by glycogen synthase kinase 3. *Genes Dev.* **10**, 1443–1454 (1996).
18. Gould, T.D., O'Donnell, K.C., Picchini, A.M. & Manji, H.K. Strain differences in lithium attenuation of D-amphetamine-induced hyperlocomotion: a mouse model for the genetics of clinical response to lithium. *Neuropsychopharmacology* **32**, 1321–1333 (2007).
19. Davies, S.P., Reddy, H., Caivano, M. & Cohen, P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* **351**, 95–105 (2000).
20. Brault, V. *et al.* Inactivation of the beta-catenin gene by Wnt1-Cre-mediated deletion results in dramatic brain malformation and failure of craniofacial development. *Development* **128**, 1253–1264 (2001).
21. Piedra, J. *et al.* Regulation of beta-catenin structure and activity by tyrosine phosphorylation. *J. Biol. Chem.* **276**, 20436–20443 (2001).
22. Bamji, S.X., Rico, B., Kimes, N. & Reichardt, L.F. BDNF mobilizes synaptic vesicles and enhances synapse formation by disrupting cadherin-beta-catenin interactions. *J. Cell Biol.* **174**, 289–299 (2006).
23. Rattiner, L.M., Davis, M., French, C.T. & Ressler, K.J. Brain-derived neurotrophic factor and tyrosine kinase receptor B involvement in amygdala-dependent fear conditioning. *J. Neurosci.* **24**, 4796–4806 (2004).
24. Gould, T.D. *et al.* Beta-catenin overexpression in the mouse brain phenocopies lithium-sensitive behaviors. *Neuropsychopharmacology* **32**, 2173–2183 (2007).
25. McGaugh, J.L. The amygdala modulates the consolidation of memories of emotionally arousing experiences. *Annu. Rev. Neurosci.* **27**, 1–28 (2004).
26. McGaugh J.L., Roozendaal, B. Role of adrenal stress hormones in forming lasting memories in the brain. *Curr. Opin. Neurobiol.* **12**, 205–210 (2002).
27. Chhatwal, J.P., Hammack, S.E., Jasnow, A.M., Rainnie, D.G. & Ressler, K.J. Identification of cell-type-specific promoters within the brain using lentiviral vectors. *Gene Ther.* **14**, 575–583 (2007).
28. Chhatwal, J.P., Stanek-Rattiner, L., Davis, M. & Ressler, K.J. Amygdala BDNF signaling is required for consolidation but not encoding of extinction. *Nat. Neurosci.* **9**, 870–872 (2006).
29. Heldt, S.A., Stanek, L., Chhatwal, J.P. & Ressler, K.J. Hippocampus-specific deletion of BDNF in adult mice impairs spatial memory and extinction of aversive memories. *Mol. Psychiatry* **12**, 656–670 (2007).
30. Naldini, L., Blomer, U., Gage, F.H., Trono, D. & Verma, I.M. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc. Natl. Acad. Sci. USA* **93**, 11382–11388 (1996).
31. Naldini, L. *et al.* In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272**, 263–267 (1996).
32. Tiscornia, G., Singer, O. & Verma, I.M. Production and purification of lentiviral vectors. *Nat. Protoc.* **1**, 241–245 (2006).
33. Zufferey, R., Donello, J.E., Trono, D. & Hope, T.J. Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J. Virol.* **73**, 2886–2892 (1999).
34. Heldt, S.A., Stanek, L., Chhatwal, J.P. & Ressler, K.J. Hippocampus-specific deletion of BDNF in adult mice impairs spatial memory and extinction of aversive memories. *Mol. Psychiatry* **12**, 656–670 (2007).
35. Jones, S.V., Heldt, S.A., Davis, M. & Ressler, K.J. Olfactory-mediated fear conditioning in mice: simultaneous measurements of fear-potentiated startle and freezing. *Behav. Neurosci.* **119**, 329–335 (2005).
36. Paxinos, G. & Watston, C. *The Mouse Brain in Stereotaxic Coordinates* (Academic, New York, 2003).