Cellular and oscillatory substrates of fear extinction learning

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The mammalian brain contains dedicated circuits for both the learned expression and suppression of fear. These circuits require precise coordination to facilitate the appropriate expression of fear behavior, but the mechanisms underlying this coordination remain unclear. Using a combination of chemogenetics, activity-based neuronal-ensemble labeling and in vivo electrophysiology, we found that fear extinction learning confers on parvalbumin-expressing (PV) interneurons in the basolateral amygdala (BLA) a dedicated role in the selective suppression of a previously encoded fear memory and BLA fear-encoding neurons. In addition, following extinction learning, PV interneurons enable a competing interaction between a 6–12 Hz oscillation and a fear-associated 3–6 Hz oscillation within the BLA. Loss of this competition increases a 3–6 Hz oscillatory signature, with BLA→medial prefrontal cortex directionality signaling the recurrence of fear expression. The discovery of cellular and oscillatory substrates of fear extinction learning that critically depend on BLA PV interneurons could inform therapies aimed at preventing the pathological recurrence of fear following extinction learning.

RESULTS
BLA PV interneurons selectively suppress conditioned freezing and fear ensemble activation following extinction

To investigate the function of BLA PV interneurons in extinction-induced suppression of conditioned fear, we infused AAV-Syn-DIO-hM4Di-mCherry virus into the BLA of PV-Cre:Fos-tTa:TetO-H2B-GFP triple-transgenic mice (PV-Cre:TetTag mice), thereby expressing the inhibitory DREADD (Designer Receptor Exclusively Activated by Designer Drug) receptor hM4Di selectively in BLA PV interneurons in the background of TetTag mice (Fig. 1a and Supplementary Fig. 1a,b). This permitted us to exert selective control over BLA PV interneuron activity while tracking the effect on functionally relevant pyramidal neuron ensembles within the BLA³. Three weeks after virus infusion, mice were subjected to contextual fear conditioning and extinction, and neurons activated during fear conditioning or extinction were tagged with GFP (Fig. 1b and Supplementary Fig. 1c,d). We previously found that tagged neurons in the BLA are excitatory pyramidal projection neurons and that BLA interneurons are not tagged⁶. Mice exhibited robust freezing responses following conditioning and suppressed freezing following extinction learning (Supplementary Fig. 1g). To selectively test the role of BLA PV interneurons in mediating the suppression of freezing following extinction, we measured freezing responses of mice in a post-extinction retrieval trial following injection of the DREADD ligand clozapine-N-oxide (CNO), which silenced BLA PV interneurons. We found that CNO injection caused significantly more freezing in the conditioned context as compared to vehicle injection (Fig. 1d and Supplementary Fig. 1h−j). This effect was not observed in an

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unconditioned neutral context, indicating that CNO injection selectively impaired the ability to suppress conditioned freezing, rather than causing an overall increase in unconditioned fear (Fig. 1d). We reasoned that perhaps extinction learning confers on the BLA PV interneuron network a specific function in fear suppression, which would be consistent with our previous structural plasticity findings. Indeed, we found that silencing of PV interneurons in the BLA resulted in increased reactivation of the FC-tagged GFP+ neurons (Fig. 1f) and quantified BLA ensembles associated with conditioned fear memories. We found that silencing of PV interneurons in the BLA resulted in increased reactivation of the FC-tagged GFP+ neurons (Fig. 1f) and quantified BLA ensembles associated with conditioned fear memories. We found that silencing of PV interneurons in the BLA resulted in increased reactivation of the FC-tagged GFP+ neurons (Fig. 1f) and quantified BLA ensembles associated with conditioned fear memories. We found that silencing of PV interneurons in the BLA resulted in increased reactivation of the FC-tagged GFP+ neurons (Fig. 1f) and quantified BLA ensembles associated with conditioned fear memories.

Figure 1 | BLA PV interneurons selectively suppress conditioned fear behavior and neuronal ensembles following extinction. (a) Bilateral infusion of AAV-Syn-DIO-hM4Di-mCherry into the BLA of PV-Cre:TetTag mice was used to selectively express DREADD receptors in BLA PV interneurons. (b) Mice were subjected to contextual fear conditioning, extinction and retrieval. Cells active during the no-doxycycline period are tagged with long-lasting H2B-GFP expression as a result of Fos-promoter driven tetracycline transactivator (tTa) expression, which enables tTa protein to bind and activate the tetO-H2B-GFP transgene. This binding is prevented in the presence of doxycycline. Cells active during the retrieval trial express short-lasting ZIF protein that can be detected in brains perfused 90 min later. PV-Cre:TetTag mice were without doxycycline chow during either the fear conditioning trials or the extinction trials, resulting in FC-tagged and EXT-tagged neurons, respectively. For the EXT-tagged experiment, extinction and retrieval was done on days 4–7 instead of days 2–5 to enable clearance of doxycycline on days 3–4. (c) Examples of GFP+ and GFP− nuclei (top and bottom, respectively) and ZIF+ nuclei (right and left, respectively) in the BLA of TetTag mice. Icons above denote the combinatorial expression for each, used hereinafter. (d,e) Silencing BLA PV interneurons results in a selective increase in conditioned fear following extinction. Mice injected with CNO 30 min before retrieval displayed increased freezing in the conditioned context (d left, Wilcoxon matched-pairs: W = 338, P < 0.0001, n = 29 mice), but not in a neutral context that was never paired with footshock (d right, Wilcoxon matched-pairs: W = 9, P = 0.6563, n = 9 mice; blue shading indicates different context). Mice injected with CNO 30 min before a retrieval trial that was not preceded by extinction trials did not show altered freezing levels in the conditioned context (e, Wilcoxon matched-pairs: W = 1, P = 0.9824, n = 12 mice). (f–h) Silencing PV interneurons selectively inhibits the BLA fear ensemble. Injection of CNO 30 min before retrieval leads to an increase in the percentage of ZIF+ cells among the FC-tagged GFP+ neurons (f left, unpaired t-test: t(13) = 2.86, P = 0.0134, vehicle (VEH) n = 7 mice, CNO n = 8 mice). The percentage reactivated GFP+ cells correlated with the behavioral effect of CNO (f right, linear regression: F(1,6) = 23.42, P = 0.0029, n = 8 mice). Injection of CNO had no effect on GFP− neurons that were not tagged during fear conditioning (g, Mann-Whitney test: U = 27, P = 0.9333, VEH n = 7 mice, CNO n = 8 mice) nor on EXT-tagged GFP+ neurons (h, unpaired t-test: t(10) = 0.4142, P = 0.6875, VEH n = 7, CNO n = 5). All box plots show median (line inside box), 25th and 75th percentiles (box edges), and minimum and maximum values (error bars).
their reactivation during PV interneuron silencing. We found that silencing BLA PV interneurons had no effect on reactivation of EXT-tagged neurons, supporting the selective role of PV interneurons in suppressing a BLA fear ensemble (Fig. 1h and Supplementary Fig. 1d). Taken together, these results indicate that BLA PV interneurons selectively suppress conditioned freezing behavior following extinction learning through the selective suppression of a neuronal correlate of the fear memory within the BLA.

A role for PV interneurons in the selective suppression of a single BLA ensemble seems counterintuitive, given that each PV interneuron in the BLA provides synaptic input to as many as 800–900 different BLA projection neurons. Silencing BLA PV interneurons would therefore be expected to disinhibit all projection neurons nonselectively. A potential substrate for this specificity is suggested by our previous finding that extinction learning causes a selective increase in perisomatic PV-positive synapses that are located around silent FC-tagged neurons. If this mechanism indeed contributes to the selective suppression of the conditioned fear ensemble, then PV interneuron silencing would have the largest disinhibiting effect on FC-tagged neurons receiving the largest amount of synaptic input from PV interneurons. To test this prediction, we analyzed the relationship between perisomatic mCherry puncta, which correlated tightly with perisomatic PV puncta, and ZIF expression (Fig. 2a,b and Supplementary Fig. 2a,b). We found that silencing of PV interneurons following CNO injection led to an induction of a positive correlation between these two measures in FC-tagged neurons, demonstrating that FC-tagged neurons receiving the most perisomatic inhibition were the most likely to be disinhibited (Fig. 2c). Combined with the lack of correlation in the vehicle group (Fig. 2c), this suggests that, following extinction, PV interneurons exert a ‘normalizing’ influence on FC-tagged neuron activation by more strongly inhibiting those FC-tagged neurons that would otherwise be the most active during the retrieval trial. Notably, this normalizing function seems specific for FC-tagged neurons, as PV interneuron silencing did not lead to positive correlations between perisomatic mCherry and ZIF in two other groups of BLA projection neurons: those not tagged during fear conditioning, and those tagged during extinction (Fig. 2d,e).

**BLA PV interneurons are necessary for a competing interaction between functionally opposed oscillations following extinction**

Though we observed that silencing PV interneurons preferentially disinhibited FC-tagged versus EXT-tagged neurons (Figs. 1f–h and 2c–e), we did not observe an overall difference in perisomatic mCherry between these two groups of BLA projection neurons (Supplementary Fig. 2c). These data argue against the simple hypothesis that the selective role of PV interneurons in suppressing the BLA fear ensemble is the result of BLA fear neurons as a whole receiving more perisomatic innervation from PV interneurons. We therefore hypothesized that static synaptic differences alone might not fully account for the selective role of BLA PV interneurons in post-extinction fear suppression and that more dynamic circuit processes must also be involved. To test this we decided to record local field potential (LFP) oscillations in the BLA, which were previously shown to correlate with fear memory retrieval. We recorded LFP oscillations in the BLA of freely behaving mice that expressed hM4Di in BLA PV interneurons and that were subjected to the same fear conditioning and extinction protocol that was used for the TetTag experiments (Fig. 3a and Supplementary Fig. 3a,b). We observed two distinct oscillations in the 3–12 Hz frequency band (Fig. 3b,c and Supplementary Figs. 3d and 4). Notably, these two oscillations seemed to respond differently to PV interneuron silencing, with an oscillation in the 3–6 Hz range increasing in power while a different oscillation in the 6–12 Hz range decreased in power (Fig. 3c). We therefore decided to quantify the power of both oscillations across conditioning, extinction and retrieval trials. We found that fear conditioning led to an increase in 3–6 Hz power (Fig. 3d,e), in agreement with a previous report. Post-extinction silencing of PV interneurons also increased the 3–6 Hz power, consistent with a role for this oscillation in producing freezing behavior (Fig. 3i). While fear conditioning had no effect on 6–12 Hz power, silencing PV interneurons significantly reduced the power of this band (Fig. 3j). We therefore reasoned that perhaps the balance of these two oscillations determined the functional output of the BLA following extinction. Consistent with this notion, the power ratio of the two bands (3–6 Hz/6–12 Hz) correlated with the amount of freezing across and within mice following extinction (Fig. 3k and Supplementary Fig. 3e,f).
This correlation could merely reflect changes in oscillations whenever mice are involved in different behaviors (freezing versus non-freezing) without the oscillations actually contributing to behavior. Arguing against this, we found that restricting the analysis of the 3–6 Hz/6–12 Hz ratio to pure freezing bouts still revealed a CNO-induced increase in the 3–6 Hz/6–12 Hz ratio (Supplementary

**Figure 3** BLA PV interneurons control the balance between two functionally opposed low-frequency oscillations. (a) Experimental design: mice were infused bilaterally with AAV-Syn-DIO-hM4Di-mCherry and simultaneously implanted with recording electrodes in BLA. Mice were then subjected to contextual fear conditioning, extinction and retrieval. (b) Example of full trial spectrograms from a single animal demonstrating a 3–6 Hz oscillation during freezing (red boxes: periods of >50% freezing per bin), as well as a shift toward increased 3–6 Hz power compared to 6–12 Hz power caused by silencing BLA PV interneurons. (c) Averaged normalized 2–12 Hz power spectra from CNO and VEH retrieval trials. Inset: running difference between power spectra from CNO trials and VEH trials, calculated by subtracting averaged VEH trial spectra from CNO trial spectra (n = 11; red vertical lines mark 3–6 Hz and 6–12 Hz intervals used for quantification; shaded bands mark s.e.m.). (d-g) Fear conditioning leads to increased freezing in the conditioned context (d, Wilcoxon matched-pairs: W = 64, P = 0.0020, n = 11 mice), increased 3–6 Hz power (e, paired t-test: t(10) = 3.481, P = 0.0059, n = 11 mice), no change in 6–12 Hz power (f, paired t-test: t(10) = 0.3747, P = 0.7157, n = 11 mice), and a trend toward an increased 3–6 Hz/6–12 Hz power ratio (g left, paired t-test: t(10) = 2.074, P = 0.0648). The 3–6 Hz/6–12 Hz power ratio does not correlate with freezing before (g right Pre, linear regression: F(1,9) = 2.299, P = 0.1638, n = 11 mice) or after (g right Post, linear regression: F(1,9) = 0.003989, P = 0.9510, n = 11 mice) fear conditioning. (h–k) Silencing PV interneurons during post-extinction retrieval leads to increased freezing in the conditioned context (h, paired t-test: t(10) = 2.867, P = 0.0167, n = 11 mice), increased 3–6 Hz power (i, Wilcoxon matched-pairs: W = 56, P = 0.0098, n = 11 mice), decreased 6–12 Hz power (j, paired t-test: t(10) = 2.427, P = 0.0356, n = 11 mice) and an increased 3–6 Hz/6–12 Hz power ratio (k left, Wilcoxon matched-pairs: W = 66, P = 0.0010, n = 11 mice). The 3–6 Hz/6–12 Hz power ratio correlates with freezing in CNO-injected mice (k right CNO, linear regression: F(1,9) = 7.423, P = 0.0234, n = 11 mice), but not in VEH-injected mice (k right VEH, linear regression: F(1,9) = 0.3594, P = 0.5636, n = 11 mice). All box plots show median (line inside box), 25th and 75th percentiles (box edges), and minimum and maximum values (error bars).
modulation of mPFC–BLA reciprocal circuits. To determine whether control over the activity of ensembles within BLA, PV interneurons following extinction is potentially critical to their function in regulating fear behavior following extinction.

Since we observed that the power of the 3–6 Hz and 6–12 Hz oscillations shifted in opposite directions following CNO injection, we decided to examine a potential dynamic interaction between these two oscillations by cross-correlating their instantaneous power envelopes. We found that after extinction, but not before, the two sub-bands exhibited a significant negative correlation with one another, implying that extinction learning results in changes to the local circuitry that allow the two oscillations to compete with each other (Fig. 4a,c,d). Given our previous findings implicating structural plasticity of PV interneurons in extinction learning\(^7\), as well as the necessity for PV interneuron activity in fear suppression following, but not before, extinction learning (Fig. 1d,e), we reasoned that perhaps PV interneurons played an integral role in the competing interaction between these two oscillations. In support of this, we found that silencing BLA PV interneurons following extinction eliminated the negative correlation between the 3–6 Hz and 6–12 Hz oscillations, indicating that the mechanism segregating them is critically dependent on PV interneuron activity (Fig. 4b–d). We did not detect a relationship between 6–12 Hz power and the 3–6 Hz : 6–12 Hz cross-correlation values (Supplementary Fig. 5), making it unlikely that the elimination of the negative correlation following CNO injection was an artifact of reduced 6–12 Hz power (Fig. 3j). Taken together, these data imply that the post-extinction state is characterized by 6–12 Hz oscillations that interact with fear-associated 3–6 Hz oscillations through a mechanism dependent on BLA PV interneurons.

BLA PV interneurons gate information flow through reciprocal BLA–mPFC circuits following extinction

One possible explanation for the selective role of BLA PV interneurons following extinction would be that the local PV network is driven in part by upstream regions that relay learning-specific information critical to the suppression of fear. To identify inputs that might instruct the post-extinction state of the PV network, we used Cre-dependent G (glycoprotein)-deleted rabies to specifically label monosynaptic inputs onto BLA PV interneurons\(^1,9\). This revealed dense input from several brain regions, including two regions in the medial prefrontal cortex (mPFC): the infralimbic cortex (ILC) and the prelimbic cortex (PLC). The ILC, a region associated with extinction learning, appeared to contain more projection neurons that directly innervate BLA PV interneurons than does the PLC, a region associated with fear learning and behavior (Fig. 5a)\(^1,11\). To further investigate the mPFC–BLA circuitry, we used Cre-dependent rabies to selectively label monosynaptic inputs onto neurons that project from BLA to mPFC. Notably, we observed mPFC projection neurons that synapse onto BLA–mPFC projecting neurons, thereby outlining a reciprocal loop between mPFC and BLA (Fig. 5b). Finally, we found that BLA→mPFC projecting neurons innervated both the PLC and ILC, with preferential targeting of superficial PLC and deep ILC (Fig. 5c). PV interneurons are therefore synaptically positioned to gate information flow across a functionally relevant BLA–mPFC circuit, a feature potentially critical to their function in regulating fear behavior following extinction.

The observed circuit design implies that, in addition to exerting control over the activity of ensembles within BLA, PV interneurons may also affect the activity of mPFC ensembles through dynamic modulation of mPFC–BLA reciprocal circuits. To determine whether BLA PV interneurons contribute to post-extinction circuit dynamics within the mPFC, we examined the effect of silencing BLA PV interneurons on LFP oscillatory activity in the mPFC using the same mice as used for the BLA analysis in Figure 3 (Fig. 6a and Supplementary Fig. 3a). Silencing BLA PV interneurons increased the 3–6 Hz/6–12 Hz power ratio in mPFC (Fig. 6b–d). The changes in
the mPFC broadly mirrored the observed effects in BLA (Fig. 3i–k), indicating that BLA output to mPFC is regulated by PV interneurons. If this is correct, then BLA PV interneuron silencing should lead to changes in ensemble activation within the mPFC. To test this, we used the same TetTag mice as used for the BLA analysis in Figure 1 (Fig. 6a). We found that silencing BLA PV interneurons, thereby disinhibiting BLA fear neurons (Fig. 1f), increased the activation of TetTagged GFP+ neurons in superficial, but not deep, PLC (Fig. 6e and Supplementary Fig. 1e). The reactivation of these PLC neurons correlated with freezing, indicating that the BLA and superficial PLC can cooperate to produce freezing behavior after extinction (Fig. 6f).

In agreement with this, we found that superficial PLC neurons tended to be TetTagged in fear-conditioned mice (Fig. 6g). In contrast to PLC, we found that silencing BLA PV interneurons resulted in a selective decrease in the activity of TetTagged GFP+ neurons in the deep ILC (Fig. 6h and Supplementary Fig. 1f). Furthermore, we found that neurons in deep ILC were preferentially TetTagged in mice placed in a novel environment without footshock, as compared to mice left in their home cages or mice that were fear conditioned, consistent with a putative role for deep ILC neurons in learned safety (Fig. 6i).

Figure 5 BLA PV interneurons participate in a reciprocal BLA–mPFC circuit. (a) Left: to trace monosynaptic inputs on BLA PV interneurons, helper virus (AAV9-Ef1α-FLEX-CTB) and mCherry-expressing rabies virus were injected into the BLA of PV-Cre mice. The example image shows mCherry-positive neurons in mPFC that synapse onto BLA PV interneurons, with the graph showing that a larger fraction of mCherry-positive neurons was located in the ILC than PLC (paired t-test: t(7) = 2.8, P = 0.0265, n = 8 mice). Right: to trace all inputs to the BLA, fluorescently labeled cholera toxin B (CTB) was injected into the BLA of wild-type mice. The example image shows CTB-positive neurons in the mPFC that project to the BLA, with the graph showing similar fractions of CTB-positive neurons located in the PLC and ILC (paired t-test: t(2) = 2.463, P = 0.1328, n = 3 mice). (b) To selectively label monosynaptic inputs onto BLA→mPFC projecting neurons, retrograde AAV9-Cre-GFP was injected into mPFC, and helper virus and mCherry-expressing rabies virus injected into BLA 3 weeks later. Bottom left: example image of injection site of mCherry-expressing rabies virus (red) in the BLA and green nuclei from retrograde AAV9-Cre-GFP virus injected into mPFC. Right: example image demonstrating mCherry-positive neurons in mPFC that synapse onto BLA→mPFC neurons injected into BLA 3 weeks later. Bottom left: example image of injection site of mCherry-expressing rabies virus (red) in the BLA and green nuclei from retrograde AAV9-Cre-GFP virus injected into mPFC. Right: example image demonstrating mCherry-positive neurons in mPFC that synapse onto BLA→mPFC projecting neurons. (c) To label BLA→mPFC projections, AAV-Cre-GFP was injected into mPFC and AAV-Syn-DIO-hM4Di-mCherry was injected into BLA. Right and bottom left: representative image and quantification showing dense innervation of superficial PL layers (L2–3) and deep IL layers (L5–6) by BLA projection neurons (n = 4; shaded bands mark s.e.m.). All box plots show median (line inside box), 25th and 75th percentiles (box edges; right graph in a has no box edges, as it represents n = 3), and minimum and maximum values (error bars).
These data support the notion that TetTagged neurons in the PLC and ILC play functionally distinct roles in the regulation of fear following extinction. Furthermore, these findings indicate that silencing PV interneurons in the BLA, thereby disinhibiting BLA fear neurons, might shift mPFC oscillatory activity through opposing changes in the activation of functionally distinct ensembles in the PLC and ILC.

Our tracing and functional data thus far point to a critical role for BLA PV interneurons in gating information flow across the BLA–mPFC circuit to regulate the expression of fear following extinction. In further support of this, we found that silencing BLA PV interneurons following extinction shifted BLA–mPFC coherence, a measure of inter-regional communication, toward the 3–6 Hz range (Fig. 7a).

To examine whether changes in BLA oscillations can have a direct impact on mPFC oscillations, we assessed directionality across the BLA–mPFC circuit using a previously described method of cross-correlating instantaneous power envelopes. We found that freezing after fear conditioning, but not after extinction, tended to be accompanied by increased mPFC→BLA directionality in the 3–6 Hz band (Supplementary Fig. 3h,i), in agreement with a previous report. In contrast, we found that post-extinction freezing was characterized by a shift toward BLA→mPFC directionality in the 3–6 Hz band (Fig. 7c,d and Supplementary Fig. 6), implying that although the circuit converges on a similar oscillatory state as assessed by a high 3–6 Hz/6–12 Hz power ratio, the pre- and post-extinction freezing states may arise from different circuit conditions. Further, we found that silencing BLA PV interneurons increased the likelihood of BLA→mPFC directionality in the 3–6 Hz band but reduced the likelihood of BLA→mPFC directionality in the 6–12 Hz band, further supporting the idea that these two oscillations have distinct functions in the post-extinction state. These data indicate that BLA PV interneurons route directional information transfer across BLA–mPFC circuits that are involved in the control of post-extinction fear expression.

DISCUSSION

Taken together, our data support a model whereby fear extinction learning induces remodeling of the BLA PV interneuron network to allow competition between an extinction memory circuit and a fear memory circuit. This competition is represented in the divergent behavior of FC-tagged and EXT-tagged neurons within BLA, as well as in the negative correlation between the 3–6 Hz and 6–12 Hz oscillations. Following extinction, this competition causes suppression of BLA fear neurons and suppression of a fear-associated 3–6 Hz oscillation. Silencing BLA PV interneurons after extinction disallows competition between the two circuits, thereby increasing the activation of the BLA fear neurons and the fear-associated 3–6 Hz oscillation, which is then signaled to the mPFC (Fig. 7f). This model has several implications.
Figure 7 BLA PV interneurons control directionality of 3–6 and 6–12 Hz oscillations following extinction. (a) Silencing BLA PV interneurons during post-extinction retrieval shifts BLA–mPFC peak coherence toward the 3–6 Hz range (paired t-test: \( t(8) = 2.486, P = 0.0378, n = 9 \) mice; shaded bands mark s.e.m.). (b) Example of a 6–12 Hz filtered trace demonstrating the extraction of instantaneous power envelopes (top), which can be cross-correlated (bottom) to determine leading (black arrows) or lagging (red arrows) relationships between two signals. (c) Averaged lags from instantaneous power cross-correlations for both 3–6 Hz and 6–12 Hz bands during the 5 s preceding and the 5 s following the onset of freezing during retrieval trials (VEH \( n = 6 \) mice, CNO \( n = 8 \) mice, 1 freezing event per mouse per trial; shaded bands mark s.e.m.). (d) The first 5 s following the onset of freezing is characterized by a shift toward a BLA lead for 3–6 Hz in both VEH and CNO groups (repeated measures two-way ANOVA: freezing \( F(1,11) = 29.12, P = 0.0002, \) CNO \( F(1,11) = 1.013, P = 0.3357, \) freezing \( \times \) CNO \( F(1,11) = 1.411, P = 0.2598, \) VEH \( n = 5 \) mice, CNO \( n = 8 \) mice; Sidak’s multiple comparisons test: pre-freeze vs. freeze for VEH \( t(11) = 2.682, P = 0.0422; \) Sidak’s multiple comparisons test: pre-freeze vs. freeze for CNO \( t(11) = 5.308, P = 0.0005 \) and a shift toward a mPFC lead for 6–12 Hz in the CNO group (repeated measures two-way ANOVA: freezing \( F(1,9) = 5.487, P = 0.0439, \) CNO \( F(1,9) = 2.263, P = 0.1668, \) freezing \( \times \) CNO \( F(1,9) = 2.355, P = 0.1592, \) VEH \( n = 4 \) mice, CNO \( n = 7 \) mice; Sidak’s multiple comparisons test: pre-freeze vs. freeze for VEH \( t(9) = 0.5063, P = 0.8953; \) Sidak’s multiple comparisons test: pre-freeze vs. freeze for CNO \( t(9) = 3.215, P = 0.0210. \) (e) Silencing PV interneurons during post-extinction retrieval increased the probability of BLA leading mPFC in the 3–6 Hz band (paired t-test, \( t(9) = 2.303, P = 0.0468, n = 10 \) mice) while decreasing the probability that it will lead in the 6–12 Hz band (Wilcoxon matched-pairs: \( W = 45, P = 0.0039, n = 9 \) mice). (f) Model based on our data and previous studies. Top: after extinction learning, BLA PV interneurons suppress conditioned freezing and fear ensemble activation by enabling a 6–12 Hz oscillation to outcompete a 3–6 Hz oscillation throughout the BLA–mPFC circuit. Bottom: chemogenetic silencing of BLA PV interneurons causes dysfunctional competition between the 6–12 and 3–6 Hz oscillations, which leads to an increase in 3–6 Hz power and BLA–mPFC directionality, an increase in the activation of fear ensembles in BLA and PLC, and an increase in conditioned freezing. All box plots show median (line inside box), 25th and 75th percentiles (box edges), and minimum and maximum values (error bars).

First, our findings reveal that a local PV interneuron network can mediate a direct interaction between a new extinction memory and a previous fear memory, which are represented by distinct network states. Recent studies have demonstrated that PV networks can adopt multiple learning-dependent network states that can modulate synaptic plasticity and circuit output7,16–18. Here we report that extinction learning confers on PV interneurons in the BLA a selective role in the suppression of conditioned fear behavior and in the silencing of a neuronal fear memory correlate within the BLA. Our findings are consistent with our previous discovery of an extinction-induced, target-specific structural plasticity of BLA PV interneurons that likely results in increased perisomatic inhibition of FC-tagged neurons7. Target-specific changes in synapse numbers and/or strength are likely to alter the balance of local ‘winner-take-all’ competitive inhibitory networks, thereby biasing the control of circuit output in favor of a select population of pyramidal neurons at the expense of others19,20. In agreement with a role for PV interneurons in mediating this competition, silencing PV interneurons during extinction retrieval altered the activity of BLA fear neurons, but not of other functional ensembles within BLA. The ability of BLA PV interneurons to dynamically regulate the activation of BLA projection neurons may be supported by their recruitment through both feedback input from BLA projection neurons and feedforward input from projection neurons outside the BLA, including mPFC and ventral hippocampus21,22. Divergent feedback input from BLA PV interneurons might contribute to lateral inhibition between anatomically and functionally distinct populations of BLA projection neurons, as was recently observed23. In addition, our tracing data indicate that BLA PV interneurons in mediating this competition, silencing PV interneurons during extinction retrieval altered the activity of BLA fear neurons, but not of other functional ensembles within BLA. The ability of BLA PV interneurons to dynamically regulate the activation of BLA projection neurons may be supported by their recruitment through both feedback input from BLA projection neurons and feedforward input from projection neurons outside the BLA, including mPFC and ventral hippocampus21,22. Divergent feedback input from BLA PV interneurons might contribute to lateral inhibition between anatomically and functionally distinct populations of BLA projection neurons, as was recently observed23. In addition, our tracing data indicate that BLA PV interneurons participate in reciprocal connections between mPFC and BLA24,25. Therefore, BLA PV interneurons could indirectly modulate their own activity via engagement of reciprocal connections with mPFC and feedforward inhibition. It remains to be determined what effect the BLA–mPFC reciprocal connections have on BLA circuit dynamics and...
whether input from mPFC is necessary to confer on the BLA PV network its post-extinction fear-suppressing function. Also, it will be of interest to investigate how the role of the BLA PV network in post-extinction fear suppression might complement other inhibitory circuits that have been implicated in post-extinction fear suppression, such as the intercalated neurons, and whether the involvement of the BLA PV network in post-extinction fear suppression is similar for contextual and cued fear.

Second, we propose that while the 3–6 Hz oscillation in the BLA–mPFC circuit consistently correlates with freezing behavior, the valence of the 6–12 Hz oscillation, and indeed its relationship with the 3–6 Hz oscillation, is dependent on previous learning. The 6–12 Hz oscillation resembles classic hippocampal theta, and hippocampal activity can contribute to both fear expression and fear suppression, indicating a lack of predefined valence. Accordingly, hippocampal neurons active during fear learning could be manipulated to change their valence through additional learning, while similar neurons in BLA could not. A recent study demonstrated that direct ventral hippocampus (VH) input to BLA is required for the expression of contextual fear, but a role for this pathway in extinction learning remains to be determined. Since VH→BLA projections directly innervate BLA PV interneurons, and can thereby modulate synaptic plasticity, it is possible that in the behaving animal VH→BLA projections can have opposing effects on amygdalar activity and behavioral output depending on previous learning and the state of the BLA PV interneuron network. It should be noted that the VH can also regulate the BLA microcircuit indirectly through projections to mPFC neurons that project to the BLA. Further studies will be necessary to determine the origin and precise function of the 6–12 Hz oscillation, as well as how this function is dependent on the BLA PV network.

Third, the increased reactivation of fear neurons within BLA coincided with a shift toward 3–6 Hz oscillations, suggesting a causal relationship between the 3–6 Hz oscillation and BLA fear neuron activation. One possible explanation would be that BLA fear neurons act as resonators that are preferentially tuned to the 3–6 Hz frequency band; conversely, they could actively participate in the production of the 3–6 Hz oscillation through engagement of local circuit mechanisms. While BLA does not appear to generate low-frequency oscillations in slice preparations, previous studies have found that projection neurons within the BLA have resonance properties aligned to the 3–6 Hz range. An interesting avenue for future studies would be to explore whether a learning experience can modulate intrinsic resonance properties of select neuronal ensembles, thereby entraining them to a particular functionally relevant frequency band. If correct, then the artificial induction of a 4 Hz or 8 Hz oscillation in BLA following fear conditioning and extinction might lead to the selective recruitment of fear or extinction neurons, respectively. Such an outcome would be consistent with the notion that the 3–12 Hz LFP oscillations recorded in the BLA reflect oscillatory activity of BLA neurons. However, pending either confirmation that BLA neurons are phase-locked to 3–12 Hz oscillations or gain-of-function manipulations of 3–12 Hz oscillatory activity within the BLA, we cannot rule out the possibility that the observed 3–12 Hz oscillations in the BLA resulted from volume conduction of oscillatory activity that occurred outside of the BLA.

Fourth, the observed relationship between LFP oscillations and freezing suggest that these oscillations contribute to freezing. Indeed, artificial induction of a 4 Hz oscillation in dorsal mPFC was found to be sufficient to synchronize mPFC single units and induce freezing in mice. It should be noted that although our correlational data suggest that, like the mPFC 4 Hz oscillation, a 3–6 Hz oscillation in the BLA could cause freezing, this remains a hypothesis that will have to be confirmed in future studies that employ frequency-specific manipulations of BLA activity. Until then, we cannot exclude the possibility that at least some of the observed oscillatory changes in our study resulted from changes in behavior, instead of the other way around.

Finally, our findings indicate that a state of fear that re-emerges after extinction can be distinct from the state of fear before extinction. In contrast to the pre-extinction state, in which mPFC activity can drive BLA during fear behavior, our findings indicate that the return of extinguished fear can be caused by a signal from the BLA to the mPFC. Based on our data, we propose that this BLA→mPFC signal shifts mPFC activity toward a pro-fear state by causing opposite changes in the activation state of PLC and ILC ensembles that are associated with promoting and suppressing fear, respectively. Accordingly, recent studies reported a role for BLA→mPFC projections in the retention and extinction of fear memories, and reported the occurrence of a BLA→PLC signal during fear states that are caused by conflicting cues. The conditioned stimulus in our behavioral protocol (i.e., context) could be considered a conflicting cue, as it was previously associated with both danger (fear conditioning) and safety (extinction). Our observation of a BLA→mPFC signal during post-extinction fear is also consistent with previous reports of latent fear circuits in the human amygdala that can contribute to the return of fear. A better mechanistic understanding of the conditions that lead to the return of fear behavior following extinction, such as during spontaneous recovery, would greatly aid the development of better treatments for anxiety disorders such as post-traumatic stress disorder. Our findings suggest that a therapy or manipulation that reduces the likelihood of BLA fear neuron reactivation, 3–6 Hz oscillatory activity and/or BLA→mPFC engagement could be beneficial for preventing the return of fear following extinction learning.

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.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

P.D., J.M., and L.G.R. conceived and designed the experiments. P.D. and Y.Z. executed the experiments. P.D., Y.Z., and L.G.R. analyzed the experiments. P.D. and L.G.R. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals. All animal procedures were performed in accordance with the NIH Health Guide for the Care and Use of Laboratory Animals and were approved by the Tufts University Institutional Animal Care and Use Committee. The TetTag mice used in this study were heterozygous for two transgenes: Fos promoter–driven tetracycline transactivator (Fos-td) and a tetr operator–driven fusion of histone2B and eGFP (tetO-H2B-GFP). PV-Cre and PV-Cre:TetTag mice used in this study were heterozygous for a PV-ires-Cre knock-in locus (B6; 129p2-Pvatalm(cre)/Arbr)). Mice had food and water ad libitum and were socially housed until the start of behavioral experiments, which was at an age of at least 12 weeks. Mice were kept on regular light-dark cycle, and all experiments were performed during the light phase.

Stereotoxic surgery. Mice were anesthetized with isoflurane, held in a stereotoxic apparatus (Kopf) and injected with virus. After injection, the needle was left in place for 10 min before slowly being retracted. The incision was sutured, and mice were weighed and monitored to ensure recovery. For DREADD experiments, stereotaxic surgery.

During the light phase.

Mice were kept on regular light-dark cycle, and all experiments were performed.

Behavior. PV-Cre and PV-Cre:TetTag mice 3–6 months old were used for the study. None of the mice had prior procedures or testing performed. Mice were randomly assigned to groups, except for ensuring an equal distribution of females and males across groups. Method of randomization was based on arbitrary numbers that were assigned at the time of weaning. The design of experiment 1 is summarized in Figure 1b. Mice were subjected to contextual fear conditioning consisting of three training trials (FC1, FC2, and FC3) with 3 h between each trial. The total duration of each training trial was 500 s. A training trial started with placing the mouse in a square chamber with grid floor (context A) (Coulbourn Instruments; H10-11RTC). At 198 s, 278 s, 358 s and 438 s, a foot shock was delivered (2 s, 0.70 mA). On days 2 and 3 (or 4 and 5 for the EXT-tagged group), mice were subjected to four extinction trials per day. Each extinction trial lasted 1,200 s, with an inter-trial interval of 2 h. For each extinction trial, mice were placed in the same box used for fear conditioning without receiving foot shocks. On days 4 and 5, mice were tested over 500 s during a single retrieval test in context A. A subset of mice in experiment 1 also were tested in a neutral context without footshock, which consisted of a square plastic box with bedding sprayed with 10% acetic acid and striped walls. CNO/VEH mice received an i.p. injection of 8–10 mg/kg clozapine-N-oxide (CNO) on day 4 and of vehicle (VEH; 5% DMSO in saline) on day 5. VEH/CNO received injections in the reverse order. CNO/VEH and VEH/CNO mice were counterbalanced within each experiment. All TetTag mice were perfused 90 min after the final retrieval trial (day 5 for FC-tagged, day 7 for EXT-tagged).

For labeling of activated cells, TetTag mice were raised on food with doxycycline (40 mg doxycycline/kg chow). For the FC-tagged group, 4 d before conditioning, mice were individually housed and doxycycline was removed from the food. After the last fear conditioning trial on day 1, mice were put on food with a high dose of doxycycline (1 g/kg) to rapidly block the tagging of neurons activated after fear conditioning. On day 2 mice were put back on the regular dose of doxycycline (40 mg/kg). For the EXT-tagged group, mice were conditioned while activated after fear conditioning. On day 2 mice were put back on the regular dose of doxycycline (40 mg/kg chow). For the FC-tagged group, 4 d before conditioning.

Tissue preparation and immunohistochemistry. Ninety minutes after retrieval, mice were deeply anesthetized with ketamine/xylazine and intracardially perfused with 0.1 M phosphate buffer (PB) followed by 4% paraformaldehyde (PFA 4%) dissolved in 0.1 M PB. Brains were extracted and post-fixed in PFA 4% for 24 h.

Brains were transferred to 30% sucrose for 48–72 h before slicing 40-μm (for hM4D)-mCherry localization and Nissl staining) or 25-μm (for immunofluorescence) coronal sections of the entire brain using a cryostat. Sections were stored in phosphate-buffered saline (PBS) with 0.025% sodium azide at 4 °C until use.

For immunofluorescence staining, sections were blocked for 1 h at room temperature in PBS-T (PBS with 0.25% Triton X-100) with 8% normal goat serum. Sections were incubated in rabbit anti-Zif268 (Santa-Cruz sc-189; polyclonal; 1:3,000) with mouse anti-PV (Millipore MAB1572; monoclonal; 1:2,000), or rabbit anti-RFP (Rockland 600–401–379; polyclonal; 1:1,500) at 4 °C for 48–72 h. Secondary antibodies (Jackson ImmunoResearch 111–505-144, goat anti-rabbit 549, 1:1,500; Jackson ImmunoResearch 115–605-146, goat anti-mouse 647, 1:500) were diluted in the blocking solution and were then applied to the sections for 2 h at room temperature, followed by three rinses for 15 min in PBS-T. Sections were mounted on slides and coverslipped, after a brief wash with 0.00005% DAPI in PBS-T to label cell nuclei, and stored at 4 °C.

Microscopy. A wide-field epifluorescence microscope (Keyence BZ-X700) was used to acquire images for electrode and injection site validation, and for TetTag data excluding perisomatic mCherry analysis. Images were obtained at 10–20× and stitched together using Keyence software. Acquisition settings were optimized for each brain region and were identical across groups. A minimum of 8 total amygdala or 6 other brain region sections (4 or 3 bilateral) per animal was analyzed, after excluding sections for quality reasons. Sections including damage from the injection site were excluded. A confocal laser-scanning microscope (Nikon A1R or Leica SPE) was used to acquire images for mCherry/PV overlap analysis and mCherry–ZIF correlation analysis. The settings for PMT, laser power, gain and offset were identical between experimental groups.

For mCherry/PV overlap, 20× z-stacks were acquired and the maximum intensity projection was used for analysis. For mCherry-ZIF correlation analysis, 20× images were used. A minimum of 4 sections containing the amygdala per animal was used for this analysis.

Quantification of activated cells. ImageJ software was used to select and count the total number of DAPI-, GFP- and ZIF-positive nuclei and nuclei double-positive for GFP and ZIF. To avoid bias, all three cell types (GFP+ZIF+, GFP+ZIF− and GFP-ZIF+) were selected from the same pictures, and the threshold settings for GFP and ZIF were identical across all mice.

Perisomatic analysis. Selection of GFP-labeled cells for confocal perisomatic analysis was designed to only include excitatory neurons as described previously. mCherry signal was distinguished from nuclear ZIF using the ImageJ image calculator function and DAPI as a mask for nuclear signal. Absolute values of fluorescence were calculated for each individual neuron in an unbiased way without knowledge of GFP fluorescence or experimental group. Values were then normalized within each section to reduce noise from imaging and tissue processing and to allow comparison across animals.

Electrophysiology. All LFP data were acquired using prefabricated headmounts (Pinnacle 8201) for 2EEG/1EMG recordings. The headmounts were affixed to the

Quantification of freezing. Freezing behavior was measured using a digital camera connected to a computer with Actimetrics FreezeFrame software. The minimum bout length was 1.5 s, and the threshold for freezing behavior was set before the onset of the experiment and was the same for all subsequent trials for each animal. Freezing scores were obtained by averaging freezing during the entire trial, unless otherwise indicated.
skull with stainless steel screws that also act as EEG reference and ground electrodes. Stainless steel wires served as the EEG electrodes, which were placed into the BLA and mPFC (BLA AP −1.35 mm, ML ±3.45 mm, DV −5.15 mm; mPFC AP +0.75, ML ±0.3 mm, DV −2.1 mm). Local field potentials were recorded using a 100× preamplifier (Pinnacle 8202-SE) at a sampling rate of 4 kHz. The data were analyzed and filtered offline using LabChart software.

All power spectra quantification data were generated using LabChart’s DataPad function, using the first 4 min of each behavioral trial. Normalized power and 3–6 Hz/6–12 Hz ratio (Fig. 3 and Supplementary Fig. 3) were calculated by subtracting the power or power ratio value for the first 2 min of the first conditioning trial, during which the animal is acclimating to the context, from the ratio for the CNO or VEH retrieval trials.

Directionality was calculated using a modified process as described by others15. In short, instantaneous amplitudes of filtered traces were generated in LabChart. Files were then exported into ClampFit and cross-correlated after being segmented into 20-s bins, and statistics were calculated using the population distribution of lags per individual trial (based on the first 4 min of each trial). For finer temporal scale directionality analysis, periods of at least 10 s of motion followed by at least 10 s of freezing (one per animal per trial) were selected to maximize signal to noise. The onset of freezing was demarcated at 0 s and directionality analysis was performed from −5 to 5 s in 1 s bins with 0.5 s steps. Lags were then averaged across animals on a per-second basis for visualization, or, for quantification, for the 5 s before freezing and the 5 s after the onset of freezing. For pure freezing analysis, separate files were created in LabChart collating all periods of pure freezing or non-freezing for a given trial using second-by-second freezing data from FreezeView. These files were then subsequently used for analysis as indicated.

Sub-band cross-correlation analysis was performed in ClampFit by cross-correlating instantaneous amplitudes of 3–6 Hz and 6–12 Hz bands over a 2-min window at beginning of the trial and for each trial per animal. Statistics were performed using the area under the curve of the cross-correlogram. Onset of freezing analysis was performed using 1-s bins from the same freezing onset epochs as described above. Spectrograms and coherence data were generated using the EEGLAB plugin in MATLAB (spectrograms: three cycle wavelets with a Hanning taper window; coherence: pop_crossf function)50. Quantification of peak coherence was performed using the first 4 min of each trial, exporting coherence curves to GraphPad Prism for analysis.

For Granger causality analysis, linear detrending and normalization were performed on the LFP signals before analysis, and the SIFT toolbox in EEGLAB was used to fit a higher order vector autoregressive model to the processes. Data were tested for stability in time and model order was determined using the Akaike information criterion. The SIFT toolbox in EEGLAB was used to calculate normalized directed transfer function (nnDTF) values for BLA→mPFC and mPFC→BLA using the first 2 min of retrieval trials (for CNO versus vehicle comparison) or using 10-s bins of periods of high or low freezing (for freezing versus no-freezing comparison).

**Exclusion of mice from analysis.** Mice were excluded from analysis if they did not show effective fear conditioning (both FC3 and EXT1 freezing less than 35%, or EXT1 freezing less than 60% of FC3 freezing; n = 3 TetTag mice excluded, n = 0 LFP mice excluded) or effective fear extinction (EXT8 freezing more than 50% of EXT1 freezing; n = 3 TetTag mice excluded, n = 0 LFP mice excluded), or if AAV-Syn-DIO-hm4Di-mCherry infusion was not targeted to the BLA (n = 4 TetTag mice excluded, n = 4 LFP mice excluded). TetTag mice were excluded from ensemble activation analysis if the average number of GFP+ cells in the each BLA section was fewer than 10 (n = 2 TetTag mice excluded). Mice were excluded from LFP analysis if electrode placement was outside of the BLA (n = 3 LFP mice excluded). This left a total of 11 mice for LFP analysis, of which 10 mice also had correct mPFC placement. Two out of the 11 mice with correct BLA electrode placement were excluded from the Post-FC cross-correlation analysis due to insufficient quality of the LFP data (Fig. 4). Of the 10 mice with correct BLA and mPFC electrode placement, 1 mouse had low-frequency noise in the recordings that interfered with the power analysis, but not with the directionality analysis. This mouse was therefore only used for directionality analysis, resulting in an n = 9 for power analysis (Fig. 6) and an n = 10 for directionality analysis (Fig. 7). For directionality analysis around freezing onset, a period of at least 10 s of motion followed by at least 10 s of freezing was used to maximize signal-to-noise ratio, and mice without such an epoch during the retrieval trial were therefore excluded from the analysis. This resulted in a higher exclusion rate for the VEH trials, since VEH-injected mice had lower overall freezing levels (Fig. 7: trials from n = 5 or 6 VEH-injected mice excluded, trials from n = 2 or 3 CNO-injected mice excluded).

**Statistics.** No statistical methods were used to predetermine sample sizes, but sample sizes are similar to those reported in previous publications6,11. Data collection and analysis were not performed blind to the conditions of the experiments. Statistical tests were performed using Prism (GraphPad) and are indicated in the figure legends. All statistical tests were two-tailed. Assumptions about normal distributions were tested using the Shapiro–Wilk normality test, and assumptions about equality of variances were tested using an F-test: normal distribution and equal variance of population values; paired t-test: normal distribution of differences; linear regression: normal distribution of residuals). All box plots show median (line inside box), 25th and 75th percentiles (box edges), and minimum and maximum values (error bars).

**Life Sciences Reporting Summary.** A summary of experimental design and software used is also provided in the Life Sciences Reporting Summary.

**Data availability.** All relevant data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Life Sciences Reporting Summary

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Experimental design

Sample size

Describe how sample size was determined. Sample sizes were based on previous experiments in our lab and published data for each of the assays.

Data exclusions

Describe any data exclusions. See "Exclusion of mice from analysis" in online methods.

Replication

Describe whether the experimental findings were reliably reproduced. All experimental findings were made using groups of mice (for group sizes see figure legends).

Randomization

Describe how samples/organisms/participants were allocated into experimental groups. Mice were randomly assigned to groups, except for ensuring equal distribution of females/males across groups. Method of randomization was based on arbitrary numbers that were assigned at the time of weaning.

Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis. Investigators were not blinded to group allocation during data collection and/or analysis. All data collection and analysis was performed using predefined pipelines without ad-hoc alterations.

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

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).

- 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

7. Software

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

SIFT and EEGLAB plugins in MATLAB, and Actimetrics FreezeFrame software.

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

8. 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, except that some antibodies are only available from a for-profit company.

Antibodies

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

Antibodies used are described in methods, and were validated based on the known cellular and subcellular localization of the epitopes.

Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

b. Describe the method of cell line authentication used.

N/A

Describe the authentication procedures for each cell line used OR declare that none of the cell lines used have been authenticated OR state that no eukaryotic cell lines were used.

c. Report whether the cell lines were tested for mycoplasma contamination.

Confirm that all cell lines tested negative for mycoplasma contamination OR describe the results of the testing for mycoplasma contamination OR declare that the cell lines were not tested for mycoplasma contamination OR state that no eukaryotic cell lines were used.

Provide a rationale for the use of commonly misidentified cell lines OR state that no commonly misidentified cell lines were used.

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.

Animals and human research participants

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

Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Mice were used (see methods for details).

Policy information about studies involving human research participants

Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

N/A