Dentate granule cell recruitment of feedforward inhibition governs engram maintenance and remote memory generalization

Nannan Guo1–3, Marta E Soden4,5, Charlotte Herber1,2, Michael TaeWoo Kim1–3, Antoine Besnard1–3, Paoyan Lin1–3, Xiang Ma6,7, Constance L Cepko6,7, Larry S Zweifel4,5 & Amar Sahay1–3,8

Memories become less precise and generalized over time as memory traces reorganize in hippocampal–cortical networks. Increased time-dependent loss of memory precision is characterized by an overgeneralization of fear in individuals with post-traumatic stress disorder (PTSD) or age-related cognitive impairments. In the hippocampal dentate gyrus (DG), memories are thought to be encoded by so-called ‘engram-bearing’ dentate granule cells (eDGCs). Here we show, using rodents, that contextual fear conditioning increases connectivity between eDGCs and inhibitory interneurons (INs) in the downstream hippocampal CA3 region. We identify actin-binding LIM protein 3 (ABLIM3) as a mossy-fiber-terminal-localized cytoskeletal factor whose levels decrease after learning. Downregulation of ABLIM3 expression in DGCs was sufficient to increase connectivity with CA3 stratum lucidum INs (SLINs), promote parvalbumin (PV)-expressing SLIN activation, enhance feedforward inhibition onto CA3 and maintain a fear memory engram in the DG over time. Furthermore, downregulation of ABLIM3 expression in DGCs conferred conditioned context-specific reactivation of memory traces in hippocampal–cortical and amygdalar networks and decreased fear memory generalization at remote (i.e., distal) time points. Consistent with the observation of age-related hyperactivity of CA3, learning failed to increase DGC–SLIN connectivity in 17-month-old mice, whereas downregulation of ABLIM3 expression was sufficient to restore DGC–SLIN connectivity, increase PV* SLIN activation and improve the precision of remote memories. These studies exemplify a connectivity-based strategy that targets a molecular brake of feedforward inhibition in DG–CA3 and may be harnessed to decrease time-dependent memory generalization in individuals with PTSD and improve memory precision in aging individuals.

Memories become less precise over time as their details are lost, a phenomenon referred to as time-dependent generalization. Time-dependent generalization of traumatic memories results in expression of fear in neutral environments at remote time points, a feature of PTSD that has been captured in numerous animal studies1–7. Memory imprecision also characterizes mild cognitive impairment (MCI) and episodic memory dysfunction during aging8,9. Recent studies have begun to identify circuit mechanisms that underlie memory precision and generalization of recent memories5,10–15. In contrast, we know much less about the circuit mechanisms governing the time-dependent erosion of details that gradually lead to generalization of remote memories1. Bridging this gap will edify new strategies to preserve memory precision during aging and curb time-dependent fear generalization in subjects with PTSD.

Unlike the generalization of recent memories, the generalization of remote memories is thought to reflect time-dependent reorganization of memory traces in hippocampal–cortical networks16–18. Growing evidence supports a continuous role for the hippocampus in maintaining the specificity of memories over time through the hippocampal–cortical interactions that underlie memory consolidation17,19–23. These observations, together with the hippocampal index theory5,21,22, suggest that new memories presumably encoded in hippocampal eDGCs24,25 may serve as indexes for the stabilization of cognate cortical representations5,16,21,22. Consistent with this idea, genetic silencing of eDGCs during learning impairs early maturation of cortical engram cells25. However, the circuit mechanisms by which eDGC connectivity dictates time-dependent reorganization of memory traces in hippocampal–cortical networks and governs remote memory generalization remain poorly understood.

DGC connectivity with SLINs has been suggested to govern the precision of recent memories11. Mossy fiber terminals (MFTs) form excitatory synapses onto thorny excrescence-like dendritic spines of CA3 neurons, and filopodia emanating from MFTs that contact SLINs are thought to mediate feedforward inhibition onto CA3 (refs. 26–28). DGC recruitment of inhibition onto CA3 is thought to determine the temporal window of spiking of CA3 neurons and potentially...
dictates CA3 activation patterns\textsuperscript{28,29}. Notably, MFT filopodia number is inversely correlated with fear generalization over time\textsuperscript{11}. Mice that lack adducin 2, a cytoskeletal factor expressed brain wide, show impaired synaptic plasticity, impaired learning and memory, failure to increase DGC–SLIN connectivity following learning and increased generalization of a fear memory 1 d after conditioning\textsuperscript{11,30,31} that was rescued by re-expression of adducin 2 in the DG. CA3 outputs and PV-expressing (PV\textsuperscript{+}) INs have been suggested to promote hippocampal–cortical communication through modulation of sharp wave ripples\textsuperscript{32–37}. On the basis of these findings, we hypothesized that DGC recruitment of inhibition onto CA3 maintains the DG engram over time, and this in turn dictates time-dependent reorganization of memory traces in hippocampal–cortical networks and decreases remote memory generalization. Critical to testing this hypothesis was being able to identify a molecular control by which DGC recruitment of inhibition onto CA3 could be acutely and selectively enhanced.

Evidence from studies in rodents, nonhuman primates and humans suggests that aging is associated with CA3 hyperexcitability, hyperactivity and inflexible remapping\textsuperscript{38–42}. Decreased DGC recruitment of inhibition onto CA3 (ref. 42), a decline in the number of SLINS\textsuperscript{43}, decreased DGC spine density\textsuperscript{43} and increased intrinsic CA3 excitability\textsuperscript{40} may all contribute to age-related changes in CA3. Whether DGC–SLIN connectivity is altered in aging and whether enhancing DGC recruitment of inhibition is sufficient to enhance memory precision in aging is not known.

RESULTS

ABLIM3 is a learning-regulated molecular brake of DGC–SLIN connectivity

We first examined DGC–SLIN connectivity of eDGCs at recent and remote time points after contextual fear conditioning. Using 3-month-old cFos-\textsuperscript{+}TataetO-Tau-LacZ transgenic mice\textsuperscript{44} (in which \(\beta\)-galactosidase (LacZ) expressed under the promoter of the \(\beta\)-actin gene (also known as \(\beta\)-actin) labels cFOS-expressing cells) in combination with a tetO-Tau-LacZ reporter line to indelibly label memory engrams by hybridization for \(\beta\)-galactosidase (GAL) in the DG (top) and CA3 (bottom; arrows) in VGLUT1 + MFTs in CA3. This experiment was replicated three times. Scale bars, 50 \(\mu\)m.

To causally assess how DGC recruitment of inhibition affected engram properties, we sought to identify a selective molecular regulator of DGC–SLIN connectivity. We examined brain-wide expression patterns of putative targets of Kruppel-like factor 9 (KLF9), a transcriptional regulator of DGC maturation\textsuperscript{45}, and identified \textit{Ablim3} as a candidate\textsuperscript{46,47}. \textit{Ablim3} is highly expressed in DGCs and is negligibly expressed in CA subfields (Fig. 1c, http://hippseq.janelia.org/full/t1/F64399A8-AF50-11E7-AD92-C04A01797C37?). Outside of the hippocampus, \textit{Ablim3} is enriched in granule cells of the main olfactory bulb and cerebellum (Allen Brain Atlas, http://mouse.brain-map.org/experiment/show/69626582\textsuperscript{47}). Immunohistochemistry analysis of brain sections from adult mice revealed that \textit{Ablim3} was absent from the CA1–CA3 subregions and molecular layers of the hippocampus but that it was exclusively localized to MFTs (labelled with vesicular glutamate transporter 1 (VGLUT1); Fig. 1c,d and Supplementary Fig. 1a). Within MFTs, \textit{Ablim3} was absent from active zones (labelled by bassoon immunoreactivity) but was located to puncta adherens junctions (PAJs), sites of MFT stabilization on dendritic shafts of CA3 neurons, as indicated by overlap with zona occludens 1 (ZO-1), a marker of PAJs (Fig. 1d, Supplementary Figs. 1a and Supplementary Fig. 1a). Western blot analysis of \textit{Ablim3} expression in the hippocampus of naive and contextual-fear-conditioned (training) mice revealed that \textit{Ablim3} levels were decreased following learning as compared to that in naive mice (Fig. 1e and Supplementary Figs. 1c). \textit{Ablim3} levels in CA1, although low, remained unchanged following learning (Supplementary Fig. 1d). On the basis of these observations and of evidence that supported a role for \textit{Ablim3} in stabilizing branched F-actin in lamellipodia\textsuperscript{46,49}, we predicted that \textit{Ablim3} levels in MFTs constrain generation of MFT filopodial contacts with SLINS.

To determine whether \textit{Ablim3} functions as a molecular brake of DGC–SLIN connectivity, we engineered lentiviruses that expressed short hairpin RNAs (shRNAs) to downregulate \textit{Ablim3} expression in non-tagged DGs 1 d after training, without any effects on the proportion of MFT filopodial contacts with PV\textsuperscript{+} SLINS (Supplementary Fig. 1b). However, the number of MFT filopodia of eDGCs decreased to levels observed in non-tagged (‘non-gram’) DGCs at the remote time point, day 16 (Fig. 1b), when memory becomes imprecise and is generalized\textsuperscript{2,3}.

© 2018 Nature America, Inc., part of Springer Nature. All rights reserved.
in vitro (Supplementary Fig. 2a). Stereotactic injections of a GFP-expressing lentivirus that also encoded one of two Ablim3-specific shRNAs (hereafter collectively referred to as LV-shRNA-GFP) into the DG substantially downregulated Ablim3 expression in DGCs (Supplementary Fig. 3a,b) and decreased ABLIM3 expression in MFTs (Fig. 1f). Lentiviral infection primarily targeted mature DGCs, as fewer than 4% of GFP-expressing cells also expressed doublecortin (DCX), a marker of immature adult-born DGCs (Supplementary Fig. 3c,d). shRNA-mediated downregulation of Ablim3 expression in DGCs using lentiviruses that encoded one of two different...
shRNAs (shAblim3-46 or shAblim3-47) increased the number of filopodia per MFT, but did not affect MFT filopodial length, MFT size or dendritic spine density of DGCs (Fig. 1g and Supplementary Fig. 2b). Downregulation of Ablim3 expression (using shAblim3-46 throughout the study unless otherwise noted) in DGCs did not affect the proportion of VGLUT1+ MFT filopodial contacts with PV+ or somatostatin-expressing (SST+) SLINs (Fig. 1h and Supplementary Fig. 2d). Stereotaxic injection of the anterograde tracer, vesicular stomatitis virus (VSV) expressing GFP (VSV-G)50, into DGCs following ABLIM3 downregulation resulted in increased trans-synaptic labeling of GABA+ and PV+ SLINs at 2 d (Fig. 1i), but not immediately (day 0), following injection (Supplementary Fig. 2f). ABLIM3 downregulation in DGCs did not affect the expression of GABA or PV in the stratum lucidum (Supplementary Fig. 2e). Together these data demonstrate that ABLIM3 is a molecular brake of DGC–SLIN connectivity and that decreasing ABLIM3 levels in DGCs of adult mice mimics a learning-induced increase in DGC–SLIN connectivity (Fig. 1j).

**ABLIM3 downregulation in DGCs promotes DGC recruitment of inhibition onto CA3**

Although it is assumed that DGC–SLIN connectivity mediates GABAergic inhibition onto CA3 (ref. 11), evidence linking acute modulation of DGC–SLIN connectivity in adult mice with electrophysiologically changes in inhibition onto CA3 is conspicuously absent28. Therefore, we asked whether increasing DGC−SLIN connectivity by downregulation of ABLIM3 expression translated into physiological changes in excitatory inputs onto PV+ INs and increased GABAergic inhibition onto CA3. Characterization of mice in which PV+ SLINs were labeled with the fluorescent reporter tdTomato (hereafter referred to as PV-Cre: Ai14 mice) revealed that 90% of tdTomato+ cells also expressed PV (Fig. 2a,b). Next we performed whole-cell recordings from tdTomato+PV+ SLINs and CA3 pyramidal neurons in brain slices that were obtained from PV-Cre:Ai14 mice following injection of LV-shRNA-GFP (which encoded shAblim3-46) or a lentivirus that encoded a nontargeting control shRNA (hereafter referred to as LV-shNT) into the DG (Fig. 2c). Downregulation of ABLIM3 expression in DGCs increased the frequency of miniature excitatory postsynaptic currents (mEPSCs) in PV+ SLINs (Fig. 2d,g), which was consistent with increased numbers of VGLUT1+ MFT filopodial contacts onto PV+ SLINs (Fig. 1g,h). We noted a decrease in the amplitude of mEPSCs in PV+ SLINs, which was suggestive of potential homeostatic changes in response to the increased excitatory drive onto these cells (Fig. 2d,g). Recordings from CA3 pyramidal neurons revealed an increase in frequency, but not in amplitude, of miniature inhibitory postsynaptic currents (mIPSCs) (Fig. 2e,g). Consistent with increased mIPSCs in CA3 neurons, increasing mossy fiber excitatory drive onto PV+ SLINs significantly enhanced the number of PV+ puncta per unit area in the CA3 pyramidal layer (Fig. 2h). Additionally, the amplitude of mEPSCs in CA3 neurons was decreased with no effect on mEPSC frequency (Fig. 2f,g).

To determine the net effect of these synaptic changes and whether ABLIM3 levels in DGCs dictated feedforward inhibition onto CA3, we co-injected a recombinant adeno-associated virus 5 (rAAV5) vector encoding channelrhodopsin (ChR2) under the calcium−calmodulin-independent protein kinase II alpha (Camk2a) promoter and lentiviruses expressing shAblim3-46 or shNT into the DG and performed whole-cell recordings in CA3 pyramidal neurons in hippocampal slices following blue-light stimulation (Fig. 3a). Decreasing ABLIM3 levels in DGCs resulted in greater recruitment of feedforward inhibition onto CA3 neurons, as evidenced by a significant decrease in the excitation−inhibition ratio (Fig. 3a). This effect was driven by an increase in evoked IPSCs in CA3 neurons (Fig. 3a,b). Bath application of DCG-IV, a group II metabotropic glutamate receptor agonist that selectively blocks evoked MFT release28,31, to hippocampal slices abolished evoked inhibitory and excitatory responses in CA3 (Fig. 3c).

**ABLIM3 downregulation in DGCs maintains engram in the DG and constrains reactivation of remote memory traces in the CA3−CA1−ACC and BLA networks**

Because DGC–SLIN connectivity of eDGCs is transiently increased relative to that in non-engram-bearing cells following learning and a return to baseline at remote time points after memory is generalized, we asked whether enhancing DGC−PV+ SLIN connectivity promoted maintenance of a fear-conditioned context (context A) engram in the DG. To this end, we injected both an AAV9 construct that encoded an enhanced yellow fluorescent protein (eYFP)-tagged channelrhodopsin (Chr2) under the control of the tetO promoter and either LV-shRNA-GFP or LV-shNT-GFP into the DG of cfos-tTA44,52 transgenic mice (which express the tetracycline trans-activator (tTA) protein under control of the promoter of the Fos oncogene) before contextual fear conditioning in context A (Fig. 4a). Following tagging of context A ensembles with Chr2−eYFP, we either did not or did activate the context A neuronal ensemble in a neutral, distinct context (context C) at day 2 and day 10 by shining blue light above the DG in both groups of mice (Fig. 4b,c). Both groups showed comparable levels of light-induced freezing behavior at day 2, which suggested that there were comparable numbers of tagged and activated DGCs in both groups of mice. However, at day 10, only the LV-shRNA-GFP group permitted optogenetic-dependent artificial recall of the contextual fear memory (Fig. 4c). Blue light treatment did not elicit freezing behavior in context C in nonconditioned, context-A-exposed mice (Fig. 4d,c). Examination of eDGCs revealed an ~50% reduction in the number of Chr2−eYFP-expressing eDGCs (Supplementary Fig. 4a)52, which could potentially explain the lack of induction of freezing behavior by blue light treatment in the control group25. Injection of an AAV9 virus encoding tetO-Chr2−mCherry and LV-shRNA-GFP or LV-shNT-GFP into the DG of cfostrT-tTA mice revealed that ABLIM3 downregulation in DGCs did not affect Chr2 stability (Supplementary Fig. 4b,c). Thus, despite decreased expression of the Chr2 tag over time and the time-dependent reduction in dendritic spine density of eDGCs25, increasing DGC recruitment of inhibition by ABLIM3 downregulation was sufficient to permit engram activation at a remote time point.

Next we asked whether enhancing DGC recruitment of feedforward inhibition by ABLIM3 downregulation affected context-specific reactivation of memory traces in hippocampal–cortical networks over time. We stereotaxically injected LV-shRNA-GFP or LV-shNT-GFP into the DG of TetTag mice, which harbor a knock-in allele encoding a histone 2B (H2B)−mCherry reporter to indelibly label cells that were activated during conditioning to context A (Supplementary Fig. 4d,e). Reactivation of the DG ensemble in context A decreased over time, indicating that an engram could gradually become silenced over time as the memory became generalized (Fig. 4d,e). Both LV-shRNA-GFP-injected and LV-shNT-GFP-injected mice showed equivalent levels of reactivation of context A ensembles in the DG when mice were re-exposed to context A and context C at immediate (day 1 post training) and remote time points (day 16) (Fig. 4e, Supplementary Fig. 5b–d and Supplementary Fig. 6a). However, ABLIM3 downregulation in DGCs significantly decreased reactivation of context A ensembles in CA3 when mice were exposed to the neutral context C at a remote, but not immediate, time point (Fig. 4e and Supplementary Fig. 6b).
Figure 2 ABLIM3 downregulation in DGCs increases mEPSCs in PV+ SLINs and PV+ puncta in CA3. (a) Representative images of genetically recombined tdTomato+ cells and PV+ cells (as assessed by staining with a PV-specific antibody) in CA3 of PV-Cre:Ai14 mice. The magnified image of the boxed area is shown on the right. Immunostaining for regulator of G-protein signaling 14 (RGS14) was performed to exclude CA2 cells from quantification of CA3 cells. (b) Pie graphs showing distribution of tdTomato+ and PV+ cells in the CA3 area, including the stratum pyramidale (SP), stratum oriens (SO), stratum radiatum (SR) and stratum lucidum (SL) (n = 2 mice). (c) Schematic of LV-shNT-GFP or LV-shRNA-GFP injections into the DG of PV-Cre:Ai14 mice (top) and representative images showing LV-shNT-GFP- or LV-shRNA-GFP-infected GFP+ MFTs in PV− Cre:Ai14 mice (bottom). Magnified images of the area in the white box are shown on the right. Immunostaining was replicated twice. Scale bars, 100 µm (left), 5 µm (right). (d) Whole-cell recording of mEPSC frequency (shNT: 3,887 events from n = 13 cells; shRNA: 3,588 events from n = 12 cells) (left) and mEPSC amplitude (shNT: 3,900 events from n = 13 cells, shRNA: 3,600 events from n = 12 cells) (right) from PV+ SLINs (n = 3 mice per group). *P < 0.0001 by Kolmogorov–Smirnov test. (e) Intracellular recording of mIPSC frequency (shNT: 2,691 events from n = 9 cells, shRNA: 2,691 events from n = 9 cells) (left) and mIPSC amplitude (shNT: 2,700 events from n = 9 cells; shRNA: 2,700 events from n = 9 cells) (right) from CA3 pyramidal neurons (n = 3 mice per group). *P < 0.0001 (left) or P = 0.0126 (right) by Kolmogorov–Smirnov test. (f) Intracellular recording of mEPSC frequency (shNT: 1,209 events from n = 9 cells; shRNA: 1,620 events from n = 12 cells) (left) and mEPSC amplitude (shNT: 1,218 events from n = 9 cells; shRNA: 1,632 events from n = 12 cells) (right) from CA3 pyramidal neurons (n = 3 mice per group). P = 0.0222 (left) or *P < 0.0001 (right) by Kolmogorov–Smirnov test. In d-f, the schematics indicate that whole-cell recordings were from from PV+ (d) or CA3 pyramidal (e,f) neurons following LV-shNT-GFP or LV-shRNA-GFP injections into the DG. (g) Schematic showing how Ablim3 knockout in DGCs increases the number of MFT filopodial contacts with PV+ SLINs to exert increased inhibition onto CA3 (left), with representative recording traces from each set of recordings (right), as in d-f. (h) Images showing PV+ puncta (arrows) in CA3 stratum pyramidale of LV-shNT-GFP- or LV-shRNA-GFP-infected mice (left) and quantification of PV+ puncta density in CA3 of LV-shRNA-GFP- or LV-shNT-GFP-infected mice (right) (n = 4 mice per group). **P < 0.01 by two-tailed unpaired t-test. Scale bars, 10 µm. Throughout, data are represented as mean ± s.e.m. See Supplementary Table 1 for detailed statistical analyses.

Immunostaining for regulator of G-protein signaling 14 (RGS14) was performed to exclude CA2 cells from quantification of CA3 cells (Supplementary Fig. 6b). Consistent with these observations, by using TetTag mice with transgenic tetO-H2B-GFP reporter alleles, we found that ABLIM3 downregulation decreased reactivation of context A ensembles in CA3 (Supplementary Fig. 5a) and CA1 when

© 2018 Nature America, Inc., part of Springer Nature. All rights reserved.
mice were placed in a neutral context C at the remote time point, day 16 (Fig. 4f,g and Supplementary Fig. 6c). Both groups of mice (LV-shRNA-GFP and LV-shNT-GFP) showed comparable numbers of labeled cells (Supplementary Fig. 5b–d) and similar percentages of chance overlap (Supplementary Fig. 5e) in the DG, CA3 and CA1.

The anterior cingulate cortex (ACC) is a storage site for remote memories. To determine whether ABLIM3 downregulation in DGcs also modulated reactivation of context A ensembles in the ACC, we used cfos-tTA transgenic mice in combination with a tetO-H2B-GFP reporter line to indelibly tag context A cortical ensembles (Fig. 4f,g). Downregulation of ABLIM3 in the DG of these mice increased reactivation of context A ensemble in the Cg1, but not Cg2, fields of the ACC following exposure to context A at a remote time point (Fig. 4g and Supplementary Figs. 5a and 6c,f). Furthermore, reactivation of the context A ensemble in Cg1 following exposure to context C was decreased (Fig. 4g and Supplementary Fig. 6e,f). Both groups of mice had comparable numbers of labeled cells in the ACC and similar percentages of chance overlap (Supplementary Fig. 5b,d,e).

The basolateral amygdala (BLA) acts downstream of the hippocampus and prefrontal cortex to govern expression of fear. Therefore, we examined activation dynamics of H2B–GFP-tagged context A ensemble in the BLA over time and following ABLIM3 downregulation in DGcs (Supplementary Fig. 6d). Notably, we found a time-dependent increase in reactivation of the H2B–GFP-tagged context A ensemble in the BLA following exposure to the neutral context (day 1 versus day 16, shNT group; Fig. 4f,g). Of note, ABLIM3 downregulation in DGcs constrained this time-dependent generalization of the fear memory trace in the BLA (day 1 versus day 16, shRNA group; Fig. 4f,g). Both groups of mice had comparable numbers of labeled cells in the BLA and similar percentages of chance overlap (Supplementary Fig. 5b,d,e).

**ABLIM3 downregulation in DGcs decreases remote memory generalization**

Next we asked whether ABLIM3 downregulation in the DG decreased generalization of remote fear memories. We stereotaxically injected LV-shRNA-GFP or LV-shNT-GFP into DG, and 2 weeks later, the mice were conditioned to three foot-shocks in context A and then exposed to context A and a neutral, distinct context C in a counterbalanced manner 1d, 10d and 16d after training. ABLIM3 downregulation in the DG significantly decreased levels of freezing behavior in context C and improved discrimination between contexts A and C at remote (significant effect on day 16, trend on day 10), but not recent (day 1), time points (Fig. 5a–d). ABLIM3 downregulation in DGcs did not affect contextual fear acquisition, locomotor activity, behavioral measures of anxiety or behavior in the tail-suspension test (Fig. 5i and Supplementary Fig. 7c–g). ABLIM3 downregulation in the DG resulted in increased sparseness of activity in CA3 following exposure to context C, but not in the home cage group, at the remote time point (Fig. 5e–g), consistent with the decreased reactivation of context A ensembles in CA3 when mice were exposed to the neutral context C at a remote time point (Fig. 4e). We used a separate cohort of mice that was not exposed to context C until day 16 and found that ABLIM3 downregulation in DGcs significantly decreased levels of freezing behavior in context C at the remote, but not recent, time point (Fig. 5h–k). These mice showed a trend toward better discrimination between contexts A and C at day 16 (Fig. 5k), Notably, and as seen in adult mice, ABLIM3 downregulation in DGcs increased activation of PV⁺, but not SST⁺, SLINs when assessed at the remote time point (Supplementary Fig. 8a–e).

**ABLIM3 downregulation in DGcs enhances feedforward inhibition onto CA3.** (a) Schematic showing injection of rAAV5-Camk2a-ChR2-eYFP and either LV-shNT-GFP or LV-shRNA-GFP into the DG and recording of blue light (473 nm)-evoked EPSCs and IPSCs in CA3 pyramidal neurons in slices (top) and quantification of the ratio of peak EPSC amplitude to peak IPSC amplitude in the CA3 of LV-shNT-GFP- or LV-shRNA-GFP-injected mice after blue light stimulation (n = 14 cells, n = 4 mice per group) (bottom). *P < 0.05. (b) Absolute values of light-evoked EPSCs (top) and IPSCs (bottom) from CA3 following blue light stimulation (n = 14 cells, n = 4 mice per group). (c) Schematic showing how Ablim3 knockdown in DGcs increases the number of MFT filopodial contacts with PV⁺ SLINs to increase feedforward inhibition onto CA3 (left) with representative recording traces from each set of recordings and traces of the same cells after perfusion of DCG-IV (right) (n = 14 cells, n = 4 mice per group). (d) Absolute values of light-evoked EPSCs, IPSCs, and a moderate reduction in the number of MFT filopodia at baseline. However, aged mice were refractory to learning-induced increases in the number of MFT filopodia that was seen in adult mice (Fig. 6b). On the basis of these observations, we asked whether we could harness ABLIM3 to restore DG–SLIN connectivity in aged mice. Injection of LV-shRNA-GFP into DG of aged mice profoundly increased the number of VGLUT2 MFT filopodial contacts with PV⁺ SLINs and the density of PV⁺ puncta in the CA3 pyramidal cell layer, without affecting MFT size or DG dendritic spine density (Fig. 6c,d).
**Figure 4** Enhancing DGC recruitment of inhibition promotes engram maintenance and governs reactivation of remote memory traces in hippocampal–cortical and BLA networks. (a) Schematic of virus-mediated ensemble labeling strategy and behavioral paradigm for optogenetic activation of DG engram cells. (b) Levels of freezing behavior following blue light stimulation at day 2 in the fear-conditioned (FC; light off, n = 8 mice; light on, n = 7 mice) and no-shock (NS; light off, n = 7 mice; light on, n = 9 mice) groups. (c) Levels of freezing behavior following blue light stimulation at day 10 in the FC and NS groups in b. In b,c, comparison in the FC group was by two-way ANOVA with repeated measures; main effect of treatment (LV-shRNA-GFP versus LV-shNT-GFP), F(1,14) = 6.972, P = 0.019, Bonferroni post hoc. (d–g) Schematic of Tet-off (TetTag) genetic system and behavioral paradigm to indelibly label ensembles in the DG, CA3, CA1, BLA and ACC regions (d,f) and reactivation as assessed by quantification of the percentage of tagged neuronal ensembles (tetO-H2B-mCherry (e) or tetO-H2B-GFP (g)) that were c-FOS+. Reactivation in the context A–context A (AA) group in the DG by two-way ANOVA, main effect of time: P < 0.001. Reactivation in the context A–context C (AC) group: in CA3 by two-way ANOVA, main effect of treatment (LV-shRNA-GFP versus LV-shNT-GFP), F(1,14) = 6.972, P = 0.019, Bonferroni post hoc; in CA1 by two-way ANOVA, day × treatment (LV-shRNA-GFP versus LV-shNT-GFP), F(1,21) = 23.27, P < 0.001, Bonferroni post hoc; in ACC-Cg1 by two-way ANOVA, day × treatment (LV-shRNA-GFP versus LV-shNT-GFP), F(1,22) = 4.318, P = 0.04, Bonferroni post hoc; in BLA by two-way ANOVA, day × treatment (LV-shRNA-GFP versus LV-shNT-GFP), F(1,22) = 32.32, P < 0.0001, Bonferroni post hoc. Throughout, data are represented as mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001. See Supplementary Table 1 for detailed statistical analyses.
and Supplementary Fig. 9a–c). Stereotactic injection of VSV-G into the DG of 17-month-old mice resulted in increased anterograde trans-synaptic labeling of GABA⁺ and PV⁺ SLINs in CA3 following ABLIM3 downregulation in DGCs (Fig. 6e). ABLIM3 downregulation in DGCs did not affect the expression of GABA or PV in CA3 (Supplementary Fig. 9d).

We next sought to determine whether increasing DGC–PV⁺ SLIN connectivity in aged mice by ABLIM3 downregulation in the DG was sufficient to improve precision of remote memories. Following ABLIM3 downregulation in the DG, 17-month-old mice showed better memory for conditioning of context A at day 10 following training, resulting in significantly higher discrimination between the conditioning
Figure 6 ABLIM3 downregulation in the DG of aged mice enhances DGC–PV+ SLIN connectivity and improves remote memory precision. (a) Schematic of LV-shNT-GFP or LV-shRNA-GFP injection into the DG of 3- and 17-month-old mice. (b) Representative images showing GFP+ MFTs with filopodial extensions (arrows) after training (left) and quantification of MFT filopodia number in 3-month-old (n = 4) and 17-month-old (n = 3) mice at baseline (naive, unpaired t-test, baseline, 3 month versus 17 month: P = 0.06) and after training (right). Comparisons by two-way ANOVA, age × baseline or learning F(1,10) = 13.03, P = 0.0048, Bonferroni’s post hoc tests: 3 month, baseline versus learning, P = 0.0001; 3 month versus 17 month, following learning, P = 0.0001. Scale bars, 5 µm. (c) Representative images (top) and quantification (bottom) showing the number of filopodia per GFP+ MFT (arrows) following LV-shNT-GFP or LV-shRNA-GFP injection into the DG of 17-month-old mice (shNT: n = 75 MFTs; shRNA: n = 97 MFTs; n = 3 mice per group). Scale bars, 5 µm. (d) Representative images (top) and quantification (bottom) showing PV+ puncta (arrows) density in CA3 stratum pyramidale of 17-month-old mice (n = 5 mice per group). Scale bars, 10 µm. (e) Representative images (left) and quantification (right) of anterograde labeled PV+ and GABA+ cells in CA3 after VSV-G injection into the DG of 3- and 17-month-old mice (n = 5 mice per group). Scale bar, 50 µm. (f) Schematic of the contextual-fear-conditioning paradigm using aged mice. (g,h) Freezing behavior levels of LV-shNT-GFP-injected (n = 7) or LV-shRNA-GFP-injected (n = 8 mice) mice during training (g) and at day 1 (left), day 10 (middle) and day 16 (right) of testing (h). Comparisons were done by two-way ANOVA with repeated measures (day 10, context × treatment (shRNA versus shNT virus), F(1,13) = 15.58, P = 0.0017. (i) Contextual discrimination ratios for day 1 (left) and day 10 (right) (shNT, n = 7 mice; shRNA, n = 8 mice). Paired t-test, day 1 versus day 10, in shNT mice, P = 0.0017. Two-way ANOVA with repeated-measures analysis of discrimination ratios, virus × day, F(1,13) = 10.74, P = 0.006. (j,k) Representative confocal images showing c-FOS+PV+ cells in CA3 area (including the stratum pyramidale (SP), stratum oriens (SO), stratum radiatum (SR) and stratum lucidum (SL)) after context A exposure at day 16 (j) and quantification of c-FOS+PV+ cells counts (left) and percentage of c-FOS+PV+ cells over total PV+ cells in CA3 area (right) (shNT, n = 7 mice; shRNA, n = 8 mice) (k). Scale bars, 100 µm (top), 50 µm (bottom). Throughout, data are represented as mean ± s.e.m. Unless otherwise specified, statistical comparisons were performed using two-tailed unpaired t-tests. *P < 0.05, **P < 0.01, ***P < 0.001. See Supplementary Table 1 for detailed statistical analyses.
context A and a distinct, neutral context C (Fig. 6f–i). Furthermore, at day 16 following training, aged mice with restored DGC–SLIN connectivity showed stronger memory for context A and increased activation of PV⁺ and CA3 pyramidal neurons, but not of SST⁺ SLINs or DGCs, following exposure to context A (Fig. 6j,k and Supplementary Fig. 9e–g), which is consistent with the modest increase in reactivation of the context A ensemble in CA3 when mice were exposed to the context A at a remote time point (P = 0.06; Fig. 4e). Increasing DGC–SLIN connectivity in aged mice by ABLIM3 downregulation in the DG did not affect locomotor activity but produced anxiolytic-like behavioral effects in the elevated plus maze and decreased immobility in the tail-suspension test (Supplementary Fig. 10).

**DISCUSSION**

Our studies causally link a MFT-localized cytoskeletal factor, ABLIM3, with DGC–SLIN connectivity, DGC recruitment of feedforward inhibition onto CA3, reorganization of remote memory traces in hippocampal–cortical–BLA networks and remote memory generalization.

ABLIM3 belongs to the ABLIM family, whose members were originally characterized as regulators of growth cone motility in axon guidance46. ABLIM3’s role in stabilization of branched F-actin in lamellipodia47–49 and its localization to PAJs, sites of structural stabilization of the MFT on the CA3 dendritic shaft, suggest that the molecular mechanisms underlying generation of growth cone filopodia during development, which are notably distinct from those underlying dendritic filopodia57, maybe re-used in controlling the generation of MFT filopodia in mature DGCs. The convergent elongation model of filopodial formation suggests a trade-off in growth cone lamellipodia versus filopodia during development57,58. Consistent with this model, retrovirus-mediated downregulation of Abilm3 expression in 4-week-old, but not 8-week-old, adult-born DGCs increased the number of MFT filopodia and decreased MFT size (N.G. and A.S., unpublished observations). ABLIM3 downregulation in mature DGCs did not significantly affect MFT size, presumably because the MFT was already stabilized onto the dendritic shaft of CA3. On the basis of these observations, we predict that factors that regulate destabilization of branched F-actin networks in MFTs of mature DGCs may dictate the number of MFT filopodial contacts with SLINs to promote recruitment of inhibition onto CA3 in response to learning. Although studies have begun to edify how afferent connectivity of eDGCs changes over time25, much less is known about how efferent connectivity of eDGCs dictates engram properties. We found that learning induced a greater increase in eDGC–SLIN connectivity than in non-eDGCs and that eDGC–SLIN connectivity decreased over time as memory became generalized. By regulating ABLIM3 expression we found that increasing DGC recruitment of inhibition onto CA3 permitted artificial recall of a memory trace by light stimulation of eDGCs at a remote time point that was otherwise not possible in the controls. These observations suggest that maintaining eDGC–SLIN connectivity may, in turn, maintain the engram in the DG. Whether eDGCs recruit hippocampal–cortical replay mechanisms59 or CA3 back-projections to maintain the engram remains to be addressed.

Increasing DGC recruitment of inhibition by ABLIM3 downregulation in DGCs governed reactivation of memory traces of the conditioned context in CA3–CA1–ACC–BLA networks at remote time points. The decrease in context-C-induced reactivation of memory trace of the conditioned context in CA3, but not DG, at the remote time point implicates ABLIM3-dependent changes in DGC–CA3 connectivity. We argue that constraining context-specific CA3 reactivation dictates reactivation of the memory trace of the conditioned context in CA1, ACC and BLA. These findings generally support the transformative view of the multiple-trace theory of systems consolidation18,19 and suggest that the memory of conditioned context is distributed over multiple traces in CA3, CA1, ACC and BLA that are linked or that is one highly distributed trace. Our findings extend recent observations that define a role for the DG engram in early maturation of prefrontal cortical engram25 to suggest that the DG engram may serve as a hippocampal index for time-dependent consolidation of cognate remote memory traces in the ACC5,16,21,22 and govern reactivation dynamics in the BLA (Supplementary Fig. 11a,b)55. At the recent time point, intact input (dendritic spines) and output (DGC–SLIN) connectivity of eDGCs ensure context-specific reactivation in CA3–CA1–ACC and BLA networks. With gradual silencing of the engram in the DG and a decrease in DGC–SLIN connectivity over time, context-specific reactivation in CA3–CA1–ACC and BLA is impaired. However, increased DGC recruitment of inhibition onto CA3 continues to maintain the DG engram and permit context-specific reactivation in CA3–CA1–ACC–BLA networks (Supplementary Fig. 11a,b). Whether DG-dependent modulation of ensemble reactivation and consolidation in BLA is independent of the ACC and is mediated by ventral CA1 (ref. 56) remains to be determined.

Notably, ABLIM3 downregulation in DGCs increased PV⁺ SLIN activation but not SST⁺ SLIN activation, despite increasing DGC connectivity with both of these SLIN populations. Future studies to dissect SLIN microcircuitry may edify the basis of preferential recruitment of PV⁺ SLINs by DGCs60. Because increasing mossy fiber excitatory drive onto PV⁺ SLINs resulted in an increase in PV density in CA3, it is plausible that PV⁺ SLIN connectivity with downstream targets in CA3, and potentially CA1, is also modified. These changes in PV⁺ SLIN connectivity over time may promote consolidation of remote memory cortical and subcortical traces through recruitment of replay mechanisms such as sharp-wave ripples32–37 (Supplementary Fig. 11).

ABLIM3 downregulation in DGCs decreased remote memory generalization. Because overgeneralization of remote traumatic memories characterizes PTSD, our findings suggest that efficient consolidation of a traumatic memory may decrease its generalization over time. It follows that therapeutic strategies that fail to completely block consolidation would exacerbate overgeneralization of fear. We suggest that mechanisms such as pattern separation may help maintain the DG engram by decreasing interference between new and old similar engrams, thereby promoting stabilization of cognate cortical and subcortical traces and decreasing remote memory generalization5,22.

Converging evidence from studies in rodents, nonhuman primates and humans suggest a role for alterations in properties and functions of the DG–CA3 circuit in cognitive impairments in aging and in MCP38–42. Although the fear-conditioning training protocol used here did not permit detection of an age-related deficit in remote memory generalization4, we observed a profound failure in learning-induced increase in DGC–SLIN connectivity in aged mice. Of note, ABLIM3 downregulation in aged mice was sufficient to restore levels of DGC–SLIN connectivity, increase PV⁺ SLIN activation and improve remote memory precision. In light of these findings, it is notable that Ablim3 expression is upregulated several fold in peripheral leukocytes of aged individuals who convert into amnestic subtype of MCI (M. Fiodaca, M.E. Mapstone, Y. Gusev, H.J. Federoff & T.B. Buck, patent application WO/2015/184107).

An excitation–inhibition imbalance in DG–CA3 may underlie memory impairments in aging and contribute to overgeneralization of fear in PTSD. By identifying a molecular regulator of DGC–SLIN connectivity, ABLIM3, we show that increasing DGC recruitment of
feedforward inhibition onto CA3 promotes engram maintenance in the DG and that this in turn governs reactivation of remote memory traces in hippocampal–cortical–BLA networks and decreases generalization of remote fear memories in adulthood and aging.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

ACKNOWLEDGMENTS

We wish to thank members of the Sahay lab for comments on the manuscript and the late N.J. Sahay for advice. We are thankful to S. Tonegawa (MIT) for the AAV9-tet-O-Chr2 construct, M.E. Greenberg (Harvard Medical School) for the lentiviral pLLX vector, J. Rajagopal (MGH) for the HEK293T cells and S. Ramirez (Boston University) for the AAV9-tet-O-Chr2-mCherry virus. I.Z. is supported by US National Institutes of Health (NIH) grant R01MH104450. C.C. is an investigator at the Howard Hughes Medical Institute. A.S. acknowledges support from the NIH Biobehavioral Research Awards for Innovative New Scientists (BRAINS; grant R01MH104175), the NIH–National Institute on Aging (NIA) grant R01AG049808, NIH grant R01MH111729, the Ellison Medical Foundation New Scholar in Aging, the Whitehall Foundation, an Incysop Decode award, a NARSAD Independent Investigator Award, Ellison Family Philanthropic support, the Blue Guitar Fund, a Harvard Neurodiscovery Center—MADRC Center Pilot Grant award, and a Harvard Stem Cell Institute Development grant and HSCI seed grant. C.H. was supported by a 2016 HSCI Harvard Internship Program Award.

AUTHOR CONTRIBUTIONS

N.G., M.E.S., C.H., M.T.K., P.L. and A.B. performed experiments; X.M. and C.L.C. performed experiments; A.S. and N.G. are named co-inventors on a patent application (US 2016/0376588 A1, PCT/US 2015/20540) relating to this study.

COMPETING INTERESTS

A.S. and N.G. are named co-inventors on a patent application (US 2016/0376588 A1, PCT/US 2015/20540) relating to this study.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html. Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Jasnow, A.M., Lynch, J.F., III, Gilman, T.L. & Riccio, D.C. Perspectives on fear generalization and its implications for emotional disorders. J. Neurosci. Res. 95, 821–835 (2017).
2. Biedenkapp, J.C. & Rudy, J.W. Context pre-exposure prevents forgetting of a contextual amnestic mild cognitive impairment. Learn. Mem. 25, 73–78 (2017).
3. Willген, B.J. & Silva, A.J. Memory for context becomes less specific with time. Learn. Mem. 14, 313–317 (2007).
4. Saul, A.M. Conditioning and time-dependent increases in context fear and generalization. Learn. Mem. 23, 379–385 (2016).
5. Besnard, A. & Sahay, A. Adult hippocampal neurogenesis, fear generalization and stress. Neuropsychopharmacology 41, 24–44 (2016).
6. Liberoz, I. & Abelson, J.L. Context processing and the neurobiology of post-traumatic stress disorder. Neuron 92, 14–30 (2016).
7. Jovanovic, T. & Ressler, K.J. How the neurocircuitry and genetics of fear inhibition as revealed by the unbiased stereological dissector technique. Hippocampus 22, 437–444 (1992).
44. Reijmers, L.G., Perkins, B.L., Matsuo, N. & Mayford, M. Localization of a stable neural correlate of associative memory. Science 317, 1230–1233 (2007).
45. Scobie, K.N. et al. Krüppel-like factor 9 is necessary for late-phase neuronal maturation in the developing dentate gyrus and during adult hippocampal neurogenesis. J. Neurosci. 29, 9875–9887 (2009).
46. Lundquist, E.A., Herman, R.K., Shaw, J.E. & Bargmann, C.I. UNC-115, a conserved protein with predicted LIM and actin-binding domains, mediates axon guidance in C. elegans. Neuron 21, 385–392 (1998).
47. Barrientos, T. et al. Two novel members of the ABLIM protein family, ABLIM-2 and -3, associate with STAP5 and directly bind F-actin. J. Biol. Chem. 282, 8393–8403 (2007).
48. Matsuda, M., Yamashita, J.K., Tsukita, S. & Furuse, M. ABLIM3 is a novel component of adherens junctions with actin-binding activity. Eur. J. Cell Biol. 89, 807–816 (2010).
49. Cao, J. et al. miR-129-3p controls cilia assembly by regulating CP110 and actin dynamics. Nat. Cell Biol. 14, 697–706 (2012).
50. Beier, K.T. et al. Anterograde or retrograde trans-synaptic labeling of CNS neurons with vesicular stomatitis virus vectors. Proc. Natl. Acad. Sci. USA 108, 15414–15419 (2011).
51. Kamiya, H., Shinozaki, H. & Yamamoto, C. Activation of metabotropic glutamate receptor type 2/3 suppresses transmission at rat hippocampal mossy fiber synapses. J. Physiol. (Lond.) 493, 447–455 (1996).
52. Liu, X. et al. Optogenetic stimulation of a hippocampal engram activates fear memory recall. Nature 484, 381–385 (2012).
53. Goshen, I. et al. Dynamics of retrieval strategies for remote memories. Cell 147, 678–689 (2011).
54. Vazdarjanova, A. & McGaugh, J.L. Basolateral amygdala is involved in modulating consolidation of memory for classical fear conditioning. J. Neurosci. 19, 6615–6622 (1999).
55. Redondo, R.L. et al. Bidirectional switch of the valence associated with a hippocampal contextual memory engram. Nature 513, 426–430 (2014).
56. Girardeau, G., Inema, I. & Buzsáki, G. Reactivations of emotional memory in the hippocampus-amygdala system during sleep. Nat. Neurosci. 20, 1634–1642 (2017).
57. Yang, C. & Svitkina, T. Filopodia initiation: focus on the Arp2–3 complex and formins. Cell Adh. Migr. 5, 402–408 (2011).
58. Mejillano, M.R. et al. Lamellipodial versus filopodial mode of the actin nanomachinery: pivotal role of the filament barbed end. Cell 118, 363–373 (2004).
59. Wilson, M.A. & McNaughton, B.L. Reactivation of hippocampal ensemble memories during sleep. Science 265, 676–679 (1994).
60. Bartos, M., Aile, H. & Vida, I. Role of microcircuit structure and input integration in hippocampal interneuron recruitment and plasticity. Neuropharmacology 60, 730–739 (2011).
ONLINE METHODS

Subjects. All mice were handled, and experiments conducted, in accordance with procedures approved by the Institutional Animal Care and Use Committees at the Massachusetts General Hospital and University of Washington, Seattle, in accordance with NIH guidelines. All mice were housed three or four per cage in a 12-h (7:00 a.m. to 7:00 p.m.) light–dark colony room at 22–24 °C with ad libitum access to food and water. Adult female C57BL/6 mice (3–4 months old) were purchased from the Jackson Laboratories. Aged female C57Bl/6 mice (17–18 months old) were obtained from the National Institute on Aging (NIA). The following mouse lines were obtained from the Jackson Laboratories: cfos-TA; tet-O-taulacZ;TA* (stock no. 008344), tet-O-H2B-GFP (stock no. 005104), and Coll1a1-tet-O-H2B-mCherry (stock no. 014592). TetTag mice were generated by crossing reporter mouse lines, tet-O-H2B-GFP mouse line or tet-O-H2B-mCherry mouse line, with mice that express tetracycline- trans-activator (tTA) protein under control of the Fos promoter (stock no. 008344, Jackson labs; outcrossed from tetO-IacZ, TA*). cfos-TA mice also contain a transgene that consists of a Fos promoter driving the expression of nucleus-localized EGFP with a 2-h half-life. All TetTag mice were maintained on a C57BL/6 background. Mice were bred and raised on a Dox-containing diet was replaced with regular diet for 3 d to open the window of the reporter before the desired experimental labeling of ensembles. The mice were maintained on a C57BL/6 background. Mice were bred and raised on a Dox-containing diet was replaced with regular diet for 3 d to open the window of the reporter before the desired experimental labeling of ensembles. Expression of the reporter was shut off by administration of a Dox-containing diet immediately following the desired labeling window. PV–Cre mice (stock no. 017320, Jackson Labs) were bred with the Ai14:tdTomato reporter mouse line (stock no. 007914, Jackson Labs) to generate mice for virus injections and in vitro electrophysiological whole-cell recordings.

Western blot analysis. To detect ABLIM3 protein levels following learning, mice were subjected to contextual fear conditioning (training, Fig. 1e) and euthanized by decapitation 1 h later to permit rapid dissolution of the hippocampus. Naïve control mice (naïve, Fig. 1e) were euthanized and dissected directly from home cages. Hippocampi and CA3 subregions were dissected on Whatman paper soaked with ice-cold PBS and snap-frozen in liquid nitrogen immediately. Tissue was homogenized in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 0.1% sodium deoxycholate, 1% Triton X-100, 50 mM Tris, pH 8.0) supplemented with 0.5 mM EDTA and Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Waltham, MA). Homogenized samples were centrifuged at 12,000 r.p.m. for 20 min at 4 °C. Protein concentrations were determined from the supernatant by the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA). Diluted samples were supplemented with SDS sample buffer (2% SDS, 10% glycerol, 0.1 M dithiothreitol, 0.01% bromophenol-blue, 62.5 mM Tris, pH 6.8) and boiled at 95 °C for 5 min. Proteins (40 µg) were resolved in 8% gels by SDS–PAGE and transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA) by electroblotting. To prevent nonspecific binding, membranes were blocked with 3% dry milk in TBS-T (150 mM NaCl, 20 mM Tris, 0.1% Tween-20) for 2 h at room temperature. Membranes were incubated in primary antibodies—rabbit anti-ABLIM3 (1:500, Abcam, ab129480) and mouse anti-GAPDH (1:2,000, Millipore, MAB374, Billerica, MA)—overnight at 4 °C. Membranes were incubated in secondary antibodies—horseradish peroxidase (HRP)-conjugated goat anti-rabbit-IgG (1:5,000, Bio-Rad Laboratories, 170-5046, Hercules, CA) and HRP-conjugated goat anti-mouse-IgG (1:5,000, Bio-Rad Laboratories, 170-5047, Hercules, CA)—for 2 h at room temperature. Membranes were imaged using the ChemiDoc XRS+ System. Images were analyzed with ImageJ software using pixel intensities. For each membrane, the relative densities of the ABLIM3 bands were calculated using the intensities of each lane’s corresponding GAPDH band.

Validation of shRNAs. Lentiviral vectors expressing shRNAs were obtained from Sigma Mission library (Sigma–Aldrich). Five shRNAs targeting mouse Ablim3 (cat. no. SHCLNG-NM_198649) were verified. A lentiviral vector expressing a nonmammalian nontarget shRNA (SHC002) was used as a negative control. To validate the efficiency of the shRNAs, each shRNA-expressing lentiviral vector was co-transfected with plasmids encoding HA-tagged full-length mouse ABLIM3 and HA-tagged GFP into 293T cells. Cell lysates were subsequently prepared for western blot analysis of ABLIM3 and GFP expression using mouse anti-ABLIM3 (Abcam, ab67721; 1:500) and chicken anti-GFP (Abcam, ab13970; 1:2,000). Actin expression (detected by anti-β-actin; Abcam, ab8227; 1:1,000) was used as a loading control. The shRNA sequences are as follows:

TRCN0000099545 (targeting Ablim3): CCGGGCGAGATTAATCCCTTGATCTCGAGGATAAAGAGGATATATGGCCTTGGTGTGTTG;
TRCN0000099546 (targeting Ablim3): CCGGGCCCTTTCCTATTGAGATACCTGAGGATATCCATCAAAGAAGGCTTGGTGTGTTG;
TRCN0000099547 (targeting Ablim3): CCGGTTGGAATGAGCTTCAATCTCGAGATAGGATCTCATTACCACTTGGTGTGTTG;
TRCN0000099548 (targeting Ablim3): TGCGCCGGCAGATCGAACTTCCATCTCGAGATGGAATGGTCCAGAAGGCTTGGTGTGTTG;
TRCN0000099549 (targeting Ablim3): CCGGCCTGAAAGGAGAAGCAAGTAGCCTCGAGGATGAGTTCTGAGAAGGCTTGGTGTGTTG.

Viral constructs. shNT and validated shRNAs against Ablim3 (shAblim3-46 and shAblim3-47) were cloned into the PLX-shRNA lentiviral vector under the control of the U6 small nuclear RNA (Rnue) promoter as previously described.24 The PLX-shRNA vector also contains a second expression cassette in which GFP is expressed from the ubiquitin C (UbC) promoter, which allows the monitoring of transfection and infection efficiency. Lentiviral stock solutions (5 × 108 to 8 × 108 U/ml) were prepared by cotransfection of pLX-shRNA lentiviral vector and packaging plasmids into HEK293T cells (gift of the Rajagopal Laboratory, MGH), followed by ultracentrifugation of viral supernatant as described24. Generation of VSV-G (viral titer was 4 × 1011 U/ml) for tracing neuronal connections in vivo was done as previously described.24 Briefly, the rVSV-EFGP construct was with the eGFP in the first genome position was amplified in baby hamster kidney BSR T7/5 cells (gift of S.P. Whelan) and purified as described previously.63 At 100% confluency BSR T7/5 cells were infected with rVSV-EFGP at a multiplicity of infection (MOI) of 0.01, and the supernatant was collected 2 d later. The supernatant was concentrated by ultracentrifugation at 21,000 r.p.m. in an SW28 rotor (Beckman Coulter) for 3 h at 4 °C. Virus pellets were resuspended overnight in phosphate-buffered saline (PBS) with 25 mM HEPES. The virus resuspension was purified on linear 15–45% sucrose gradients in the NTE buffer (10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA). The sucrose gradient was centrifuged at 25,000 r.p.m. in an SW41 rotor (Beckman Coulter) for 5 h at 4 °C. The virus-containing band was collected and then aliquoted and frozen in –80 °C before use. The rVSV-EFGP virus was titrated with BSR T7/5 cells and a Dulbecco’s modified Eagle’s medium (DMEM)–agarose (0.33%) overlay, and the titer was found to be 4 × 1011 U/ml. The PAAV-TRE-ChR2-eYFP construct was obtained from Dr. Sussumu Tonegawa (MIT)25. The RAAV vectors were serotyped with AAV9 coat proteins and packaged by the Boston Children’s Hospital Viral Core at the Harvard Medical School. Viral titer was 4.956 × 1014 genome copies/ml for AAV9-TRE-ChR2-eYFP. The titer for AAV9-TRE-Chr2-eYFP was 8 × 1012 U/ml (a gift from Dr. Steve Ramirez). rAAV5-Camk2a-hChr2(H134R)-eYFP virus was generated at the University of North Carolina at Chapel Hill (UNC) Vector Core, and the viral titer was 8.5 × 1012 genome copies/ml.

Stereotactic viral injection. Mice were anaesthetized with ketamine and xylazine (10 mg/ml and 1.6 mg/ml, respectively). Mice were placed in the stereotactic apparatus, and a small hole was drilled at each injection location. The needle was slowly lowered into the target site and remained there for 5 min before injection. Virus injection was performed using a Hamilton microsyringe (0.1 µl/min) into the dorsal DG using the following coordinates: adult (3–4 months old) and aged (17–18 months old) mice, anterioposterior (AP) = −1.8 mm from bregma; lateral (ML) = ±1.5 mm; depth (DV) = 2.5 mm; 4–5-week-old juvenile mice, anterioposterior = −1.7 mm from bregma; lateral = ±1.4 mm; depth = −2.4 mm. Injection needles were left in place for additional 10 min after injection to ensure even distribution of the virus and were then slowly withdrawn. The skin incision was carefully sutured after viral injection to minimize inflammation. 0.5 µl LV-shNT-GFP or LV-shNT-EB2-GFP was injected into the DG using a Hamilton microsyringe (0.1 µl/min).
LV-shRNA-GFP (unless otherwise specified, shAblim3-46 was used throughout the study), or AAV9-TR-Chr2-eYFP was injected bilaterally into dorsal DG. 0.1 µl diluted VSVG (1:10 of original virus stock) was injected unilaterally into dorsal DG. Mice were given carprofen (5 mg/kg) before and during surgery, and 24 h later, to minimize discomfort. All injection sites were verified histologically. Only mice with virus expression that was limited to the targeted regions were included for analysis.

**Optical fiber implant.** A virus cocktail (0.5 µl of LV-shNT-GFP or of LV-shNT-GFP + LV-shRNA-GFP and 0.5 µl of AAV9-TR-Chr2-eYFP or AAV9-TR-Chr2-mcherry) was injected bilaterally into the dorsal DG of adult (3- to 4-month-old) cfos-tTA mice. One week after the injections, optical fiber implant surgeries were performed to the same mice. A 200-µm core, 0.37 numerical aperture (NA) multimode fiber (ThorLabs) was threaded through and glued with epoxy to a 230-µm core stainless steel or zirconia multimode ferrule (Fiber Instrument Sales and Precision Fiber Products), polished and cut for implantation. Optical patch cables were generated the same way with the free end connected to a multimode FC ferrule assembly for connecting to a 1X2 optical rotary joint (Doric Lenses). The other end of the rotary joint was connected via a patch cable to either a 100 mW 473-nm laser diode (OEM laser systems) via a noncontact-style laser-to-fiber coupler (OZ optics). Mice were anesthetized with ketamine and xylazine (10 mg/ml and 1.6 mg/ml, respectively) and placed in the stereotaxic apparatus, and the optical fiber was lowered into the injection site (–1.8 mm AP; –1.75 mm ML; –1.75 mm DV). The implant was secured to the skull with two jewelry screws, adhesive super glue (Loctite Adhesives) and dental cement. Mice were given carprofen (5 mg/kg) before, during and after surgery to minimize discomfort. Mice were allowed to recover for 2 weeks before behavioral experiments.

**Whole-cell electrophysiological recordings.** Two weeks after injection of LV-shNT-GFP or LV-shRNA-GFP into 4- to 5-week-old P7-P14 Ai11 mice, mice were used for slice electrophysiology. Briefly, 300-µm-thick hippocampal sections were cut in an ice slush solution containing: 85 mM NaCl, 75 mM sucrose, 2.5 mM KCl, 1.3 mM NaH₂PO₄, 24 mM NaHCO₃, 0.5 mM CaCl₂, 4 mM MgCl₂ and 25 mM D-glucose. Slices were allowed to recover for 30 min at 31.5 °C in ACSF containing: 126 mM NaCl, 2.5 mM KCl, 1.1 mM NaH₂PO₄, 26.2 mM NaHCO₃, 2.5 mM CaCl₂, 1.3 mM MgSO₄ and 11 mM D-glucose. Whole-cell recordings were made using an Axopatch 700b amplifier (Molecular Devices) with filtering at 1 kHz using 4- to 6-ΩM electrodes. For mEPSCs, electrodes were filled with an internal solution containing: 120 mM CsMeSO₄, 5 mM MgCl₂, 8 mM NaCl, 1 mM MgCl₂, 10 mM HEPES, 1 mM QX-314, 0.5 mM Na₃GTP and 2 mM Mg-ATP, pH 7.2–7.3; 280–290 mOsm. For analysis of light-evoked EPSCs and IPSCs in CA3, a virus cocktail containing 0.5 µl LV-shNT-GFP or LV-shRNA-GFP and 0.3 µl rAAV5-Chr2-eYFP (1:10 of original dilution) was injected unilaterally into the dorsal DG of adult (3- to 4-month-old) B6 mice that were subsequently used for slice electrophysiology. Electrodes were filled with an internal solution containing: 132 mM CsMeSO₄, 8 mM CsCl, 10 mM HEPES, 1 mM EGTA, 0.5 mM CaCl₂, 10 mM glucose and 1 mM QX-314, pH 7.2–7.3; 280–290 mOsm.

Cutting and ACSF solutions were continually bubbled with O₂ and CO₂, and Cutting and ACSF solutions were continually bubbled with O₂ and CO₂, and 25 mM D-glucose. Slices were allowed to recover for 30 min at 31.5 °C in ACSF containing: 126 mM NaCl, 2.5 mM KCl, 1.1 mM NaH₂PO₄, 26.2 mM NaHCO₃, 2.5 mM CaCl₂, 1.3 mM MgSO₄ and 11 mM D-glucose. Whole-cell recordings were made using an Axopatch 700b amplifier (Molecular Devices) with filtering at 1 kHz using 4- to 6-ΩM electrodes. For mEPSCs, electrodes were filled with an internal solution containing: 120 mM CsMeSO₄, 5 mM MgCl₂, 8 mM NaCl, 1 mM MgCl₂, 10 mM HEPES, 1 mM QX-314, 0.5 mM Na₃GTP and 2 mM Mg-ATP, pH 7.2–7.3; 280–290 mOsm. For analysis of light-evoked EPSCs and IPSCs in CA3, a virus cocktail containing 0.5 µl LV-shNT-GFP or LV-shRNA-GFP and 0.3 µl rAAV5-Chr2-eYFP (1:10 of original dilution) was injected unilaterally into the dorsal DG of adult (3- to 4-month-old) B6 mice that were subsequently used for slice electrophysiology. Electrodes were filled with an internal solution containing: 132 mM CsMeSO₄, 8 mM CsCl, 10 mM HEPES, 1 mM EGTA, 0.5 mM CaCl₂, 10 mM glucose and 1 mM QX-314, pH 7.2–7.3; 280–290 mOsm.

Cutting and ACSF solutions were continually bubbled with O₂ and CO₂, and all recordings were made in ACSF at 32 °C that was continually perfused over slices at a rate of ~2 ml/min. mEPSC recordings were made in the presence of tetrodotoxin (500 nM) and picrotoxin (100 µM). mIPSC recordings were made in the presence of tetrodotoxin and kynurenic acid (2 mM). For mEPSC and mIPSC recordings, neurons were held in voltage clamp at −70 mV. Miniature events were analyzed using MiniAnalysis software (Synaptosoft) using automatic detection with visual confirmation of events. For light-evoked EPSCs, neurons were held at −70 mV, and for light-evoked IPSCs, neurons were held at 0 mV. Responses were elicited with a 5-µs blue light pulse delivered at 0.1 Hz via a fiber optic placed above the DG in-slice. Peak current amplitudes were measured from an average of at least ten traces.

**Behavioral procedures.** Two weeks after the stereotactic injection of virus or optical fiber implant, mice were subjected to behavioral experiments. For the cohorts of adult C57Bl/6 mice (3–4 months old) and aged C57Bl/6 mice (17–18 months old) that were injected with LV-shNT-GFP or LV-shRNA-GFP, behavioral tasks were performed in the following order: open-field test (day 1), dark-light test and elevated plus-maze test (day 2), and tail-suspension test (day 3). Contextual fear conditioning was performed 2 d after the tail-suspension test. For the cohorts of TetTag mice, mice were raised and maintained on a Dox-containing diet (40 mg/kg chow). Two weeks after surgery, mice were habituated to transportation and external environmental cues by being carted out of the animal facility into the experimental rooms and handled for 5 min in the experimental room each day for 3 d before the experiment. Following the third habituation session, mice were fed regular food without Dox for 3 d until contextual fear-conditioning training. After the training trial, mice were returned to their home cage and placed on Dox-containing chow to turn off reporter gene expression. For the cohorts used for the optogenetic engram-stimulation experiment, a similar protocol was used as described above except that mice were taken off Dox for 2 d before contextual fear conditioning. Following behavioral testing, brain sections were examined to confirm efficient virus-mediated labeling of target areas. Mice with mistargeted virus labeling were excluded before behavior quantification.

**Open-field paradigm.** The open-field paradigm was performed as done previously. Mice were kept in a quiet holding area for at least 30 min before the test. Motor activity over 30 min was quantified in four Plexiglas open-field boxes of 41 × 41 cm (Kinder Scientific) with 16 sets of double-stacked pulse-modulated infrared photobeams equally spaced on every wall (128 total) to record x–y ambulatory movements. The software-defined grid lines divided each open field into a center and surrounding regions, with the periphery consisting of the 10 cm closest to the wall around the entire perimeter. Dependent measures were the total distance traveled (in centimeters), time spent in the center (in seconds) and rearing events through a 30-min recording period.

**Light–dark test.** The light–dark test was conducted in the open-field chamber as described above, but with a dark plastic box that was opaque to visible light but transparent to infrared covering one-half of the chamber area, thus creating dark and light compartments of equal size. An opening at floor level in the center of one wall of the dark compartment allowed passage between the light and dark compartments. The light compartment was brightly illuminated. Mice were kept in a quiet holding area for at least 30 min before the test. Between each trial, the whole apparatus was cleaned. At the beginning of the test, the mouse was placed in the dark compartment and allowed to freely explore both compartments for 10 min. Ambulation distance and time spent in the dark and light compartments were recorded.

**Elevated plus-maze test.** The elevated plus-maze test was performed as done previously. The elevated plus maze consisted of black Plexiglas apparatus with four arms (16 cm long and 5 cm wide) set in a cross from a neutral central square (5 cm × 5 cm) placed 1 m above the floor. Two opposing arms were delimited by vertical walls (closed arms), whereas the two other opposing arms had unprotected edges (open arms). Mice were placed in the center, and their behavior was recorded for 5 min via a video camera system (ViewPoint, Lyon, France) located above the maze. Cumulative time spent in the open and closed arms were scored manually by investigators who were blind to the treatment conditions. An arm visit was recorded when the mouse placed both its forepaws in the arm.

**Tail-suspension test (TST).** Mice were suspended 50 cm above a solid surface by the use of adhesive tape applied to the tail. A "cushioned" surface with mouse bedding below the TST was placed to help prevent injury to the falling mouse. The animal's behavior was recorded for 6 min using an automated video-tracking system. Struggling and climbing behaviors were analyzed using View-Point Life Sciences software.

**Contextual fear conditioning.** Conditioning was conducted in Coulbourn Habestit fear-conditioning chambers with clear front and back Plexiglas walls, aluminum side walls and stainless-steel bars as floor, as done previously. The chamber was lit from above with a light, ventilated with a fan and encased.

---

doi:10.1038/nm.4491

© 2018 Nature America, Inc., part of Springer Nature. All rights reserved.
by a sound-dampening cubicle. On the days of training and testing, mice were brought out of the animal facility and allowed to habituate for 1 h outside the testing room before starting the experiment. Mouse behavior was recorded by digital video cameras mounted above the conditioning chamber. For the training context (designated ‘context A’ throughout), the fan and lights were left on, stainless-steel bars were exposed, and ethanol was used as an olfactory cue. Mice were brought into the behavioral room in a standard housing cage. For neutral context (designated ‘context C’ throughout), mice were brought into the behavioral room in cardboard buckets. Microslides were placed in individual round white cardboard paper chambers inside a large plastic container. On the training day, mice were kept in a quiet holding area for at least 1 h before the training. During training, mice were placed for 180 s in the conditioning chamber and given three shocks (2 s each, 0.75 mA) at 180 s, 240 s and 300 s. Mice were taken out 60 s after termination of the third footshock and kept in a quiet area for additional 30 min before being returned to the animal facility. For memory acquisition, levels of freezing behavior were quantified over the initial 180-s period before the shock (baseline) and 60 s after each footshock. Freezeframe and Freezeview software (Actimetrics) were used for recording and analyzing freezing behavior, respectively.

For the multiple-exposure paradigm (days 1, 10 and 16 after training), mice were exposed to the training context (context A) and a neutral context (context C) for 3 min in a counterbalanced design with the second exposure 2 h following the initial test. Freezing behavior levels were quantified over the 3-min exposure period. For the limited-exposure paradigm, mice were exposed to the training context (context A) for 3 min on day 1. Then the same animals were exposed to the habitation context (context B) and a neutral context (context C) for 3 min in a counterbalanced design at day 16. Freezing behavior levels were quantified over the 3-min exposure period. Mice were perfused 1 h after context exposure to examine neuronal activation. cfos-tTA tetO-TauLucZ mice were perfused 24 h or 16 d after contextual fear conditioning and indelible labeling to examine engram connectivity. cfos-tTA tetO-H2B-GFP and cFos-tTA tetO-H2B-Tet-tag mice harboring tetO-H2B-GFP or tetO-H2B-mCherry alleles (cfos-tTA:tetO-H2B-GFP and cFos-tTA tetO-H2B-mCherry mice) were perfused 24 h or 16 d after contextual fear conditioning (context A) or a neutral context (context C) for 3 min at day 1 or day 16 after contextual fear conditioning. Mice were perfused 1 h after context exposure to examine neuronal ensemble reactivation at different time points.

For the optical-stimulation experiments, mice were allowed to explore context C for 6 min 2 d and 10 d after contextual fear conditioning training. Prior to each test session, fiber implants were attached to the patch cables via a zirconia sleeve, and mice were left for 30–60 s in a transition cage that was similar to their home cage. The patch cables were interfaced to an FC/PC rotary joint (Doric lenses) that was attached to the other end to a laser diode. The 6-min session was divided into two 3-min epochs, the first was a light-off epoch, and the second was a light-on epoch. During the light-on epochs, mice received blue light stimulation (473 nm, 9 mW, 20 Hz, 15 ms) for a 3-min duration. At the end of 6 min the mouse was immediately detached from the laser and returned to its home cage. The no shock (NS) group went through the same habituation, training and test sessions as the experimental group except that no foot shocks were given during the training session. Light stimulation during test sessions interfered with motion detection of the program, and, therefore, experimenters manually scored the freezing behavior while blinded to the identity of the group.

In situ hybridization (ISH). ISH was performed using digoxigenin-labeled riboprobes on 35-μm cryosections generated from perfused tissue as described previously. Premixed RNA labeling nucleotide mixes containing digoxigenin-labeled UTP (Roche Molecular Biochemicals) were used to generate RNA riboprobes. An Ablim3–specific antisense probe sequence was obtained from Allen Brain Atlas (probe RP_051214_03_D07). A sense probe was generated by using a NanoMate instrument before the addition of formamide. Briefly, sections were mounted on charged glass (Superfrost Plus) slides and postfixed in 4% paraformaldehyde (PFA). Sections were then washed in DEPC-treated PBS, treated with protease K (40 μg/ml final), washed again in DEPC-treated PBS and then acetylated. Following prehybridization, sections were incubated with the riboprobe overnight at 58 °C and washed in decreasing concentrations of SSC buffer; immunological detection was carried out with anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche). Color reaction was carried out with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) (Roche). Color reaction times were identical for both treatment groups. Images were acquired with an epifluorescence microscope (Nikon) using a 10× objective lens. For quantification, 2–4 color images per region per mouse were analyzed using the mean intensity function in Image J. All images were captured using the same light intensity and exposure times. The mean intensity of the region of interest (minus mean intensity of a selected background region) was averaged across images for each mouse and each treatment group.

Immunohistochemistry. 35-μm cryosections obtained from perfused tissue were stored in PBS with 0.01% sodium azide at 4 °C. For immunostaining, floating sections were washed in PBS, blocked (in PBS containing 0.3% Triton X-100 and 10% normal donkey serum) and incubated with primary antibody overnight at 4 °C (rabbit anti-c-FOS, Calbiochem PC38, 1:10,000 (discontinued); different batches of rabbit anti-c-FOS, Santa Cruz SC52, 1:2,000; rabbit anti-GFP, Life Technologies A11122, 1:500; guinea pig anti-GABA, Millipore AB175, 1:500; mouse anti-PV, Millipore MAB1572, 1:2,000; mouse anti-SST, Santa Cruz, sc-55565, 1:100; mouse anti-RGS14, BioLegend N133/21, 1:400; chicken anti-β-galactosidase, Abcam, 1:1,000; guinea pig anti-VGLUT1, Synaptic Systems 135304, 1:3,000; mouse anti-NeuN, Millipore MAB377; goat anti-doublecortin (DCX), sc-8066, Santa Cruz, 1:500). On day 2, sections were rinsed three times for 10 min in PBS and incubated for 90 min with fluorescent-labeled secondary antibody (Alexa-Fluor-488-conjugated donkey anti-rabbit IgG, Jackson ImmunoResearch 711-545-152, 1:500; Cy3-conjugated donkey anti-rabbit-IgG, Jackson ImmunoResearch 711-165-152, 1:500; Cy5-conjugated donkey anti-rabbit-IgG, Jackson ImmunoResearch 711-175-152, 1:500; Alexa-Fluor-488–conjugated donkey anti-mouse-IgG, Jackson ImmunoResearch 715-545-151, 1:500; Cy3-conjugated donkey anti-mouse-IgG, Jackson ImmunoResearch 715-165-151, 1:500; Cy5-conjugated donkey anti-mouse-IgG, Jackson ImmunoResearch 715-605-151, 1:500; Cy3-conjugated donkey anti-goat-IgG, Jackson ImmunoResearch 705-165-147, 1:500; Cy3-conjugated donkey anti-guinea-pig-IgG, Jackson ImmunoResearch 706-165-148, 1:500). Sections were rinsed three times for 10 min each in PBS before mounting on to glass slides and covellsplited with mounting medium containing DAPI (Fluoromount with DAPI, Southern Biotech). ABLIM3–specific immunostaining was performed as described previously. Briefly, to analyze the distribution of ABLIM3 in the hippocampus, fresh-frozen sections were mounted on glass slides, air-dried and sequentially fixed in 95% ethanol at −20 °C for 30 min and 100% acetone at room temperature for 1 min. The slides were blocked with 1% BSA in PBS for 30 min and incubated with primary antibodies at room temperature for 2 h (mouse anti-ABLIM3, Abcam ab67721, 1:100; guinea pig anti-VGLUT1, Synaptic Systems 135304, 1:3,000; rabbit anti-Bassoon, Abcam ab10426, 1:1,000; rabbit anti-ZO-1, Invitrogen 402200, 1:500). After washing with PBS, the slides were incubated with fluorescent-label-conjugated secondary antibodies (Alexa-Fluor-488–conjugated donkey anti-mouse-IgG, Jackson ImmunoResearch, 715-545-151, 1:500; Cy3-conjugated donkey anti-guinea-pig-IgG, Jackson ImmunoResearch, 706-165-148, 1:500; Cy5-conjugated donkey anti-rabbit-IgG, Jackson ImmunoResearch, 711-175-152, 1:500) for 30 min and then coverslipped with mounting medium containing DAPI (Fluoromount with DAPI, Southern Biotech).

Image acquisition and analysis. Images were obtained from one set of brain sections (six sets generated for each brain) for each immunostaining experiment (set of antigens). All analysis was performed by an experimenter who was blinded to group identity.

Image analysis of mossy fiber terminals. A Nikon A1R Si confocal laser, a TIE inverted research microscope and NIS Elements software were used to capture z-stacks for MFT imaging using a 60× objective plus 10× digital zoom, as we previously published. Mossy fiber axons of dentate granule cells were imaged in the stratum lucidum of the CA3ab subfield. Images were...
obtained from high-resolution (2,048 resolution) image z-stacks with a step size of 0.3 µm. The area of individual MFTs was assessed at the widest point in the z-stack using ImageJ image>stacks>Z projection, followed by unbiased area selection with the tracing tool. MFTs were defined as having a cross-sectional area of 4 µm² or greater from LV-shNT-GFP- or LV-shRNA-GFP-injected samples. MFT filopodia extensions were also quantified. MFT filopodia extensions were defined as protrusions that exceeded 1 µm in length and that possessed an end-swelling varicosity structure. At least 20–30 MFTs from each animal were imaged for MFT and MFT filopodia measurements.

**Image analysis of dendritic spines.** For quantification of dendritic spines, confocal z-stack images were acquired using a Nikon A1R Si confocal laser, a TiE inverted research microscope and NIS Elements software. Imaging was performed using a 60× objective lens and 6× digital zoom. For spine imaging, confocal z-stacks (2,048 resolution) with 0.3-µm step size were taken centered on the dendritic segment. The z-stacks were flattened using the maximum-intensity projection, and flattened images were quantified using ImageJ. For spine density, spines were counted manually for at least 100 µm of dendritic length per region per mouse. The outer molecular layer was defined as the outer one-third of the molecular layer of the DG.

To quantify c-FOS⁺ cells and overlap in antigens (GFP and GABA, GFP and PV, c-FOS and PV, c-FOS and SST, c-FOS and H2B–mCherry, H2B–GFP and c-FOS, TdTomato and PV, and DCX and GFP), z-stack images were acquired with a Nikon A1R Si confocal laser and a TiE inverted research microscope using a 20× objective lens. Images (1,024 resolution) were acquired as 11-z-stack images with ImageJ, and the cell signal intensity over the background within the same image was computed. Colocalization was determined by assessment of overlap of two independent fluorescence signals. Double-positive cells were selected in the maximum-intensity projection image using a multipoint tool and manually confirmed within each z-stack image that also contained DAPI signal.

For overlap by chance quantification, 50 DAPI⁺ cells were randomly selected within the manually defined ROI on each image used for quantification of reactivation. Because of the high cell density in the DG, 75 DAPI⁺ cells were randomly selected for chance-overlap quantification in the DG. The number of c-FOS⁺ and GFP⁺ cells among the randomly selected cells were then determined by examination of overlap in the appropriate channels for each TetTag system. Chance overlap was defined as the product of the probability of a randomly selected cell being c-FOS⁺ and the probability of such a cell being GFP⁺ (%c-FOS⁺ × %GFP⁺). A minimum of four regions in different hemi-sections were analyzed per mouse to calculate an ‘average chance overlap’ per mouse.

To quantify PV puncta density in CA3, images were acquired with a Nikon A1R Si confocal laser and a TiE inverted research microscope using a 60× objective lens plus 4× digital zoom. Puncta number was counted in each image, and the area per image was measured with ImageJ. Image size was constant for each section (2,812.5 µm²) and among all of the animals. Puncta density was defined as the number of puncta per 1,000 µm². For each mouse, puncta density was averaged from three images of the CA3ab region.

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism software. Comparison of two groups was performed using a two-tailed Student’s t-test unless otherwise specified. Comparison of one group across time was performed using a one-way ANOVA with repeated measure. Comparison of two groups across treatment condition or time was performed using a two-way repeated-measure ANOVA, and main effects or interactions were followed by Bonferroni post hoc analysis. mEPSCs and mIPSCs were analyzed using Kolmogrov–Smirnov tests with significance set at *P* < 0.0001. In the text and figure legends, ‘n’ indicates number of mice per group. Detailed statistical analyses can be found in [Supplementary Table 1](#supplementary) and the Life Sciences Reporting Summary.

**Life Sciences Reporting Summary.** Further information on experimental design is available in the Life Sciences Reporting Summary.

**Data availability.** Details of shRNA sequences and genetically modified mice can be found in the Online Methods. This information is sufficient for interpretation and replication of the findings.

61. Zhou, Z. et al. Brain-specific phosphorylation of MeCP2 regulates activity-dependent Bdnf transcription, dendritic growth and spine maturation. Neuron **52**, 255–269 (2006).
62. Lois, C., Hong, E.J., Pease, S., Brown, E.J. & Baltimore, D. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science **295**, 868–872 (2002).
63. Drokhlyansky, E. et al. The brain parenchyma has a type I interferon response that can limit virus spread. Proc. Natl. Acad. Sci. USA **114**, E95–E104 (2017).
64. McKown, K.M. et al. Modulating neuronal competition dynamics in the dentate gyrus to rejuvenate aging memory circuits. Neuron **91**, 1356–1373 (2016).
65. Ikra, T. et al. Adult neurogenesis modifies excitability of the dentate gyrus. Front. Neural Circuits **7**, 204 (2013).
Experimental design

1. Sample size

Describe how sample size was determined.

We performed pilot experiments to determine sample sizes for measuring fear generalization.

Data exclusions

Describe any data exclusions.

Criteria were pre-established. Specifically: Mis-hits were excluded (see Methods). Additionally, Two mice were excluded from CA1-AA-day16 shNT and CA1-AC-day16 shNT group analysis because there were no tagged cells detectable in CA1 pyramidal layer. This is stated in the legend for Supplementary Fig 5.

Replication

Describe whether the experimental findings were reliably reproduced.

1. abLIM3 downregulation increases DGC recruitment of inhibition. This effect was replicated in multiple cohorts of mice and in two labs.
2. abLIM3 downregulation in DGCs decreases time-dependent fear generalization - This effect was observed in multiple groups of mice.
3. abLIM3 downregulation governs reactivation dynamics in hippocampal-cortical networks. This effect was replicated using multiple Tet-Tag reporters.

Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

Mice from Jackson or NIA or from breeders at MGH were randomly assigned to treatment groups. Age-matched mice arrived from JAX as 4/cage and were randomly assigned to shRNA vs. NTshRNA.

Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

1. All experiments were performed blinded to treatment. 2. Data was randomly selected by PI and scored by scientist in the lab not working on the experiment.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.
6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

| n/a   | Confirmed |
|-------|-----------|
|       |           |

- The exact sample size \( (n) \) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.).
- A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly.
- A statement indicating how many times each experiment was replicated.
- The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section).
- A description of any assumptions or corrections, such as an adjustment for multiple comparisons.
- The test results (e.g. \( P \) values) given as exact values whenever possible and with confidence intervals noted.
- A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range).
- Clearly defined error bars.

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

Software

Describe the software used to analyze the data in this study.

Prism software was used for statistical analysis of data.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

No restrictions

Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Primary antibodies:
1. c-fos: Rabbit anti-c-fos, Calbiochem PC38, 1:10,000 – discontinued
   Different batches of rabbit, Santa Cruz SC52, 1:2,000
2. GFP: Rabbit anti-GFP, Life Technologies A11122, 1:500
3. GABA: Guinea-pig anti-GABA, Millipore AB175, 1:500
4. PV: mouse anti-PV, Millipore MAB1572, 1:2,000
5. SST: mouse anti-SST, Santa Cruz, sc-55565, 1:100
6. RGS14: mouse anti-RGS14, Biolegend N133/21, 1:400
7. β-gal: chicken anti-β-galactosidase, Abcam, 1:1000
8. VGLUT1: Guinea pig anti-VGLUT1, Synaptic Systems 135304, 1:3000
9. NeuN: Mouse anti-NeuN, Millipore MAB377
10. DCX: Goat anti-Doublecortin (DCX) sc-8066, Santa Cruz, 1:500
11. abLIM3: mouse anti-abLIM3, Abcam ab67721, 1:100
12. Bassoon: rabbit anti-bassoon, Abcam ab110426, 1:1000
13. ZO-1: rabbit anti-ZO-1 antibody, Invitrogen 402200, 1:500

Secondaries: Fluorescent-label-coupled secondary antibody (Jackson ImmunoResearch, 1:500).
10. Eukaryotic cell lines
   a. State the source of each eukaryotic cell line used.
      HEK293T (ATCC)
   b. Describe the method of cell line authentication used.
      Commercially available and extensively validated, https://www.atcc.org/en/Products/All/CRL-3216.aspx
   c. Report whether the cell lines were tested for mycoplasma contamination.
      This cell line tested negative for mycoplasma contamination.
   d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by iCLAC, provide a scientific rationale for their use.
      No commonly misidentified cell lines were used.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals
   Provide details on animals and/or animal-derived materials used in the study.
   All animals were handled and experiments were conducted in accordance with procedures approved by the Institutional Animal Care and Use Committees at the Massachusetts General Hospital and University of Washington, Seattle, in accordance with NIH guidelines. All mice were housed three-four per cage in a 12 hr (7:00 a.m. to 7:00 p.m.) light/dark colony room at 22°C–24°C with ad libitum access to food and water. Adult female C57Bl/6 mice (3-4 months old) were purchased from Jackson lab. Aged female C57Bl/6 mice (17-18 months old) were obtained from National Institute on Aging (NIA). The following mouse lines were obtained from Jackson Labs: cfas-tTA: tetO-taulacZ,tTA* (Stock No: 008344, Jackson labs), tetO-H2B-GFP (Stock No: 005104, Jackson labs), Col1a1-tetO-H2B-mCherry (Stock No: 014592, Jackson labs). TetTag mice were generated by crossing reporter mouse lines, tetO-H2B-GFP mouse line or tetO-H2B-mCherry mouse line, with mice that express tetracycline-trans-activator (tTA) protein under control of the c-fos promoter (Stock No: 008344, Jackson labs; outcrossed from tetO-lacZ, tTA*). c-fos-tTA mice also contain a transgene consisting of a c-fos promoter driving the expression of nuclear-localized 2-h half-life EGFP (shEGFP). All TetTag mice were maintained on a C57BL/6 background. Mice were bred and raised on doxycycline (dox) diet (40 mg kg\(^{-1}\) chow) to prevent any reporter expression prior to desired experimental labeling of ensembles. Dox diet was replaced with regular diet for 3 days to open the window for activity-dependent labeling of ensembles. Expression of reporter was shut off by administration of dox diet immediately following desired labeling window. PV-Cre mice (stock No: 017320, Jackson labs) were bred with Ai14: tdTomato reporter mouse line (stock No: 007914, Jackson labs) to generate mice for viral injection and in vitro electrophysiological whole cell recordings.

Policy information about studies involving human research participants

12. Description of human research participants
   Describe the covariate-relevant population characteristics of the human research participants.
   The study did not involve human research participants.