Manipulating fear associations via optogenetic modulation of amygdala inputs to prefrontal cortex

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Fear-related disorders are thought to reflect strong and persistent fear memories. The basolateral amygdala (BLA) and the medial prefrontal cortex (mPFC) form strong reciprocal synaptic connections that play a key role in acquisition and extinction of fear memories. While synaptic contacts of BLA cells onto mPFC neurons are likely to play a crucial role in this process, the BLA connects with several additional nuclei within the fear circuit that could relay fear-associated information to the mPFC, and the contribution of direct monosynaptic BLA–mPFC inputs is not yet clear. Here we establish an optogenetic stimulation protocol that induces synaptic depression in BLA–mPFC synapses. In behaving mice, optogenetic high-frequency stimulation of BLA inputs to mPFC interfered with retention of cued associations, attenuated previously acquired cue-associated responses in mPFC neurons and facilitated extinction. Our findings demonstrate the contribution of BLA inputs to mPFC in forming and maintaining cued fear associations.

The amygdala plays an essential role in acquisition, retention and expression of aversive memories\(^1\)\(^-\)\(^3\). A widely supported model suggests that the balance between expression and suppression of learned fear responses is modulated by inputs to the amygdala from two sub-regions of the mPFC: the prelimbic cortex (PL), which supports fear extinction\(^4\)\(^-\)\(^5\). Whereas most studies to date have focused on mPFC projections to the BLA and their role in emotional modulation and extinction learning\(^6\)\(^-\)\(^9\), recent work has demonstrated that the reciprocal pathway, comprised of BLA projections to mPFC neurons, is also important for coding fear-associated cues in the mPFC and further contributes to fear expression\(^1\)\(^,\)\(^4\)\(^,\)\(^1\)\(^0\)\(^-\)\(^1\)\(^3\). These two pathways might therefore contribute to maintaining the delicate balance between fear and safety representations in the mammalian brain.

The mPFC receives strong synaptic inputs from the BLA that preferentially target BLA-projecting neurons within the mPFC\(^1\)\(^4\). Distinct groups of BLA neurons project to the PL and the IL\(^1\)\(^5\), and they presumably carry information that is relevant to fear-learning and anxiety\(^1\)\(^1\)\(^,\)\(^1\)\(^3\)\(^,\)\(^1\)\(^6\). Functionally, lesions in\(^1\)\(^7\) and inactivation\(^1\)\(^3\)\(^,\)\(^1\)\(^6\) of the BLA attenuate fear-associated responses of mPFC neurons, and information at precise temporal resolution across this circuit differentiates successful from unsuccessful learning\(^1\)\(^8\). Moreover, the activity of BLA neurons projecting to the mPFC (BLA–mPFC neurons) shows strong correlations with fear acquisition and extinction\(^1\)\(^3\), and silencing of PL–projecting BLA neurons during extinction learning facilitates long-term extinction memory\(^1\)\(^5\).

However, the mPFC receives synaptic input from several other structures within the fear-learning circuitry, including the ventral hippocampus and the mediodorsal thalamus\(^1\)\(^9\), and these regions are in turn innervated by the BLA\(^2\)\(^0\)\(^,\)\(^2\)\(^1\). It is therefore unclear whether and to what extent the information is transmitted directly from the BLA, or alternatively through such indirect pathways, to support acquisition or extinction of learned fear responses. Here we sought to elucidate the role of this direct monosynaptic connection in learning and extinction of conditioned fear.

To specifically target BLA input to the mPFC, we developed a protocol that allows attenuation of synaptic strength in this projection. Synaptic plasticity can be induced by electrical microstimulation protocols\(^2\)\(^2\)\(^,\)\(^2\)\(^3\), and such manipulations in the BLA–mPFC circuit have been shown to alter learned fear associations\(^2\)\(^4\)\(^,\)\(^2\)\(^5\). However, in pathways that involve reciprocal synaptic projections, electrical microstimulation can activate distal cells in connected regions due to activation of axonal fibers of passage\(^2\)\(^6\). Inhibitory optogenetic tools can indeed be used to silence synaptic terminals with spatial specificity\(^2\)\(^7\)\(^,\)\(^2\)\(^8\), but this approach suffers from several limitations\(^2\)\(^9\), particularly for sustained minutes-long inhibition\(^3\)\(^0\). To overcome these limitations, we developed an optogenetic protocol that induces long-term depression in monosynaptic BLA inputs to mPFC neurons. We showed that this protocol can effectively attenuate the response of mPFC neurons to BLA input both in the acute slice preparation and in awake, freely-moving mice, and we then examined the role of this pathway in the acquisition and extinction of fear associations.

RESULTS

Repetitive optogenetic stimulation of the BLA–mPFC pathway with ChETA\(_{TC}\) induces synaptic depression

We first set out to explore optogenetic protocols that allow specific attenuation of synaptic transmission in this pathway\(^3\)\(^1\)\(^-\)\(^3\)\(^3\). We injected mice with viral vectors encoding the fast channelrhodopsin variant ChETA\(_{TC}\) under a calmodulin-dependent protein kinase (CaMKII\(\alpha\)) promoter (rAAV5-CaMKII\(\alpha\)-ChR2(E123T/T159C)-P2A-eYFP) and identified BLA-derived eYFP-positive axons in the mPFC\(^1\)\(^4\) (Fig. 1a). Using whole-cell current-clamp recordings, we characterized the intrinsic properties of 239 mPFC layer 2/3 neurons and their responses.

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to light-evoked excitation of BLA afferents (Supplementary Fig. 1a). Based on after-hyperpolarization amplitudes and action potential widths, we determined 31 cells to be fast-spiking, putative inhibitory interneurons (FS). The remaining 208 cells clustered around a mean action potential width of 0.977 ± 0.046 ms and 3.5 ± 1.18 mV after-hyperpolarization and were grouped as regular-spiking, putative excitatory cells (RS; Fig. 1b).

Of all recorded neurons, 94 showed optically-evoked excitatory postsynaptic potentials (oEPSPs). Both the responsive and non-responsive populations contained similar fractions of FS cells (11.7% and 13.8% FS cells in the responsive and nonresponsive groups, respectively; Fig. 1b). Average oEPSP amplitudes in FS and RS neurons showed no significant difference in size (n = 8 FS, 3.48 ± 0.11 mV; n = 49 RS, 3.85 ± 0.53 mV; Student's unpaired t-test, P = 0.26, t55 = -0.224; Fig. 1c). Light-evoked EPSPs showed short latencies and low jitter, consistent with monosynaptic transmission. The response jitter was not statistically different between the FS and RS neuronal populations (median, 0.203 ms in RS cells and 0.244 ms in FS cells; Wilcoxon W = 3,505, P = 0.52), while latency to oEPSC showed a trend toward lower latency in the FS population (median, 3.85 ms in RS cells and 3.28 ms in FS cells; Wilcoxon test, W = 3,680, P = 0.106; Fig. 1d), reflecting a synaptic mechanism of feedforward inhibition in the BLA–mPFC projection.

We next probed the response of BLA–mPFC afferents to optogenetic high-frequency stimulation (oHFS) protocols. Baseline responses were recorded at 0.2 Hz at a range of light power densities (Supplementary Fig. 1a,b). We presented 15 oHFS trains ('short oHFS'; 1-s bursts of 100 Hz, 3-ms blue light pulses repeated every 60 s for 15 min) with a single 3-ms test pulse after each oHFS train to measure synaptic strength. Following this protocol, we resumed the presentation of 0.2-Hz light pulses to examine oHFS-induced changes in synaptic strength. We observed a reduction in the amplitude of post-oHFS synaptic responses (Fig. 1e,f; F4,54 = 8.419, P < 0.0001), with specific reductions between the first and last 5-min bins (Tukey honest significant difference (HSD) post hoc test, P < 0.05). Throughout the experiment, evoked responses in oHFS recordings remained smaller than those in which oHFS was omitted (Fig. 1g: significant main effect for experiment, F2,104 = 148.532; P < 0.0001, with Tukey HSD post hoc test; P < 0.0005).

To achieve more persistent synaptic depression, we examined a longer stimulation protocol ('long oHFS'; 9-s bursts of 100 Hz, 3-ms blue light pulses every 60 s for 15 min). This protocol led to a more pronounced light-evoked responses immediately following the first 5-min bin (Tukey's HSD post hoc test, all P < 0.001) and reached a near-complete elimination of light-evoked synaptic responses after nine repetitions (Fig. 1f). Synaptic depression following long oHFS persisted for the entire duration of the recording (60 min; Fig. 1g; Tukey's HSD post hoc test, P < 0.0005). Notably, spontaneous EPSPs in the poststimulation period showed no reduction when compared with the prestimulation baseline period (Supplementary Fig. 1c), indicating that non-BLA inputs were not affected by the oHFS protocols.

To further characterize the synaptic changes, we performed voltage-clamp recordings in mPFC neurons while stimulating BLA afferents with pairs of light pulses (Δt = 100 ms) repeated at 0.2 Hz (Fig. 1h). We calculated the paired-pulse ratio as a measure of release probability before and after induction of synaptic depression using the oHFS protocol. The stimulation protocol was repeated until oEPSCs showed a twofold reduction in amplitude (Fig. 1h,i; paired Student's t-test P(oEPSC1) = 0.0022, t9 = 5.0993, P(oEPSC2) = 0.0074, t9 = 3.9654; Supplementary Fig. 1d; n = 7 cells). However, paired-pulse ratios remained constant (P = 0.9482, t9 = -0.0678, paired Student's t-test; Fig. 1j and Supplementary Fig. 1d), suggesting that the release probability of individual synapses was not affected. Notably, we observed a significant increase in oEPSC failure rate for the first pulse when compared to pre-oHFS baseline (P(oEPSC1) = 0.0324, t9 = -2.7716, paired Student's t-test; Supplementary Fig. 1e), indicating a potential reduction in presynaptic excitability following oHFS. Synaptic release from BLA–mPFC terminals showed strong, rapid adaptation during the oHFS stimulation train, such that the response in postsynaptic mPFC cells was similar for a single light pulse and for an entire oHFS train, suggesting an overall similar amount of glutamate release in both (Supplementary Fig. 2). These results indicate that synaptic depletion did not play a major role in the long-term oHFS-mediated depression. Taken together, the findings suggest that high-frequency stimulation of BLA–mPFC terminals using the channelrhodopsin variant ChETA TC induced robust synaptic depression that was associated with reduced presynaptic excitability.

Optogenetic high-frequency stimulation of BLA–mPFC terminals induces synaptic depression in freely moving mice

Our ex vivo findings indicated that repetitive optogenetic stimulation could elicit synaptic depression in BLA–mPFC synapses. We next asked whether we could use the same protocol to modulate synaptic strength in behaving animals. To record the responses of mPFC neurons to BLA input, we constructed movable fiberoptic-coupled microwire arrays and recorded single units from mice unilaterally injected with rAAV5-CaMKIIα-ChETA TC-P2A-eYFP in the BLA (Fig. 2a and Supplementary Fig. 3). Units were characterized as responsive if they exhibited a short-latency increase in firing rate (FR) to 3-ms light pulses delivered at 0.2 Hz through the optical fiber during the baseline testing period. A substantial proportion (21%) of all recorded units (oHFS, 57 units; no oHFS, 38 units; n = 5 mice) showed reliable light-evoked responses and were used for further analyses.

We applied the same oHFS protocol as in the aforementioned ex vivo experiments (9-s bursts of 3-ms pulses at 100 Hz, repeated once per min for 15 min). The oHFS schedule caused a reduction in evoked responses in the vast majority of responsive units (46 of 57 units, 80.7%; Fig. 2b,c; paired t-test, t56 = 6.142, P < 0.0001); whereas control recordings in which no oHFS protocol was applied showed stable responses 15 min after baseline testing (Fig. 2b,c; t56 = -1.628, P = 0.112). During oHFS trains, the FR of responsive units increased only slightly (Supplementary Fig. 4a). Single-unit FRs during the oHFS train were not significantly different from baseline (Supplementary Fig. 4a; main effect of time, F2,54 = 2.53, P = 0.08). This is consistent with the strongly adapting synaptic response recorded ex vivo during oHFS (Supplementary Fig. 2). Together, these results indicate that oHFS-induced changes in synaptic strength did not result from recurrent activity of intracortical synapses or from depletion of BLA–mPFC terminals and further support a presynaptic mechanism for oHFS-induced synaptic depression.

Light-evoked responses to BLA–mPFC stimulation gradually recovered following the oHFS protocol, reaching 60% of baseline evoked-response amplitude after 90 min (averaged over all light intensities; Fig. 2d; two-way ANOVA; significant group × time interaction F12, 23,380 = 6.922, P < 0.001 with all post hoc P < 0.001). The distribution of evoked response magnitudes, which showed strongly reduced responses immediately following oHFS, was statistically indistinguishable from control ninety minutes after oHFS (T90; n = 27; Supplementary Fig. 4b). Units recorded in both the PL and IL subregions showed similar oHFS-induced attenuation of responses.
Notably, despite the changes in evoked responses to BLA–mPFC axon stimulation, the spontaneous FR did not change when measured at the same time points (Fig. 2d; F_{4,220} = 0.05, P = 0.99). This is consistent with our in vitro results and further suggests that non-BLA inputs to mPFC neurons are not affected by the oHFS protocol.

We next asked whether oHFS differentially impacts putative excitatory and inhibitory neurons. The basal FR of single units was
correlated with the change in their response to oHFS (Fig. 2c; \( r = 0.46, P < 0.001 \)). Units with firing rates above the median were less affected by the oHFS than cells with lower firing rates. This difference occurred only in the oHFS group and not in control animals in which no oHFS was applied (Fig. 2e; significant experiment × cell-type interaction; \( F_{1,88} = 7.965, P = 0.0059 \); \textit{post hoc} tests, \( P = 0.0079 \) only for oHFS). This implies that the effect of oHFS could be amplified, at least in part, through its differential effects on RS and FS populations.

Previous work has demonstrated that ChR2-mediated high-frequency stimulation strongly attenuates at higher frequencies\(^{34}\). We hypothesized a similar effect here, which would effectively limit antidromic spiking of stimulated axons and restrict the impact of oHFS to the mPFC. We recorded from the BLA while stimulating ChETA\(_{-TC}\)-expressing BLA axons in the mPFC (Supplementary Fig. 5a) and obtained baseline responses to a 5-Hz stimulation followed by three oHFS trains (Supplementary Fig. 5b). BLA units did not consistently follow the oHFS. In fact, these units showed no significant increase in firing rate during the 9-s oHFS protocol compared to their pre-oHFS firing rate (\( F_{2,94} = 1.0651, P = 0.35 \); Supplementary Fig. 5c,d), indicating that, in contrast with 5-Hz stimulation, 100-Hz oHFS at axonal terminals did not efficiently back-propagate to BLA somata (Supplementary Fig. 5e). Short-latency responses (under 11 ms) were less strongly modulated following oHFS at the mPFC, while the longer-latency responses depressed strongly (Supplementary Fig. 5b), resulting in a negative correlation between latency and oHFS-induced change (Supplementary Fig. 5f; \( r = -0.335 P < 0.001 \)). The selective depression of longer-latency responses indicates that oHFS at BLA–mPFC terminals mainly influenced polysynaptic responses of BLA neurons to afferent inputs from the mPFC.

This experiment provided evidence for minimal antidromic propagation of the oHFS to the somata of mPFC-projecting neurons in the BLA (BLA–mPFC cells). To better understand whether any residual back-propagation could in fact influence the output of the BLA to other brain regions through collateral axons, we performed a viral vector tracing experiment in which BLA–mPFC cells were labeled with eYFP (Supplementary Fig. 6a,b). Previous work demonstrated minimal overlap between BLA cells projecting to the ventral hippocampus and the mPFC\(^{15}\). Our analysis of projection patterns to some of the major BLA targets in the brain further revealed that BLA–mPFC cells were more specific in their projection patterns than the general BLA population (labeled using a nonspecific CaMKII\textalpha-eYFP vector). Axonal projections of BLA–mPFC cells were mainly found in the mPFC and were largely absent from the nucleus accumbens, lateral septum and mediodorsal thalamus (Supplementary Fig. 6c). These findings indicate that BLA–mPFC neurons were target-specific, a specialization that might point to a unique role for this projection in carrying acquired information to the cortex.

**Optogenetic high-frequency stimulation of BLA–PL synapses disrupts the retention of cued fear representations**

We asked whether attenuation of BLA–mPFC synaptic transmission leads to changes in the representation of cued fear in mPFC neurons. Mice were injected unilaterally with ChETA\(_{-TC}\) vectors in the BLA and implanted with fixed multielectrode arrays targeting the ipsilateral mPFC (Fig. 3a). Mice were fear-conditioned using six pairings of an AAV5-CaMKII\textalpha-ChETA\(_{-P2A}\)-eYFP and implanted above the ipsilateral PL with a movable optrode drive. (a) Top: raster plot overlaid with peristimulus time histograms (PSTH, red) showing the spiking of all responsive units before (T\(_{\text{pre}}\)) and immediately after (T\(_{1}\)) 15-min oHFS (right) or control protocols (left). Bottom: evoked FR for each responsive unit before (FR\(_{\text{pre}}\)) vs. immediately following (FR\(_{\text{oHFS}}\)) the stimulation or control protocols (see main text for statistics). (b) Mean evoked FR of all responsive units for oHFS and control protocols, as in a. (c) Top: light-evoked FR in response to test pulses at four different light intensities before (T\(_{\text{pre}}\)) and up to 90 min (T\(_{0}–T_{90}\)) following oHFS of BLA–mPFC projections (see text for statistics). Bottom: spontaneous FR of all responsive units in the oHFS experiment during the same time points used for recording evoked responses. (d) Left: scatter plot depicting the correlation between the basal unit firing rate and the oHFS-induced change in evoked response. The change is calculated as \((\text{FR}_{T_{1}} – \text{FR}_{\text{pre}})/(\text{FR}_{\text{pre}} + \text{FR}_{T_{10}})\). Right: mean change in light-evoked spiking in units with low (<5 Hz) and high (>5 Hz) baseline FR during the control (gray) and oHFS (blue) protocols. Shading or error bars in all panels indicate s.e.m.; *P < 0.05.

control group (\( n = 5 \)) expressed ChETA\(_{-TC}\) and underwent a similar experiment, excluding the delivery of oHFS trains. We recorded 89 mPFC units in the oHFS group, 50 (56%) of which displayed CS-evoked FR increases, and 71 in the control group, 47 (66%) of which displayed CS-evoked responses.

Whereas conditioned freezing during the extinction session diminished over time (ANOVA main effect of trial, \( F_{19,152} = 1.79663, P = 0.0027 \)), there was no difference in freezing between the control and oHFS groups (ANOVA, main effect of group: \( F_{1,8} = 1.32, P = 0.28 \); Supplementary Fig. 7), consistent with previous work showing no behavioral effects of unilateral manipulation in amygdala and mPFC\(^{7,35}\). To ascertain that the oHFS effect was similar in these recordings to the oHFS described above (Fig. 2), we examined the responses of single units before and after the oHFS protocol (Fig. 3b). Indeed, units that showed light-evoked firing during the baseline testing period (7 of 89 in oHFS and 9 of 71 in control) showed attenuated...
light-evoked responses following oHFS compared to control (Fig. 3c,d), confirming that oHFS led to attenuation of synaptic transmission at BLA–mPFC terminals.

In mice that were exposed to oHFS of BLA–mPFC projections during the test, the magnitude of cue-evoked responses was significantly reduced after the oHFS schedule was initiated (Fig. 3e,f). While both the oHFS group and controls showed a reduction in CS-evoked response with behavioral extinction (ANOVA main effect of time-bin, $F_{3,1392} = 27.759, P < 0.00001$), the reduction occurred earlier in the oHFS group (Fig. 3f). These findings are consistent with a role for the direct BLA–mPFC pathway in transmitting learned CS–US associations to the mPFC15–17. Furthermore, because behavioral responses were similar in control and oHFS mice (due to the unilateral manipulation), we conclude that the reduction in cue-evoked responses originated from the attenuation of BLA input to the mPFC.

Optogenetic high-frequency stimulation of BLA–PL synapses impairs cued fear consolidation

Given the fear-promoting role of PL4,5,10, we asked whether the information carried by the direct inputs of BLA to PL is necessary for fear learning. We bilaterally injected mice with rAAV5-CaMKII$\alpha$-ChETA$\alpha$-P2A-eYFP into the BLA and implanted optical fibers above the midline area containing superficial layer neurons of the PL region. Control mice were injected into the BLA with rAAV5-CaMKII$\alpha$-eYFP (no-opsin control, $n = 10$) or injected with rAAV5-CaMKII$\alpha$-ChETA$\alpha$-P2A-eYFP but stimulated with yellow light during the experiment (yellow light control; $n = 10$; see Online Methods for details). Eight weeks later, all mice underwent oHFS followed by fear conditioning in context A (Fig. 4a). Both groups (ChETA$\alpha$, $n = 18$; control, $n = 20$) increased freezing during acquisition with no significant differences between the control and oHFS groups (Fig. 4a; main effect of trial, $F_{3,180} = 17.62, P < 0.0001$; post hoc all $P < 0.01$; group effect and group × trial interaction both nonsignificant; $P = 0.386$ and $P = 0.464$ respectively). To test for fear recall and extinction, mice underwent extinction training in context B on the following day. Mice that underwent oHFS before acquisition exhibited reduced freezing during fear recall (main effects of group: $F_{1,36} = 4.08, P = 0.05$; trial, $F_{1,36} = 27.5986, P < 0.001$; trial × group interaction, $F_{1,36} = 3.1636, P = 0.08$), with the reduction appearing during early extinction training (Fig. 4b; $P = 0.0125$, interaction post hoc) but not during the late stage. This suggests that reduced synaptic strength in BLA–PL synapses during fear acquisition interfered with later recall of cued fear associations. Consistent with this notion, we observed no difference between the oHFS and control mice during an extinction retrieval experiment performed the following day (Fig. 4c). The short oHFS protocol (Fig. 1e–g) was only mildly effective (Supplementary Fig. 8). These findings are consistent with a role for BLA–PL synapses in the long-term consolidation of fear but not in extinction memories.

We next asked whether contextual associations were affected by the attenuation of synaptic strength in the BLA–mPFC pathway. In mice that had previously undergone cued fear learning and extinction, exposure to the fear acquisition context (context A) led to similar freezing levels in oHFS and control mice ($t_{13} = 0.0321, P = 0.9361$; Supplementary Fig. 9a). Moreover, in mice that had been fear-conditioned to contextual cues alone, oHFS delivered before fear acquisition did not lead to changes in freezing during acquisition nor upon context exposure the following day (Supplementary Fig. 9b). These results indicate that contextual memory was not influenced by oHFS of BLA–mPFC synapses during acquisition, consistent with specific transmission of cued fear associations in the BLA–mPFC pathway. To test whether the effects of BLA–mPFC oHFS are specific to fear learning, we assayed anxiety-related behaviors in mice following oHFS of BLA-to-mPFC projections. In the open field test, mice that underwent oHFS of BLA–PL projections showed no change in either locomotor activity or time spent in the center of the arena (Supplementary Fig. 9c). We also found no change in anxiety-related measures in the elevated plus maze (Supplementary Fig. 9d). These results further suggest that the BLA–mPFC projection contributes specifically to cued-fear associations and plays a lesser role in contextual learning and unconditioned anxiety.

Optogenetic high-frequency stimulation of BLA–PL synapses facilitates extinction of previously acquired fear associations

Given the well-established role of the PL in promoting fear and opposing extinction24,36–38, we examined the contribution of BLA–PL synapses to the maintenance and learning of extinction. We applied the oHFS protocol immediately before extinction training, 24 h after fear acquisition ($n = 13$ ChETA$\alpha$ mice, $n = 13$ control mice). Both groups increased freezing during acquisition, with no significant differences between the control and oHFS groups (Fig. 4d; main effect of trial $F_{5,390} = 31.2146, P < 0.0001$). During extinction training, freezing in all groups was higher in the first trial than during late trials (all...
In contrast to the PL, the IL subregion of the mPFC contributes to extinction and its maintenance. For example, if BLA–IL input contributes to extinction, depression of these synapses would lead to impaired extinction learning. Conversely, BLA input to IL might signal the previously acquired fear association, and the extinction-promoting role of IL could result from integration of fear and safety cues within the region. To test these two opposing scenarios, we repeated the experiments with fibers implanted above the IL region. When oHFS was applied to BLA–IL inputs before fear acquisition (Fig. 5a), mice showed reduced freezing responses on the following day during extinction training (Fig. 5b). Two-way ANOVA main effect for group, $F_{1,16} = 12.795, P = 0.0025$, and a trend of reduced freezing during extinction retrieval test on the third day (Fig. 5c; $F_{1,16} = 4.2758, P = 0.05523$). Performing oHFS on BLA–IL inputs immediately before extinction training, 24 h after fear acquisition (Fig. 5d), led to reduced cue-associated freezing responses both during extinction learning (Fig. 5e; two-way ANOVA main effect for group, $F_{1,17} = 14.2605, P = 0.0015$) and during extinction retrieval testing (Fig. 5f; $F_{1,17} = 14.3307, P = 0.0015$). This suggests that, under these conditions, BLA input to the IL played an extinction-opposing role during extinction learning. Together, our findings support a fear-promoting role for BLA–mPFC inputs and therefore suggest that extinction-promoting activity of IL resulted from integration of information from additional nodes in the fear-learning circuit.
DISCUSSION

The present study was designed to examine the contribution of monosynaptic transmission from the BLA to the mPFC in acquisition and extinction of fear associations. Although recent work has implicated BLA contributions to coding in the mPFC and highlighted the potential role of this information in fear expression\(^{9,10,13}\), it has remained unclear to what extent such information is transmitted through monosynaptic BLA–mPFC projections or mediated via other indirect pathways. We employed a combination of anatomical tracing, electrophysiological recordings and optogenetic manipulation, by which we unveiled the contribution of the BLA–mPFC synapse in acquisition, retention and extinction of fear associations.

The BLA projects heavily to several subregions of the mPFC\(^{19}\) but also has dense projections to other key regions that are involved in conditioned value associations\(^{10}\). Previous studies have used lesions and pharmacological manipulations to examine the contribution of BLA activity to the coding of fear-associated cues in the mPFC and to fear learning\(^{16,17}\). However, silencing or lesioning the BLA is unclear to what extent such information is transmitted through synaptic plasticity can be effectively induced using repetitive electrical microstimulation\(^{22,23}\). High-frequency stimulation (typically at 100 Hz) has been used to induce long term potentiation, while...
continuous low-frequency (1 Hz) stimulation is typically used to achieve long term depression (LTD)40. However, because electrical microstimulation cannot be directed at specific synapses, recent studies have used optogenetic techniques to manipulate synaptic strength31,33. Whereas some of these studies reported results comparable with electrical microstimulation in defined pathways31,41, other studies have shown that short-term plasticity induced by optogenetic stimulation can greatly vary based on synapse type and the location of stimulation32. Indeed, previous studies have demonstrated low-frequency-stimulation-induced potentiation43,44 and high-frequency-stimulation-triggered synaptic depression in various other synapses45,46. It has also been demonstrated that optogenetic stimulation schedules induce synaptic plasticity differently from anatomically matched electrical stimulation protocols32. Thus, our finding that an oHFS protocol induced synaptic depression here could stem from differences in the ways electrical and optogenetic stimulations recruit the network or from mechanisms that are specific to the pathway we examined here, and further studies will need to address this in detail.

To test the functional role of BLA projections to the PL in fear memory acquisition and consolidation, we applied the long oHFS protocol to BLA–mPFC inputs before fear acquisition. Whereas there was no change in immediate freezing during learning itself, there was a substantial reduction in freezing during the early phase of the extinction–retention test. These results suggest that the activity in the BLA–PL projection during fear acquisition was required for later recall of learned CS–US associations. Although context–US associations involve the amygdala and other forebrain circuits31,47,48, our results indicate that monosynaptic BLA input to the PL subregion is not required for the maintenance of contextual associations. Notably, recent work has shown that BLA outputs to the entorhinal cortex are required for contextual fear learning49, consistent with a functional segregation of BLA outputs for contextual and cued associations. Based on our findings of the attenuation of cued fear by oHFS BLA–PL synapses, we further hypothesized that BLA input to PL could modulate the fear-promoting role of PL during extinction learning24,36–38, namely in opposing extinction. Indeed, we observed that extinction learning was facilitated following oHFS of these inputs. This result suggests that the BLA–PL projection supports and actively maintains the aversive memory during the extinction process. Consistent with this notion, fear-encoding neurons have been shown to be directly project to the mPFC12.

Previous work has assigned the PL with a bear-promoting role through its projections to the amygdala, while the IL has been linked to extinction of learned fear4,5. A recent study demonstrated that BLA neurons projecting to the PL and IL are differentially activated by fear and extinction learning, respectively15. However, whether the information transmitted to the IL through this direct synaptic pathway is in itself extinction- or fear-promoting remains to be determined. Two scenarios are possible in this respect: in one, the BLA transmits fear- and extinction-related information to the mPFC, and this information is routed differentially to the PL and IL to support fear and extinction-promoting activity of these two regions; in the second scenario, both types of fear-related information are received in PL and IL, and local-circuit processing combined with information from other nodes of the fear circuit transforms this information within the PL and IL to allow the differential contributions of these two regions to fear and extinction learning. Senn et al. used a differential fear-conditioning model to examine the role of PL- and IL-projecting BLA cells in extinction learning, demonstrating reduced extinction of fear responses to cues paired with inhibition of IL-projecting neurons compared with extinction of responses to cues paired with excitation of the same neurons, providing support for the first model15. Our results in this study, using a simpler fear-conditioning protocol, lend support to the second alternative and indicated that amygdala input to the mPFC was pivotal in maintaining information about fear associations. Differences in experimental design might account for these different outcomes, and future work will further refine our understanding of this complex circuit under different behavioral settings. Our results suggest that reciprocal information transfer regarding fear associations was balanced within the circuit and was not carried by specific regions alone.

In summary, our findings shed light on the role of BLA–mPFC synapses in fear acquisition and extinction. We developed a protocol for optogenetically induced LTD, which causes presynaptic attenuation through a reduction in presynaptic excitability. Using this protocol, we found that whereas attenuation of BLA–PL and BLA–IL synapses before acquisition led to reduced freezing during retention, the same manipulation before extinction led to more efficient reduction in fear expression and enhanced extinction. Our results therefore suggest that monosynaptic BLA input to both these mPFC subregions provided cue-related information that was used to maintain the fear response and supports resistance to extinction learning. Modulation of fear memory and its extinction therefore requires a balance between both directions of the amygdala–mPFC pathway, and future manipulations should take this into account and potentially utilize it to suggest clinical approaches.

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

O.K., M.P. and O.Y. designed and planned the experiments. O.K. carried out in vitro electrophysiology and behavioral experiments. M.P. conducted the in vitro electrophysiology and anatomical tracing experiments. A.S. helped with behavioral experiments. R.P. contributed to ideas and discussions. O.K., M.P. and O.Y. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Sterotoxic surgery, viral injections and optrode implants. Five- to six-week-old C57BL/6J male mice were used for stereotoxic viral injection. All mice were anesthetized with an intraperitoneal injection of ketamine–xylazine mixture and placed into a stereotoxic frame (David Kopf Instruments); 1% isoflurane was then provided to maintain a deep anesthetized state over the course of the surgery. Virus was injected (Nanojell syringes, World Precision Instruments) either into the BLA (anterior–posterior (AP), –1.34 mm from bregma; mediolateral (ML), ±3.38 mm; dorsoventral (DV), –4.8 mm) or above the prelimbic (PL) part of the mPFC (AP, +2 mm; ML, ±0.25 mm; DV, –2.25 mm). The total volume of viral suspension per injection site was 600 nl, at an injection rate of 100 nl per min. After injection, the needle was left in place for an additional 5 min and then slowly withdrawn. After viral injections, the skin was glued together with Vetbond adhesive (3M). Mice were allowed to recover for at least 8 weeks to allow axonal expression of opsin. Mice were used for either slice recording, fiber optic implantation for behavior experiments or optrode drive implantation for in vivo electrophysiology. For fiber optic implantation, a ferrule-terminated implanted optical fiber (ThorLabs) was placed at a midline position above the PL subregions (AP: +2 mm; PL: 0 mm; DV: –2 mm) and secured to the skull using with Metabond (Parkell) and dental acrylic. For optrode drive implantation, the drive (described below) was lowered to above either right or left PL (AP: +2 mm; ML: ±0.25 mm; DV: –2 mm). Prior to the permanent attachment of the optrode to the skull, the optrode was protected with Kwik-Kast silicone elastomer (World Precision Instruments) and secured using dental acrylic. Mice were allowed to recover for at least 2 weeks before experiments. The locations of implanted optical fibers were validated using histology for all experimental mice (Supplementary Fig. 10). For the BLA projection tracing experiment, mice were injected with LT-HSV-hEF1-ΔIO-eYFP into the BLA. For all other experiments, mice were injected in the BLA with either rAAV5-CaMKIIe-ChETA\textsubscript{TC}-p2a-eYFP (refs. 51,52) or rAAV5-CaMKIIe-eYFP.

Behavioral experiments. All experiments were approved by the Weizmann Institute Animal Care and Use Committee (IACUC). Mice were housed in groups of 3–5 on a 12-h light/dark cycle with ad libitum food and water. All behavioral experiments took place during the dark cycle.

Pre-acquisition oHFS. The experiment included two repetitions: in the first, all mice were injected with viral vectors to express ChETA\textsubscript{TC} in the BLA and were assigned to either the oHFS (i.e., receiving 470-nm light) or the control group (receiving yellow 560-nm light) before the fear acquisition experiment. In the second replication experiment, the oHFS group expressed ChETA\textsubscript{TC} and the control group expressed eYFP in the BLA. Both groups were stimulated with 470-nm light. No significant differences were observed between these two control groups and the data from these mice was pooled together (t\textsubscript{p} = 0.85, P = 0.405). On the first training day (day 1), mice in both the oHFS and control groups underwent oHFS in a designated cage and were then placed in the fear conditioning chamber (TSE systems) in context A. Mice were presented with six pairings of the CS (a 5-Hz train of 100 ms, 5-kHz tones lasting for 30 s) and US (continuous 0.5-mA foot shock for 1 s), with 1.5-min intervals between US presentations. On day 2, mice were placed in the fear conditioning chamber in the same context (context A) for 10 min while freezing behavior was recorded as described above. Freezing score was calculated as the percentage of total time during which mice remained immobile.

Pre-extinction oHFS. The oHFS group expressed ChETA\textsubscript{TC} in the BLA and control animals expressed eYFP in the BLA. The experiment was performed as described above, with oHFS in both groups performed on day 2, before placing the mice in context B.

Open field and elevated plus maze experiments. The open field test was conducted in an open plastic arena (50 × 50 × 40 cm). Mice underwent oHFS and were then placed in the center of the chamber and allowed to freely explore for 10 min. Activity in the center and periphery of the field was recorded and measured using an automated video-tracking system (Biobase, Bonn, Germany). Percentage of time in center was defined as percent time spent in the central 30 × 30 cm area of the open field. Total velocity was calculated as centimeters traveled per second.

Elevated plus maze. The elevated plus maze was made of gray PVC and consisted of two closed arms and two open arms (30 × 5 cm) extending from a central platform (5 × 5 × 5 cm) at 90°. The maze was positioned 30 cm above the floor. Having undergone oHFS in a designated cage, mice were then placed on the central platform of the chamber and allowed to freely explore for 60 s. Activity in the open arms, closed arms and center of the field was recorded and measured using an automated video-tracking system (Biobase, Bonn, Germany). Percentage of time in each region was defined as the percent of total test time that was spent in that region of the maze. Total velocity was calculated as centimeters traveled per second.

Acute slice electrophysiology. Eight weeks after virus injection, mice were deeply anesthetized with pentobarbital (0.4 mg g\textsuperscript{-1} body weight) and the brain was extracted. In total, 75 male C57/Bl6 mice were used for acute slice electrophysiology. Next, 300-µm-thick coronal sections of the mPFC were prepared in ice-cold cutting solution (in mM: 11 n-glucose, 234 sucrose, 2.5 KCl, 1.25 NaH\textsubscript{2}PO\textsubscript{4}, 10 MgSO\textsubscript{4}, 0.5 CaCl\textsubscript{2}, 26 NaHCO\textsubscript{3} 340 mOsm/kg, aerated with 95% O\textsubscript{2} and 5% CO\textsubscript{2} using a vibratome (Leica VT1200 S). Slices were then incubated at 32 °C for 30 min in high-osmolarity artificial cerebrospinal fluid (aCSF; in mM: 3.24 KCl, 11.88 glucose, 123 NaCl, 28.1 NaHCO\textsubscript{3}, 1.35 NaH\textsubscript{2}PO\textsubscript{4}, 1.08 MgCl\textsubscript{2}, 2.16 CaCl\textsubscript{2}, 320 mOsm kg\textsuperscript{-1}, aerated with 95% O\textsubscript{2} and 5% CO\textsubscript{2} at 32 °C). Following recovery, slices were kept at room temperature until use. The recording chamber was perfused with oxygenated aCSF at a rate of 1.5–2 mL min\textsuperscript{-1} and maintained at 32 °C. Borosilicate glass pipettes (Sutter Instrument BF100-58-10) with resistances 4–6 MΩ were pulled using a laser micropipette puller (Sutter Instrument Model P-2000) and filled with intracellular solution (in mM: 135 potassium-glucuronate, 4 KCl, 2 NaCl, 10 HEPES, 4 EGTA, 4 MgATP, 0.3 NaGTP, 280 mOsm kg\textsuperscript{-1}, pH adjusted to 7.3 with KOH). Neurons were patched under visual guidance using infrared differential interference contrast microscopy (Scientifica SliceScope) and an Andor Clara CCD camera. Recordings were carried out using a Multiclamp 700B amplifier (Molecular Devices). Acute coronal mPFC slices contained eYFP-labeled axons concentrated in deep and superficial layers, as previously reported\textsuperscript{14}. Optical activation of ChETA\textsubscript{TC}-expressing neurons was performed with 445 ± 20 nm, 3-ms light pulses at 2–18 mW mm\textsuperscript{-2} (Lumencor Spectra X), delivered through the microscope illumination path. After establishing the whole-cell configuration, 300-ms current injections were used to probe the action potential threshold. Electrical properties such as after-hyperpolarization potential (AHP), action potential width and spike rate were calculated at 25 ± 10 mV above rheobase.

For titrating light-evoked responses, we used a protocol consisting of a light pulse (3 ms) train of 0.2 Hz for 30 s at 10 different light intensities. Only cells that were recorded through an entire light titration protocol and showed a sigmoidal light-response curve were included in the analysis of ePSC properties (Fig. 1c).
Responses were extracted in a 10-ms detection window after light onset. Light-evoked responses were fitted with a sigmoidal dose–response curve (Supplementary Fig. 2b) to estimate light intensity at which half maximal responses were evoked. The oHFS protocols were applied at this light intensity once per min over a period of 15 min. Thirty seconds after the beginning of each oHFS light pulse train, a single 3-ms test pulse was given in order to test the synaptic response. After the completion of the entire oHFS protocol, cells were probed every 10 min with the light titration protocol to quantify the changes in synaptic response. Spontaneous EPSP data were acquired during the intervals between repetitions of the light titration protocols. Data were preprocessed with a 400-Hz low-pass 4-pole Butterworth filter. EPSPs were detected as peaks in the moving variance transformation (window size 0.5 ms) that were twofold higher than the s.d. of the trace.

To determine the paired-pulse ratio, evoked responses were titrated and the light intensity that resulted in half-maximal response was identified. Pairs of consecutive 3-ms light pulses were spaced 100 ms apart and repeated every 5 s for 5 min as baseline. oHFS was repeated until single light pulse responses at half-maximal intensity were decreased by approximately 50%. The paired-pulse (PP) light protocol was then resumed for an additional 20 min. For quantifying response amplitude, failure rates and paired pulse ratios (PPR), 5 min of recording data after oHFS were compared with the 5 min of baseline. The amplitudes of responses in Figure 11 are normalized to the first 100 s (20 PP pairs) of the unpaired data. All PPR data shown are represented in 50-s bins. The threshold for light-evoked EPSC responses was set to twice the s.d. computed from a 20 ms baseline taken immediately before the light pulse.

**In vivo photostimulation and electrophysiology.** All electrophysiological recordings in awake, freely moving mice were performed using an optrode drive consisting of an electrode bundle of 16 microwires (25-μm diameter straightened tungsten wires; Wiretronic Inc.) attached to an 18-pin electrical connector, concentrically arranged around an optical fiber in a mechanically adjustable drive43. Extracellular waveform signals were collected using the Digital Lynx integrated hardware and software system (Neuralynx Inc.). The electrical signal was filtered (600–6,000 Hz) and amplified using a HS-18-CNR-LED unity-gain head-stage amplifier.

The electrode–fiber assembly was lowered using the mechanical drive to a new recording site at the end of each recording day, leaving at least 20 h before the experiment to ensure stable recordings. Optical stimulation was applied through a ferrule-terminated optical fiber (Thorlabs) attached to the patch-chord by a zirconia sleeve (Thorlabs). For optical stimulation of BLA-derived terminals in the mPFC of ChETAz-expressing mice, we used a blue diode laser (λ = 460 nm, Omicron Laserage GmbH). Light transmission for each optrode drive was measured with a calibrated power meter (Thorlabs) at the tip of the optical fiber before implanting. Light power was then measured daily before experiments at the tip of the optical patch cord. Light through the patch-chord at 100% intensity was adjusted to 16–20 mW mm−2 at the tip of the implanted fiber.

On each recording day, a baseline testing period in which light pulses were delivered at 0.2 Hz was followed by a 15-min stimulation protocol, which was succeeded by resumed testing. During the testing periods, single light pulses at 0.2 Hz were delivered through the optical fiber. Thirty single pulses were given at each of four different intensities (25%, 50%, 75% and 100% light intensity) in order to avoid potential ceiling effects resulting from response saturation. The plasticity-inducing protocol consisted of 15 repetitions of 9-s, 100-Hz pulse trains. Neural data were sorted manually using OfflineSorter 3.2.4 (Plexon) and analyzed in Matlab (Version 2012b, MathWorks). Evoked responses were calculated as the firing rate (FR) during the 10 ms after light pulse onset and compared to the FR 10 ms before the light pulse. To calculate the changes in FR during oHFS, the FR of each unit before, during and after oHFS was normalized to its average FR during the first ten minutes of recording. To correlate the changes in FR with basal FR, a change index was computed as ((baseline response – post-oHFS response)/ (baseline response + post-oHFS response)), yielding a number between −1 and 1 that quantified the magnitude and direction of the oHFS-induced change.

**In vivo photostimulation and electrophysiology in mice during extinction training.** The experiment included two groups, both expressing ChETAz in the BLA and implanted with fixed optrode arrays in the mPFC. Mice in both the oHFS and control group were placed in the fear conditioning chamber (Med Associates) in context A. Mice were presented with six pairings of the CS (50-ms long 5-kHz tones, delivered at 2 Hz for 30 s) and US (continuous 0.5-mA foot shock for 1 s). Each CS coterminated with the US, with a 60-s interval between CS–US pairings. On day 2, mice were connected to the head-stage amplifier and the optical patch chord and then placed in a different chamber (context B). Mice were allowed 20 min of habituation and then presented with 20 repetitions of the CS, separated by 60-s intervals. Following the fifth CS, only mice in the experimental group received the oHFS schedule (as described above), one train after each CS presentation for a total of 15 trains. The control group received the same amount of CS presentations without oHFS. Neural data were sorted manually using Offline Sorter 3.2.4 (Plexon) and analyzed in Matlab (Version 2012b, MathWorks).

**Histology and microscopy.** Mice were deeply anesthetized using pentobarbital (0.4 mg g−1 body weight) and perfused transcardially with ice-cold phosphate buffered saline (PBS, pH 7.4) followed by a solution of 4% paraformaldehyde in PBS. After overnight postfixation at 4 °C, brains were moved to 20% sucrose (Leica Microsystems) and collected in cryoprotectant solution (25% glycerol, 30% ethylene glycol in PBS pH 6.7). Free-floating sections were mounted on gelatin-coated slides, dehydrated and embedded in DABCO mounting medium (Sigma). Three-dimensional z-stacks and two-dimensional overview images (tiles) were acquired using a LSM 700 confocal microscope (Carl Zeiss). Acquisition settings were kept constant in each experiment for comparison between mice.

For quantification of BLA fluorescence, the BLA regions in both hemispheres were selected as regions of interest (ROI) based on anatomical features in coacquired DAPI staining images. Enhanced YFP fluorescence images were scaled with bilinear interpolation to the same image size. Images where then filtered with a Fiji44 rolling-ball threshold plugin using 10-μm diameter as an input. Axonal tube-like structures were further enhanced by applying the Fiji ‘tubeness’ plug-in on all images45. Converting the 8-bit images to binary images with the same threshold for HSV and eYFP allowed us to calculate the axonal density for selected regions.

**CLARITY imaging.** Mice were stereotaxically injected bilaterally with rAAV5-CaMKIIe-eYFP to the BLA (as described above). Eight weeks later, brains were removed and were put through a clearing protocol described elsewhere46. Following clearing, entire brains were imaged using a light-sheet microscope (UltraMicroscope from LaVision BioTec GmbH, Germany; magnification, 0.63x; NA, 0.5; excitation wavelength, 488 nm). Images were processed using Imaris software (Bitplane, Switzerland).

**Data analysis and statistics.** Connectivity experiment. Results of the first historical experiments were analyzed using multiple Wilcoxon tests with Bonferroni alpha correction.

**Acute brain slice recordings.** Average amplitudes of light-evoked EPSPs at 18 mW mm−2 were compared using an unpaired Student’s t-test. For measuring changes in response over the course of the oHFS stimulation train, we binned the responses within the 15 min of stimulation to 5-min bins and used a two-way repeated measures ANOVA with the test pulse-evoked oEPSP amplitude as dependent variable and with time-bin and experiment (control, short oHFS, long oHFS) as independent variables. All significant interactions were followed by post hoc comparisons. For all comparisons, significance was set at P < 0.05.

To quantify changes in response following the oHFS schedule, we used two-way ANOVA with the average response to light (at the intensity that evoked half-maximal oEPSP amplitudes before schedule) as the dependent variable and time-bin (repeated measure, from T0 to T15) and experiment (control, short oHFS, long oHFS) as independent variables. All significant interactions were followed by post hoc comparisons, and for all comparisons, significance was set at P < 0.05.

**Single unit electrophysiology in awake, behaving mice.** Response FR was computed as the average FR in the 10 ms immediately following light pulse onset. Responsive units were defined by comparing the average evoked FR to the average baseline FR taken 10 ms before light stimulation onset, using a paired t-test. For measuring the difference in evoked FR before and after oHFS and control schedules, we used a paired t-test for each schedule. To measure changes in FR in the different trials, we used two-way ANOVA with the response FR as the dependent
variable, with time and light intensity as independent variables. All significant interactions were followed by post hoc comparisons, and for all comparisons, significance was set at $P < 0.05$.

**Single unit electrophysiology in awake, fear-conditioned mice.** CS-evoked FR was computed as the average FR during the 100 ms from tone onset. Responsive units were defined by comparing FR to the baseline taken 100 ms before tone onset, using a paired t-test. As CS consisted of 50 ms tones delivered at 2 Hz for 30 s, each CS consisted of 60 tone presentations used for comparing tone response to baseline. For measuring change in FR, an average baseline FR was measured for each CS presentation, and the response FR was then normalized to the baseline and presented as percent change. Trials were binned to baseline (trials 1–5) and oHFS (trials 6–20) periods, and the average response in each period was computed. For measuring the difference in tone response before and after oHFS (trials 1–5 versus trials 16–20), we used a paired Wilcoxon test (signed rank) with Bonferroni correction. To measure changes in FR in the different trials, we used two-way ANOVA with the response FR as dependent variable and trial (repeated measure) and experiment group as independent variables. All significant interactions were followed by post hoc comparisons, and for all comparisons, significance was set at $P < 0.05$.

**Behavioral testing.** For fear conditioning and extinction, all results were analyzed using ANOVA with percent time freezing as the dependent variable. Significant interactions were followed by post hoc comparisons, and for all comparisons, significance was set at $P < 0.05$. For the preacquisition oHFS experiment, we used two-way ANOVA with individual trials or binned trials (early vs. late) as repeated measures and group (experiment vs. control) as independent variables. For the preextinction oHFS experiment, we added the experiment type as a factor in order to compare between experiments and conducted three-way ANOVA analysis. For both context experiments and open field tests, we used a t-test analysis. For correlating expression levels with freezing in behavioral tests, we used Spearman’s correlation and its significance test.

For all parametric tests, the sample distribution of the data was assumed to be normal, but this was not formally tested. Nonparametric tests were used when such assumption could not be made. Unless specifically noted otherwise, all tests comparing two groups are two-tailed. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications. For the exact number of animals used in each experiment, see the relevant results section or figure legend. Blinding and randomization were not performed, but automated analysis was used whenever possible. Statistical comparisons were performed using parametric and nonparametric statistical tests unless otherwise noted and all were two-sided. Data points were excluded only in cases in which a single data point differed by at least 5 s.d. from the average computed without it.

A **Supplementary Methods Checklist is available.**

**Data availability.** The data collected in this study are available from the corresponding author upon reasonable request.