Direct dorsal hippocampal–prelimbic cortex connections strengthen fear memories

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The ability to regulate the consolidation and strengthening of memories for threatening experiences is critical for mental health, and its dysregulation may lead to psychopathologies. Re-exposure to the context in which the threat was experienced can either increase or decrease fear response through distinct processes known, respectively, as reconsolidation or extinction.

Using a context retrieval-dependent memory-enhancement model in rats, we report that memory strengthens through activation of direct projections from dorsal hippocampus to prelimbic (PL) cortex and activation of critical PL molecular mechanisms that are not required for extinction. Furthermore, while sustained PL brain-derived neurotrophic factor (BDNF) expression is required for memory consolidation, retrieval engages PL BDNF to regulate excitatory and inhibitory synaptic proteins neuroligin 1 and neuroligin 2, which promote memory strengthening while inhibiting extinction. Thus, context retrieval-mediated fear-memory enhancement results from a concerted action of mechanisms that strengthen memory through reconsolidation while suppressing extinction.

Memory consolidation, the process of stabilization and storage of long-term memories1–2, and its modulation are fundamental functions for survival. In contextual fear memories, this process engages a functional crosstalk between the dorsal hippocampus (dHC) and cortical regions3–6. Although hippocampal molecular mechanisms underlying long-term memory consolidation have been relatively well characterized, the related cortical mechanisms remain largely unknown.

Memory consolidation can be modulated through context retrieval, the re-experience of contextual stimuli without reinforcement, to either strengthen or weaken memory retention via reconsolidation or extinction processes, respectively. Reconsolidation, the process of restabilizing a memory after it has been destabilized by retrieval, mediates memory strengthening7,8, whereas extinction entails new learning that results in a decrease of the conditioned fear response9,10. The two processes employ distinct mechanisms and can be doubly dissociated11,12.

Using the contextual fear-based inhibitory avoidance (IA) task in rats, we previously found that brief, nonreinforced context retrievals strengthen the memory through reconsolidation7, which requires de novo translation in the basolateral amygdala (BLA)13,14. Recently, Fukushima et al.9 reported that IA memory enhancement evoked by context retrieval in mice requires amygdala, dHC and medial prefrontal cortex (mPFC) through the simultaneous activation of calcineurin-induced proteasome-dependent protein degradation and the transcription factor cAMP-responsive element binding protein (CREB). However, important questions remain to be addressed: why do retrieval events, in certain conditions, lead to memory enhancement rather than extinction? How does dHC and mPFC generate memory consolidation and enhancement? Are there subregions of the mPFC critically implicated in memory enhancement versus extinction? Is there a functional link between reconsolidation and extinction? And finally, which molecular, cellular and behavioral mechanisms mediate memory enhancement? The answers to these questions will elucidate circuitry and molecular mechanisms underlying fear memory strengthening or weakening, important information for investigating abnormal fear responses and hopefully identifying corrective approaches.

Here we employed a protocol based on three retrievals (3Rs) following IA training in rats7, to identify hippocampal–cortical functional circuitry and mechanisms of context retrieval-induced memory strengthening. We show that 3Rs enhance fear memory by engaging direct functional connectivity between the dHC and the PL subregion of mPFC and by engaging two types of biological mechanisms in the PL cortex: one that promotes memory strengthening and another that inhibits extinction.

**RESULTS**

**Activity-regulated cytoskeleton-associated protein Arc in dHC and mPFC is required for memory enhancement**

Rats were trained in the IA task or remained in the home cage (nontrained, naive group, N). Memory reactivation started 2 d after training and consisted of a total of three brief (10-s) re-exposures to the context (the lit compartment of the IA box), given with an interval between re-exposures of 2 d (3Rs). Rats that were trained but did not undergo memory reactivation (nonreactivated group, NoR) were used as controls. We also included another control group of rats, which was trained and exposed three times to a new, different context (3Cs) instead of the reactivation trials. The 3Rs protocol, given during the first week after training, was previously shown to produce significant memory enhancement through memory reconsolidation7.

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First, we confirmed that the 3Rs protocol led to significant memory enhancement (Fig. 1a). Western blot analyses then showed that 3Rs had significantly increased levels of the activity and/or plasticity marker Arc (also called Arg3.1) in the dHC, BLA, mPFC and anterior cingulate cortex (ACC) 1 h after the last context retrieval, compared to both N and NoR conditions (Fig. 1b).

Compared to a control scrambled oligodeoxynucleotide (ODN), a bilateral injection of antisense ODN against Arc 1 h before each retrieval in the dHC completely blunted retrieval-mediated memory enhancement, without affecting either the memory in the absence of retrievals (NoR) or memory retrieval per se (Fig. 1c and Supplementary Fig. 1a). In line with previous studies on protein synthesis inhibition8,13, Arc antisense injection into the BLA significantly disrupted the memory (compared to scrambled ODN-injected 3Rs or NoR groups; Fig. 1c). This disruption persisted for 1 week, and memory was not rescued by a reminder shock given in a different context (Supplementary Fig. 1b), a protocol that reinstates extinguished fear memories7. This suggests that BLA mechanisms of de novo gene expression, including Arc translation, mediate IA memory reconsolidation. Similarly to what was found with the dHC, Arc antisense injections into the PL or the infralimbic (IL) subregions of mPFC before each retrieval completely blocked retrieval-mediated memory enhancement without affecting the memory in the NoR groups (Fig. 1c). Finally, Arc antisense injections in the ACC had no effect on retrieval-mediated memory enhancement, indicating that not all prefrontal cortical regions are similarly engaged (Fig. 1c).

We concluded that context retrieval during the first week following IA training induces Arc expression in multiple brain regions, which is critical for processing contextual fear memories. While Arc induction in the dHC, PL and IL mediates memory enhancement, Arc induction in the BLA mediates memory reconsolidation.

Retrieval-mediated Arc induction in dHC controls molecular mechanisms in mPFC underlying memory enhancement

We next identified additional molecular correlates of memory enhancement in the dHC and mPFC. Western blot analyses were employed to quantify the relative activation of two mechanisms critical for long-term plasticity and memory formation: the phosphorylation of the transcription factor CREB at Ser133 (pCREB), and the phosphorylation of the actin severing protein cofilin at Ser3 (p-cofilin)16,17. The 3Rs protocol significantly increased both pCREB and p-cofilin, but not total CREB and cofilin, in both dHC and mPFC compared to N, NoR and 3Cs (Fig. 2a and Supplementary Fig. 2). Although the 3Cs protocol did not produce memory enhancement (Fig. 1a), it significantly increased Arc in both dHC and mPFC (Fig. 2a). Furthermore, a single retrieval (1R) given at 6 d after training produced a significant increase in Arc but only a trend toward an increase in pCREB or p-cofilin in both dHC and mPFC (Fig. 2a). These data suggest that the induction of pCREB and p-cofilin, but not of Arc, correlates with memory enhancement.

Given the behavioral and molecular similarities between dHC and mPFC, we next asked whether they functionally interact to promote memory strengthening. Previous studies suggested that hippocampal input to cortical regions is important for memory consolidation and strengthening through sleep and/or slow-wave oscillations during rest periods after training18. Hence, we tested whether molecular changes evoked by the 3Rs protocol in the dHC directly control changes occurring in the mPFC. Arc antisense or scrambled control ODN was bilaterally injected into the dHC before each retrieval to block hippocampal Arc induction. Control rats (NoR group) received similar injections at matched time points in the absence of the 3Rs protocol.

Figure 1 Arc in the dHC and mPFC is required for memory enhancement. (a) Mean latency ± s.e.m. of rats trained (Tr) and re-exposed three times to the training context (3Rs) or to a novel context (context B; 3Cs) for 10 s. NoR: nonretrieval, rats trained and kept in the home cage. Memory retention was tested (T) at 8 d after training (one-way ANOVA followed by Newman-Keuls post hoc test, $F_{2,25} = 20.3, P < 0.0001, n = 11, 9, 8$; three independent experiments). (b) Cropped examples and relative quantitative western blot analyses of dHC, BLA, mPFC and ACC extracts obtained from rats killed for analysis (red arrows) 1 h after 3Rs or at matched time points in the NoR group. Naive rats (N) served as control. Data are presented as mean percentage ± s.e.m. of the mean values of the N group (one-way ANOVA followed by Newman-Keuls post hoc test, $F_{2,25} = 12.53, P = 0.0002, n = 9, 10, 7$; BLA $F_{2,25} = 3.619, P = 0.0438, n = 9, 7, 9$; mPFC $F_{2,22} = 34.77, P < 0.0001, n = 9, 8, 8$; ACC $F_{2,26} = 26.15, P < 0.0001, n = 9, 10, 7$; three independent experiments). (c) Mean latency ± s.e.m. of rats injected (black arrows) with Arc antisense (AS) or scrambled (SC) ODNs into the dHC, BLA, PL, IL or ACC 1 h before each 10-s retrieval or at matched time points in the NoR group (one-way ANOVA followed by Newman-Keuls post hoc test; dHC $F_{3,24} = 5.564, P = 0.0048, n = 8, 7, 7, 6$; BLA $F_{3,29} = 12.2, P < 0.0001, n = 8, 8, 9, 8$; PL $F_{3,33} = 11.38, P < 0.0001, n = 9, 6, 11, 11$; IL $F_{3,28} = 3.693, P = 0.0234, n = 8, 5, 9, 10$; ACC $F_{2,14} = 6.555, P = 0.0097, n = 6, 5, 6$; three independent experiments). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Histological images showing the injection sites are presented in Supplementary Figure 10. Full-length blots are presented in Supplementary Figure 11.
Figure 2 Context retrieval-mediated Arc induction in the dHC controls molecular changes in the mPFC. (a) Cropped examples and relative quantitative western blots analyses of dHC and mPFC extracts obtained from rats trained (Tr) and killed for analysis (red arrows) 1 h after one or three memory retrievals (1R or 3Rs), three exposures to a novel context (3Cs; context B) or at the matched time point in the NoR group. Naive rats (N) served as reference control. Data are presented as mean percentage ± s.e.m. of the mean values of the N group (one-way ANOVA followed by Newman-Keuls post hoc test; dHC: Arc F(3,29) = 7.25, P = 0.0003, n = 9, 10, 5, 7, 6; pCREB F(3,30) = 4.444, P = 0.0061, n = 7, 8, 6, 7, 7; p-cofilin F(3,28) = 2.757, P = 0.0474, n = 9, 6, 5, 7, 6; mPFC: Arc F(3,30) = 23.31, P < 0.0001, n = 9, 8, 4, 8, 6; pCREB F(3,26) = 4.745, P = 0.0052, n = 8, 8, 4, 5, 6; p-cofilin F(3,33) = 5.041, P = 0.0028, n = 10, 8, 5, 7, 8; three independent experiments). (b) Cropped examples and relative quantitative western blot analyses of dHC or mPFC extracts obtained from rats killed for analysis (red arrows) 1 h after three memory retrievals (3Rs), three exposures to a novel context (3Cs; context B) or at the matched time point in the NoR group. Rats received a bilateral injection (black arrows) of Arc antisense (AS) or scrambled (SC) ODNs into dHC 1 h before each retrieval or at the matched time points in the NoR group. Data are presented as mean percentage ± s.e.m. of the mean values of the N group (one-way ANOVA followed by Newman-Keuls post hoc test; dHC: Arc F(3,29) = 9.867, P = 0.0001, n = 8, 6, 8, 11; pCREB F(3,28) = 4.165, P = 0.0147, n = 8, 5, 8, 11; p-cofilin F(3,25) = 4.927, P = 0.008, n = 9, 4, 6, 10; mPFC: Arc F(3,21) = 6.173, P = 0.0036, n = 9, 5, 5, 6; pCREB F(3,22) = 3.693, P = 0.0239, n = 9, 5, 8, 9; p-cofilin F(3,20) = 4.209, P = 0.0137, n = 9, 5, 8, 11; three independent experiments). *P < 0.05, **P < 0.01, ***P < 0.001. Full-length blots are presented in Supplementary Figure 11.
dHC and mPFC protein extracts, obtained 1 h after the last retrieval or at the matched time point for the NoR group, were examined using western blot analyses. Compared to scrambled controls, Arc antisense completely blocked the 3Rs-evoked increase of Arc, pCREB and p-cofilin not only in the dHC (Fig. 2b) but also—remarkably—in the mPFC (Fig. 2b). Arc antisense injections into the dHC of NoR rats did not change the levels of any of these proteins in either dHC or mPFC (Fig. 2b). Thus, functional crosstalk between the dHC and the mPFC underlies retrieval-mediated memory enhancement.

Direct functional dHC-to-PL cortex projections mediate memory enhancement

We next asked whether neuronal activity of direct projections from dHC to specific subregions of the mPFC plays a critical role in memory enhancement. Within the mPFC, the IL cortex is known to mediate fear extinction, while the PL cortex plays a critical role in the expression of conditioned fear. Here we tested whether the neuronal activity of direct projections from dHC to PL or IL cortex is involved in memory strengthening evoked by the 3Rs protocol. To verify the specificity of effects on memory strengthening, we also investigated the role of the same neuronal activation on extinction, which was evoked by confining the animals in the dark compartment of the IA box for 5 min in the absence of footshock following a standard IA test (Ext). Western blot analyses measuring Arc, pCREB and p-cofilin confirmed that the dHC was activated with both 3Rs protocols, as well as with Ext given 6 d after IA training; in fact, these markers were significantly induced with either 3Rs protocols or extinction, compared to what we found in the N and NoR groups (P = 0.0002 for Arc, P = 0.0003 for pCREB, and P = 0.0011 for p-cofilin; Supplementary Fig. 3).

Virus-mediated expression of designer receptors exclusively activated by designer drugs (DREADD) in dHC projection neurons was combined with local infusion of DREADD ligand clozapine-N-oxide (CNO) to silence neurotransmission in the PL cortex, Adeno-associated virus 8 (AAV8)-expressing Gi-coupled DREADD hM4Di (AAV8/hSyn-HA-hM4Di-IRES-mCitrine), which silences neurotransmission in the presence of CNO, was injected bilaterally into the dHC. In addition to hM4Di, this viral vector also expressed a fluorescent protein, mCitrine, independent of hemagglutinin (HA)-hM4Di in the infected neurons using the internal ribosome entry site (IRES). Unlike HA-hM4Di, which is a transmembrane protein labeling neuronal processes including long projections, mCitrine is a soluble protein and labels the somata and proximal processes of the infected cells. Four to six weeks after viral injection, injection of dHC but not of ventral hippocampal neurons was revealed by somatic mCitrine expression. Expression of hM4Di was detected in both the somata and neurites of infected dHC neurons but not in the ventral hippocampus (Supplementary Fig. 4a). Consistent with previous reports using a variety of retrograde tracing techniques showing a small population of dHC neurons projecting to the mPFC, in the animals injected with AAV8/hSyn-HA-hM4Di-IRES-mCitrine into the dHC, the PL cortex showed sparse hM4Di expression, indicating direct projections from the dHC to the PL cortex (Supplementary Fig. 4b). No mCitrine-labeled neurons were detected in the PL cortex, excluding off-target AAV infection. This direct projection from dHC to PL cortex was further verified and confirmed using retrograde tracer cholera toxin B. Injection of cholera toxin B into the PL cortex resulted in labeling of CA2 neurons in the rostral sections of dHC and additional sparse labeling of CA1 neurons in the caudal dHC sections (Supplementary Fig. 5a,b).

Bilateral injection of CNO into the PL cortex of rats expressing hM4Di in dHC neurons significantly reduced 3Rs-evoked Arc induction in the PL cortex, compared to control groups (vehicle-injected group, or rats expressing GFP in place of hM4Di in dHC neurons and injected with CNO; P < 0.0001). No effect on Arc levels was found in the NoR group or in the IL cortex (Supplementary Fig. 5c). Compared to vehicle, CNO injection into the PL cortex completely blocked 3Rs-evoked memory enhancement. This effect persisted and did not change after a reminder shock, indicating that memory blockade was not due to a facilitated extinction but rather to a disruption of memory strengthening (Fig. 3a). No effect of CNO injection into the PL cortex was found in the NoR group. Furthermore, injection of CNO into the PL cortex in rats expressing the control virus AAV8/hSyn-GFP in the dHC had no effect on memory retention, excluding nonspecific behavioral effects caused by the virus and/or CNO (Fig. 3a).

To determine whether the dHC-to-PL direct projections are selectively involved in retrieval-mediated memory enhancement, we tested whether blocking the same neuronal activity had any effect on extinction.
Rats bilaterally infected with AAV8/hSyn-HA-hM4Di-IRES-mCitrine in the dHC and injected with CNO in the PL cortex displayed extinction similarly to vehicle-injected controls (Fig. 3b). These data suggest that direct projections from dHC to PL cortex are recruited in memory strengthening but are not involved in extinction.

Furthermore, as we found that dHC also sends direct projections to the IL cortex of mPFC (Supplementary Fig. 4b), a region known to mediate extinction, we employed the DREADD system to examine the role of neuronal activity of direct dHC-to-IL cortex projections in extinction. Extinction was completely blocked by a bilateral CNO injection into the IL cortex of rats whose dHC were infected with AAV8/hSyn-HA-hM4Di-IRES-mCitrine, compared to control virus AAV8/hSyn-GFP (Fig. 3b).

Collectively, these data showed that retrieval-evoked memory enhancement requires the activation of direct functional projections from the dHC to PL cortex. This activation functionally engages Arc and leads to phosphorylation of CREB and coflin in the PL cortex. In contrast, extinction engages direct functional projections from the dHC to IL cortex.

**PL BDNF mediates memory consolidation and extinction inhibition**

Next we investigated PL cortical mechanisms underlying retrieval-mediated memory enhancement. Given that the induction of pCREB and p-coflin correlates with memory enhancement and that CREB-dependent gene expression, as well as synaptic structural changes accompanying synaptic plasticity and memory consolidation, is regulated by BDNF27,28, we hypothesized that BDNF is an upstream critical mediator of memory enhancement. Western blot analyses showed that the levels of BDNF and of the phosphorylation of its receptor TrkB (pTrkB), but not total TrkB levels, were significantly increased in the mPFC 6 d after IA training compared to the N group (Fig. 4a and Supplementary Fig. 2). These upregulations were observed starting 30 min after training and persisted for at least 1 week (Fig. 4b). Similar increases were also observed in the 3Rs group but not in the 1R or 3Cs groups (Fig. 4a and Supplementary Fig. 2).

To block the functional role of BDNF in the PL cortex during either memory consolidation or enhancement, a functional anti-BDNF blocking antibody was injected bilaterally into the PL cortex 30 min before each retrieval or at matched time points in the absence of retrievals (NoR). Selective targeting of PL cortex injection is shown in Supplementary Figure 6a.

Compared to IgG controls, the anti-BDNF injections in the absence of retrieval significantly disrupted memory retention when tested 8 d after training. The impairment persisted as shown by another test 5 d later. The memory impairment also remained after a reminder shock, suggesting that memory consolidation requires a long-lasting BDNF-dependent function in the PL cortex (Fig. 4c). In contrast, in the rats that underwent 3Rs, PL anti-BDNF injection before each retrieval produced a distinctive outcome compared to injections of IgG: it selectively blunted memory enhancement without decreasing retention below that evoked by training (Fig. 4c). This effect persisted, as shown by another test 5 d later. However, a reminder shock fully rescued memory performance (Fig. 4c), indicating that the decrease in memory retention by anti-BDNF reflected a facilitated extinction, rather than a disruption of memory. This effect of BDNF was different than that of other PL cortical mechanisms, such as the 3Rs-evoked Arc expression, which selectively targeted memory strengthening but did not affect extinction (Fig. 1c and Supplementary Fig. 6b). Thus, these data imply that parallel molecular mechanisms are engaged in the PL cortex following context retrievals; these mechanisms, in concert, promote memory strengthening and extinction inhibition.
BDNF in PL cortex regulates neuroligin 1/neuroligin 2 ratio to promote memory consolidation or enhancement

BDNF signaling can regulate both excitatory and inhibitory synapse formation and function. Given that the balance between excitation and inhibition in the mPFC may critically contribute to fear memory expression and extinction, we first investigated whether a change in the ratio of excitatory/inhibitory synapses accompanies 3Rs-mediated IA memory enhancement. Toward this end, we employed western blot analyses to determine whether BDNF in the PL cortex regulates the expression of neuroligin 1 (NLGN1) and neuroligin 2 (NLGN2), markers of maturation of excitatory and inhibitory synapses, respectively.

Like BDNF and pTrkB, both NLGN1 and NLGN2 in the mPFC significantly increased 6 d after IA training compared to naive conditions (Fig. 5a), suggesting that both excitatory and inhibitory synapse maturation accompanies memory consolidation. Following 1R and 3Rs protocols, but not after 3Cs, NLGN1 remained increased, while NLGN2 returned to its baseline level after context retrieval or exposure to 3Cs (Fig. 5a). Thus, the 3Rs protocol significantly changed the ratio of NLGN1/NLGN2 (Fig. 5a) in favor of an overall enhancement of excitatory synapse maturation, thereby suggesting its functional contribution. Consistent with the conclusion that 3Rs enhance excitatory synapse maturation, glutamate receptor 1 (GluA1) increased after 1R and 3Rs, and glutamate receptor 2 (GluA2) increased after 3Rs (Supplementary Fig. 7). Hence, IA training produced a long-lasting increase in the levels of proteins associated with both inhibitory and excitatory synapse formation and maturation in the mPFC; furthermore, context retrieval, but not exposure to a different context, increased the excitatory-to-inhibitory synapse ratio.

Blocking BDNF with anti-BDNF antibody in the absence of retrieval blunted the training-induced increase of both NLGN1 and NLGN2 (Fig. 5b). However, blocking BDNF at each retrieval trial significantly blocked the NLGN1 induction but did not reverse the NLGN2 decrease, suggesting that the latter is mediated by BDNF-independent mechanisms (Fig. 5b). In sum, blocking BDNF significantly reversed the increased NLGN1/NLGN2 ratio after 3Rs (Fig. 5b). We concluded that BDNF signaling in the PL cortex differentially regulates inhibitory and excitatory synapse maturation after training to promote memory consolidation or after context retrieval to promote enhancement.

The role of NLGN1 and NLGN2 in memory consolidation, strengthening and extinction inhibition

We next examined the functional requirements for NLGN1 and NLGN2 in the PL cortex during either memory consolidation or 3Rs-evoked memory enhancement. The effect of bilateral injection into the PL cortex of the functional competitor extracellular domain of either NLGN1 or NLGN2 30 min before each retrieval, or at the matched time points in the NoR group, was tested 8 d after training. Blocking NLGN1 had no effect on memory retention in the NoR group but completely reversed retrieval-mediated memory enhancement (Fig. 6a). This blockade persisted as shown by another test 5 d later, and the retention was not rescued by a reminder shock. Hence, functionally blocking NLGN1 produced a behavioral effect similar to that found when Arc expression was blocked (Fig. 1c and Supplementary Fig. 6b).

In contrast, functional disruption of NLGN2 in the NoR group significantly and persistently disrupted memory retention tested 8 d after training and again 5 d later. The impairment remained following a reminder shock (Fig. 6a), indicating a persistent role of PL inhibitory synapses in IA memory consolidation. On the other hand, disrupting NLGN2 function at each retrieval trial blunted memory enhancement without further disrupting the memory. The blunting effect persisted over time, but notably, a reminder shock fully rescued memory enhancement (Fig. 6a), suggesting that, as with anti-BDNF, disrupting NLGN2 function promoted extinction.

To further understand the differential roles of NLGN1 and NLGN2 in memory enhancement or extinction inhibition, respectively, we investigated the activation of the PL and IL subregions of the mPFC using Arc expression as a readout. A bilateral injection of an NLGN1 inhibitor in the PL cortex 30 min before each retrieval trial significantly reduced Arc expression, whereas injection of NLGN2 inhibitor significantly increased Arc expression (Fig. 6b). These data confirmed previous findings that NLGN1 and NLGN2 differentially mediate excitatory and inhibitory synaptical activity, hence promoting or inhibiting neuronal activation, respectively. In agreement with our behavioral results, these data also suggest that while NLGN1 and Arc promote memory strengthening via neuronal activation (Figs. 1c and 6a and Supplementary Fig. 6b), NLGN2 inhibits neuronal activation.
and targets a different PL cortical mechanism, i.e., as indicated by our behavioral data, extinction inhibition (Fig. 6a).

To support this conclusion, we tested whether NLGN2 blockade in the PL cortex caused neuronal activation in the subregion known to mediate extinction: the IL cortex. Indeed, blocking NLGN2 in the PL cortex in the 3Rs condition increased Arc levels in both the PL and IL cortices, with the strongest effect in the IL cortex (Fig. 6b). Moreover, blocking PL NLGN1 in the 3Rs condition also led to IL cortex activation, but to a lesser degree (Fig. 6b). We concluded that NLGN1 and NLGN2 act in opposite directions in the PL cortex to functionally regulate IL cortex activation, suggesting that they likely target different neuronal populations. Together with the behavioral outcome, these data suggest that PL NLGN2 inhibits PL cortical neurons whose function is to activate the IL cortex. Thus, this PL NLGN2-mediated inhibition ultimately suppresses extinction.

To further support this conclusion, we measured the levels of neuronal activation in an extinction paradigm. We predicted that with extinction there should be a decrease in NLGN2 in the PL cortex and more activation of the IL cortex. We therefore compared the activation of the PL and IL cortices in the 3Rs protocol, which, as shown, evokes memory enhancement (3Rs-Enh), with a similar protocol that has previously shown to evoke IA extinction given 4 weeks after training (3Rs-Ext)\(^7\). Neuronal activation was measured by the expression of Arc.

**Figure 6** NLGN1 and NLGN2 have distinct roles in memory strengthening and extinction suppression. (a) Mean latency ± s.e.m. of rats trained (Tr) and given three memory retrievals (3Rs) or left in the home cage without retrieval (NoR) after training, as tested in the schema (T1–T3) and exposed to a reminder footshock (RS) where indicated. NLGN1 or NLGN2 function was blocked with recombinant extracellular domain of NLGN1 or NLGN2 (NLGN1inh or NLGN2inh) injected (black arrows) into PL 30 min before each 10-s retrieval or at the matched time points in the N group (two-way ANOVA followed by Bonferroni post hoc test; block NLGN1: treatment \(F_{2,69} = 18.84, P < 0.0001\); testing \(F_{2,69} = 2.69, P = 0.0752\); interaction \(F_{6,69} = 0.52, P = 0.7924\); \(n = 7, 8, 6, 6\); block NLGN2: treatment \(F_{2,84} = 61.46, P < 0.0001\); testing \(F_{2,84} = 6.03, P = 0.0036\); interaction \(F_{6,84} = 3.13, P = 0.0081\); \(n = 10, 7, 9, 6\); three independent experiments). (b) Examples and quantification of immunofluorescent staining of Arc in the IL and PL obtained from rats killed for analysis (red arrow) 1 h after 3Rs from the rats injected (black arrows) with control vehicle (veh), NLGN1inh or NLGN2inh into PL. Data are presented as mean percentage ± s.e.m. of the mean values of the 3Rs + veh group (two-way ANOVA followed by Bonferroni post hoc test; treatment \(F_{2,18} = 63.02, P < 0.0001\); region \(F_{1,18} = 6.25, P = 0.0223\); interaction \(F_{2,18} = 2.95, P = 0.0777\); \(n = 4, 4, 4\); two independent experiments). *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\). Histological images showing the injection sites are presented in Supplementary Figure 10.

**Figure 7** Changes in NLGN1 and NLGN2 in the mPFC correlate with retrieval-mediated memory strengthening or extinction. (a) The schemas of the experimental paradigms are shown on the top. Examples and quantification of immunofluorescence staining of Arc in the PL and IL from rats trained (Tr) and killed for analysis (red arrows) 1 h after 3Rs starting 2 d (enhancement model, 3Rs-Enh; \(n = 5, 5, 6\)) versus 4 weeks (extinction model, 3Rs-Ext; \(n = 5, 6, 6\)) after training or in the matching N and NoR groups. Data are presented as mean percentage ± s.e.m. of the mean values of the N group (two-way ANOVA followed by Bonferroni post hoc test; PL: treatment \(F_{2,27} = 36.98, P < 0.0001\); time \(F_{1,27} = 4.06, P = 0.0538\); interaction \(F_{2,27} = 2.35, P = 0.1145\); IL: treatment \(F_{2,27} = 62.13, P < 0.0001\); time \(F_{1,27} = 6.16, P = 0.0196\); interaction: \(F_{2,27} = 5.47, P = 0.0101\); two independent experiments). (b) Cropped examples and relative quantitative western blot analyses of mPFC extracts obtained from rats killed for analysis (red arrows) 1 h after 3Rs-Ext or at the matched time point in the NoR group. Naive rats (N) served as reference control. Data are presented as mean percentage ± s.e.m. of the mean values of the N group. The ratio of NLGN1/NLGN2 is shown to be higher in the extinction model compared to the enhancement model. Analysis was performed using two-way ANOVA, followed by Bonferroni post hoc test; NLGN1 \(F_{2,13} = 4.011, P = 0.0440\), \(n = 6, 5, 5\); NLGN2 \(F_{2,13} = 2.908, P = 0.0904\), \(n = 6, 5, 5\); ratio of NLGN1/NLGN2: \(F_{2,13} = 3.715, P = 0.0530\), \(n = 6, 5, 5\); two independent experiments). *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\). Full-length blots are presented in Supplementary Figure 12.
The number of Arc-positive cells significantly increased in the PL and the IL cortices after both enhancement and extinction protocols; however, the increase was significantly higher in the IL cortex after 3Rs-Ext compared to 3Rs-Enh, consistent with previous findings that IL cortex activation is critical for memory extinction.19,20 (Fig. 7a).

Furthermore, western blot analysis of 3Rs-Enh and 3Rs-Ext revealed opposing trends in NLGN1 and NLGN2 expression regulation: while in the first week after training, as well as with 3Rs-Enh, NLGN1 was significantly increased in the mPFC (Fig. 5a); 32 d later, NLGN1 significantly decreased below the level in control N rats (Fig. 7b). NLGN2, which was increased (and required for memory formation) in the first week following training, decreased below control N levels 32 d later. Compared to NoR, 3Rs-Ext did not significantly change the levels of NLGN1 and NLGN2 (Fig. 7b).

**BDNF and NLGN2 in the PL cortex inhibit extinction**

To further dissect the PL molecular mechanisms involved in 3Rs-evoked memory enhancement compared to extinction, we additionally investigated the Ext protocol. N and NoR groups served as controls. Western blot analyses revealed that changes in Arc, pCREB, p-cofilin, BDNF, pTrkB, NLGN1 and NLGN2 in the PL cortex following 3Rs were similar to those previously reported with the whole mPFC. In contrast, extinction resulted in an increase in Arc in the PL cortex but no changes in pCREB, p-cofilin, BDNF, pTrkB and NLGN1 (Supplementary Fig. 8). This suggested that extinction correlates with a very different pattern of PL cortical molecular changes.

Bilateral injections into the PL cortex of Arc antisense, anti-BDNF or inhibitors of NLGN1 or NLGN2 before extinction learning (all of which, as shown above, blunted retrieval-mediated memory enhancement; Figs. 1c, 4c and 6a) had no effect on memory retrieval or extinction (Fig. 8), suggesting that differential molecular mechanisms are involved in retrieval-mediated memory enhancement versus extinction. Finally, to test whether blocking PL BDNF or NLGN2 enhances extinction, as suggested by our experiments shown in Figures 4c and 6a, we employed a weak extinction protocol. In this protocol, during testing the rats were confined to the dark compartment of the IA box for 1 min upon entering. As shown in Figure 8c,d, blocking PL BDNF or NLGN2 significantly enhanced extinction, supporting the conclusion that these mechanisms can indeed inhibit extinction. Collectively, these data indicate that Arc, BDNF, NLGN1 and NLGN2 in the PL cortex are specifically engaged in retrieval-mediated memory enhancement and that BDNF and NLGN2 in the PL cortex contribute to memory enhancement by inhibiting extinction.

**DISCUSSION**

The consolidation and retrieval-dependent modulation of long-term fear memories greatly influence the regulation of emotions and the development of psychopathologies. Here we provide an innovative understanding of the circuitry and molecular mechanisms underlying consolidation, retrieval-dependent threat memory enhancement and extinction.

First, we showed that IA consolidation required several days of persistent BDNF upregulation in the PL cortex. These data significantly extend the findings that BDNF and TrkB play a critical role in the PL cortex for both appetitive and fear learning in mice33,34. We also showed that PL BDNF controlled the upregulation of both NLGN1 and NLGN2 in the PL cortex. To our knowledge, our data are the first to report changes of endogenous NLGN1 and NLGN2 following training in the PL cortex and their dependence on PL BDNF. Furthermore, and in line with a recent report employing a conditional NLGN2 knockout in mPFC35, our results reveal a critical role for NLGN2 in the PL cortex during the first week of IA memory consolidation, suggesting that the sustained inhibitory synapse function is an essential
mechanism of memory consolidation. These results are important in light of the imbalance of the excitation-to-inhibition ratio documented in cortical areas in models of neuropsychiatry disorders.

Second, we showed that context retrieval following a recently formed aversive memory engages BLA for memory reconsolidation and activates a direct monosynaptic input from the dHC to the PL cortex. Fukushima et al. recently reported that the dHC and mPFC mediate IA memory enhancement in mice through CREB-mediated gene expression and calcineurin-induced proteasome-dependent protein degradation. Our data significantly extend this information in several ways by showing that (i) direct functional projections from the dHC to PL cortex are necessary for memory strengthening, which occurs via Arc-dependent mechanisms in both regions. To our knowledge, this is the first evidence showing the existence of functional dHC-to-PL cortex direct projections in context retrieval-mediated memory processing. (ii) The dHC activation controls PL cortical molecular mechanisms, including the induction of pCREB, p-cofilin, BDNF, pTrkB and NLGN1, to specifically promote context retrieval-induced memory enhancement. These changes are not involved in context retrieval-induced extinction. (iii) NLGN1 mediates memory strengthening. In parallel, BDNF and NLGN2 modulate memory strengthening by suppressing extinction. In agreement, we also found that blocking BDNF or NLGN2 in the PL cortex not only did not block extinction but actually facilitated it. These data indicate, for the first time to our knowledge, that reconsolidation and extinction are co-regulated and are functionally cooperative processes.

Our data also showed that context retrieval shifts the role of PL BDNF-dependent mechanisms in processing hippocampal-dependent fear memory. Its functional role is required for several days after training to promote memory consolidation; however, with context retrieval, BDNF becomes engaged in inhibiting extinction. We speculate that this shift occurs as a result of the activation of different memory traces that takes place with training versus context retrieval. BDNF, being a common, fundamental plasticity mechanism, likely affects the active trace, thereby promoting distinct molecular and behavioral outcomes in the two behavioral conditions (training versus retrieval). Retrievals, and hence memory enhancement, also resulted in the return to baseline of NLGN2 but not of NLGN1, which produced a net increase in the excitatory over inhibitory synapse ratio. This decrease in inhibitory synapses with retrievals was not BDNF-dependent and can be explained by invoking a regulation of distinct populations of inhibitory synapses.

Several studies have reported that BDNF is a critical mechanism of memory extinction, which is known to mainly involve the IL cortex. How can BDNF be involved in memory enhancement as well as extinction, despite these two processes being mediated by different mechanisms and circuits? We suggest that retrieval protocols and the age of the memory dictate which distinct neural circuit is activated within the dHC, BLA and mPFC neuronal subpopulations to evoke either memory extinction or memory strengthening through reconsolidation. We speculate that such differential regulations may involve modulation of excitatory and inhibitory connections between the PL and the IL cortices, a hypothesis in agreement with the model proposed by Miller and Cohen and by Baldi and Bucherelli. Our speculation is also supported by our findings that memory extinction, compared to enhancement, resulted in greater activation of IL than PL cortex. Extinction also correlated with decreases in NLGN1 and NLGN2 in the mPFC, thus not changing its excitatory/inhibitory synapse ratio.

Based on our functional results targeting BDNF, NLGN1 and NLGN2 in the PL cortex, we propose, in agreement with Marquis et al. and Ragozzino, that the PL cortex plays an important role in detecting and processing mismatches, thus contributing to behavioral flexibility, i.e., the first encoding of the context associated with a footshock at training is revised at retrieval by context exposure in the absence of footshock. We suggest that this mismatch is accompanied by a significant shift in the molecular regulation of plasticity (through BDNF) that targets the active trace. We also suggest that, in addition to the mismatch detection, other mechanisms, such as the levels of arousal and trace-storage network distribution, contribute to determine when or if the memory is strengthened or extinguished.

In conclusion, the PL cortex and the input it receives from the dHC are important parts of the circuitry that mediates and modulates the strength of hippocampal-dependent fear memory. These mechanisms may represent important targets for threat-induced psychopathologies.

METHODS

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

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

X.Y., D.K.-L., A.T., M.C.I. and C.M.A. designed and developed this study. X.Y., D.K.-L., A.T. and M.C.I. carried out the experiments. X.Y., D.K.-L., A.T. and C.M.A. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONTINE METHODS

Animals. Adult male Long-Evans rats weighing 200–250 g (age 2–4 months) were used for the experiments. Rats were doubly or individually housed after surgery in the New York University animal facility and maintained on a 12-h light/dark cycle with ad libitum access to food and water. Experiments were performed during the light cycle. All rats were handled for 3 min per d for 5 d before any procedure. All protocols complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the New York University Animal Welfare Committee.

Cannulae implants. Rats were anesthetized with ketamine (75 mg/kg) and xylazine (10 mg/kg). Stainless steel cannulae (22 gauge for dHC, 26 gauge for other brain regions) were implanted stereotactically and bilaterally to target dHC (4.0 mm posterior to bregma, 2.6 mm lateral from midline and 2.0 mm ventral), BLA (2.8 mm posterior to bregma, 5.3 mm lateral from midline, 6.25 mm ventral), PL (14° angle toward midline, 2.8 mm anterior to bregma, 1.45 mm lateral from midline, 2.2 mm ventral), IL (30° angle toward midline, 2.8 mm anterior to bregma, 3.1 mm lateral from midline, 3.3 mm ventral) and ACC (26 gauge, 2.6 mm anterior to bregma, 0.6 mm lateral from midline, 1.3 mm ventral). Rats were given Buprenex (0.1 mg/kg, twice per d for 3 d before and after surgery) or meloxicam (3 mg/kg, once before surgery) for postoperative analgesic treatment and allowed to recover for at least 8 d before training.

Inhibitory avoidance (IA). The IA chamber (Med Associates, St. Albans, VT) consisted of a rectangular Perspex box, divided into a safe (lit) compartment and a shock (dark) compartment. Foot shocks were delivered to the grid floor of the shock compartment via a constant-current scrambler circuit. The two compartments were separated by a sliding door. The chamber was located in a sound-attenuated room illuminated by dim red light. During the training session, each rat was placed in the safe compartment with its head facing away from the door. After 10 s the door was automatically opened, allowing the rat access to the shock compartment, and a 2-s 0.6-mA foot shock was administered. Latency to enter the shock compartment was taken as a measure of acquisition. Ten seconds after delivery of the foot shock, the rat was returned to its home cage. Memory retention was tested at the indicated time points as described in each experiment and performed by placing the rat back into the safe compartment and measuring the latency to enter the shock compartment without administering foot shock. Testing was terminated at 900 s and performed blind to treatments. Memory reactivation consisted of 10-s exposures to the safe compartment. In control experiments, 10-s exposures to a different context (context B) were used. This context (control context used for the 3Cs exposures) consisted of a square chamber (Med Associates, St. Albans, VT) with three transparent walls, an opaque Perspex wall and a floor grid with narrow spacing, located in a separate, well-lit room. To test whether memory impairment was due to extinction, animals underwent a 2-s 0.6-mA reminder foot shock in the control context. Naïve rats were handled but otherwise remained in the home cage. For memory extinction, rats were tested for memory retention in the IA box, followed by confining the animals in the dark compartment of the IA box for 5 min or 1 min (weak extinction) in the absence of foot shock, as specified.

Oligodeoxynucleotides and drug injections. Arc antisense (Arc AS; 5′-GTC CAGCTCCATGCTCGCC-3′) or relative scrambled ODNs (Arc SC; 5′-CGTGC CACCTCTGGACCTTC-3′) were dissolved in PBS pH 7.4. The control Arc scrambled ODN contained the same base composition but in a randomized order and showed no homology to any mammalian sequence in the GenBank database. The ODNs were phosphorothioated on the three terminal bases at each end to protect against nuclease degradation. The ODNs were reverse-phase cartridge-purified and purchased from Gene Link (Hawthorne, NY). Rats were injected with 2 nmoL of ODNs per side in the dHC (in 1 μL), PL (in 0.3 μL), IL (in 0.2 μL), BLA (in 0.5 μL) or ACC (0.5 μL) 1 h before each 10-s context reactivation trial or at the matched time points in the NoR group. The sheep functionally blocking antibody to BDNF (Millipore, Billerica, MA) or control sheep IgG (Sigma-Aldrich, St. Louis, MO) was dissolved in PBS pH 7.4 and injected at 0.3 μg in 0.3 μL per injection per side into PL. Recombinant extracellular domains of NLGN1 or NLGN2 (R&D systems, Minneapolis, MN) were dissolved in PBS pH 7.4 and injected at 0.12 μg in 0.3 μL per injection per side into PL. These inhibitors were injected 30 min before each 10-s context reactivation trial or at the matched time points for the NoR group. The infusion needles (28 gauge for dHC; 33 gauge for other brain regions) extended 1.5 mm beyond the cannula. Injections were carried out bilaterally with an infusion pump at a rate of 0.333 μL/min with 10-μL Hamilton syringes (for dHC, BLA and ACC) or 0.2 μL/min with 1-μL Hamilton syringes (for PL and IL). The injection needle was left in place for 2 min following the nannopump (KD Scientific, Holliston, MA) was stereotactically inserted into the dHC (4.2 mm posterior to bregma, 2.6 mm lateral from midline and 3.2 mm ventral). AAV8/hSyn-IA-hM4Di-ires-mCitrine or AAV8/hSyn-GFP (2.1 × 1012 genomic copies/mL, 2 μL per side; UNC Vector Core, Chapter Hill, NC) was microinjected at a rate of 0.4 μL/min. The needle was left in place an additional 5 min following microinjection to ensure complete diffusion of the AAV and then slowly retracted. The scalp was sutured. Three weeks after AAV injection, the rats received stereotactic cannula implants.
bilateral targeting the PL cortex, as described above. Buprenex (0.1 mg/kg) or meloxicam (3 mg/kg) was used as analgesic treatments after both surgeries, and rats were allowed to recover for 6–13 days before training. CNO (Enzo Life Sciences, Farmingdale, NY) was dissolved in PBS pH 7.4 and injected at 500 µM in 0.3 µL per injection per side into PL 30 min before each 10-s context reactivation trial or at the matched time points for the NoR group, as described for the other treatments above. After behavioral experiments, the rats were anesthetized with an i.p. injection of 750 mg/kg chloral hydrate and transcerebrally perfused with 4% paraformaldehyde in PBS pH 7.4, and their brains were postfixed in this solution overnight at 4 °C, followed by PBS pH 7.4 with 30% sucrose for 72 h. We collected 30-µm brain sections by cryosection for free-floating immunofluorescent staining. For Arc staining, antigen retrieval was performed by boiling the brain sections in nanopure H2O for 5 min. The sections were then incubated with the blocking solution (PBS pH 7.4 with 0.25% Triton X-100, 4% normal goat serum, 1% bovine serum albumin) for 1 h at room temperature (20–22 °C). They were then stained with rabbit anti-HA antibody (1:500, Cell Signaling, cat# 3724, Danvers, MA) and chicken anti-GFP antibody (1:1,000, Aves Labs, cat# GFP-1020, Tigard, OR), diluted in the blocking solution for 48 h at 4 °C, followed by subsequent staining with goat anti-rabbit Alexa Fluor 568 and anti-chicken Alexa Fluor 488 antibodies (1:800, Invitrogen, Waltham, MA) for 2 h at room temperature. The sections were mounted with Prolong Diamond antifade mountant with DAPI (Invitrogen, Waltham, MA). Images were collected by an Olympus VS120 virtual slide microscope (Olympus, Tokyo, Japan) and Leica TCS SP5 confocal microscope (Leica, Wetzlar, Germany) at 20× magnification. Quantification was performed using the ImageJ software (US National Institutes of Health) by experimenters blind to the experimental conditions and using automated custom macro programs. Briefly, all images in an experiment were processed using the same parameters to remove background and outlier noise. Arc-positive neurons were counted automatically by the Analyze Particles function using a threshold and parameters to differentiate the cytoplasmic Arc staining from the dendritic staining. The same parameters were applied to all the images. The numbers of Arc-positive neurons were then normalized by the size of the region of interest.

**Cholera toxin subunit B (CTB) injection.** Rats were anesthetized with ketamine (75 mg/kg) and xylazine (10 mg/kg). The skull was exposed and holes were drilled in the skull bilaterally above the PL. A Hamilton syringe with a 33-gauge needle (75 mg/kg) and xylazine (10 mg/kg). The skull was exposed and holes were drilled bilaterally targeting the PL cortex, as described above. Buprenex (0.1 mg/kg) or meloxicam (3 mg/kg) was used as analgesic treatments after both surgeries, and rats were allowed to recover for 6–13 days before training. CNO (Enzo Life Sciences, Farmingdale, NY) was dissolved in PBS pH 7.4 and injected at 500 µM in 0.3 µL per injection per side into PL 30 min before each 10-s context reactivation trial or at the matched time points for the NoR group, as described for the other treatments above. After behavioral experiments, the rats were anesthetized with an i.p. injection of 750 mg/kg chloral hydrate and transcerebrally perfused with 4% paraformaldehyde in PBS pH 7.4, and their brains were postfixed in this solution overnight at 4 °C, followed by PBS pH 7.4 with 30% sucrose for 72 h. We collected 30-µm brain sections by cryosection for free-floating immunofluorescent staining. For Arc staining, antigen retrieval was performed by boiling the brain sections in nanopure H2O for 5 min. The sections were then incubated with the blocking solution (PBS pH 7.4 with 0.25% Triton X-100, 4% normal goat serum, 1% bovine serum albumin) for 2 h at room temperature, followed by staining with rabbit anti-Arc antibody (1:2,000, Synaptic System, cat# 156 003, Gottingen, Germany) diluted in the blocking solution for 48 h at 4 °C. Subsequently, the brain sections were stained with goat anti-rabbit Alexa Fluor 568 antibody (1:800, Invitrogen, Waltham, MA) for 2 h at room temperature and mounted with Prolong Diamond antifade mountant with DAPI (Invitrogen, Waltham, MA). Three sections around bregma +3.2mm, +2.8mm and +2.5mm, representing rostral, medial and caudal mPFC, were used for each set of staining. Two images per side of the PL and one image per side of the IL cortex for each animal were captured by a Leica TCS SP5 confocal microscope (Leica, Wetzlar, Germany) at 20× magnification.

**Statistical analyses.** Data were analyzed with the Prism 7 (GraphPad Software Inc.). No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those generally employed in the field. Statistical analyses were designed using an assumption of normal distribution and similar variance among groups, but this was not formally tested. The data were analyzed by one- or two-way ANOVAs followed by post hoc tests. One-way ANOVAs followed by Newman–Keuls post hoc tests were performed when comparing groups for which a pairwise post hoc analysis of each group was required. Two-way ANOVAs followed by Bonferroni post hoc tests were used when two factors (such as treatment and testing) were compared. When two groups were compared, Student’s t-tests were used. All analyses are two-tailed. The significance of the results was accepted at P < 0.05. A Supplementary Methods Checklist is available.

**Data availability.** The data that support the findings of this study are available from the corresponding author upon reasonable request.