Hebbian and neuromodulatory mechanisms interact to trigger associative memory formation

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A long-standing hypothesis termed "Hebbian plasticity" suggests that memories are formed through strengthening of synaptic connections between neurons with correlated activity. In contrast, other theories propose that coactivation of Hebbian and neuromodulatory processes produce the synaptic strengthening that underlies memory formation. Using optogenetics we directly tested whether Hebbian plasticity alone is both necessary and sufficient to produce physiological changes mediating actual memory formation in behaving animals. Our previous work with this method suggested that Hebbian mechanisms are sufficient to produce aversive associative learning under artificial conditions involving strong, iterative training. Here we systematically tested whether Hebbian mechanisms are necessary and sufficient to produce associative learning under more moderate training conditions that are similar to those that occur in daily life. We measured neural plasticity in the lateral amygdala, a brain region important for associative memory storage about danger. Our findings provide evidence that Hebbian mechanisms are necessary to produce neural plasticity in the lateral amygdala and behavioral memory formation. However, under these conditions Hebbian mechanisms alone were not sufficient to produce these physiological and behavioral effects unless neuromodulatory systems were coactivated. These results provide insight into how aversive experiences trigger memories and suggest that combined Hebbian and neuromodulatory processes interact to engage associative aversive learning.

Hebbian plasticity | amygdala | neuromodulation | instructive signals | associative learning

Hebbian plasticity refers to the strengthening of a presynaptic input onto a postsynaptic neuron when both pre- and postsynaptic neurons are coactive (1). This was originally proposed as a mechanism for memory formation. Findings from in vitro and in vivo physiological studies suggest that Hebbian processes control synaptic strengthening (2–10). However, other results and theories suggest that Hebbian mechanisms alone are not normally sufficient for producing synaptic plasticity and that synaptic strengthening mediating memory formation involves interactions between Hebbian and neuromodulatory mechanisms (3, 4, 7, 11–19). Although molecules that may mediate Hebbian processes in memory formation have been identified (3, 11, 16, 17, 20–22), it has been difficult to directly test whether Hebbian plasticity alone or in combination with neuromodulation is necessary and sufficient to produce neural plasticity and memories in behaving animals (especially in mammals). This is because of technical limitations in controlling correlated activity between pre- and postsynaptic neurons involved in memory storage in a temporally/spatially precise manner while measuring behavioral memory formation and neural plasticity.

To overcome these problems, we used optogenetic techniques to directly manipulate Hebbian mechanisms in pyramidal neurons in the lateral nucleus of the amygdala (LA), a cell population important for storing aversive memories. Pavlovian auditory threat (fear) conditioning (23, 24) is a form of associative learning during which a neutral auditory conditioned stimulus (CS) is temporally paired with an aversive unconditioned stimulus (US), often a mild electric shock (17, 20, 21, 25–27). Following training, the auditory CS comes to elicit behavioral defense responses (such as freezing) and supporting physiological changes controlled by the autonomic nervous and endocrine systems. These conditioned responses can be used to measure the associative memory created by CS–US pairing.

This form of aversive Pavlovian conditioning is a particularly useful model for testing the Hebbian hypothesis because a critical site of associative plasticity underlying the learning has been identified in the LA (17, 22, 28). LA neurons receive convergent input from the auditory system and from aversive nociceptive circuits (29, 30). Auditory inputs to LA neurons are potentiated during threat conditioning (31–34), possibly as a result of auditory-evoked presynaptic activity occurring convergently and contemporaneously with strong activation of postsynaptic LA pyramidal neurons by the aversive shock US (i.e., a Hebbian mechanism). If these neural and behavioral changes are the result of Hebbian plasticity, then activity in LA pyramidal neurons specifically during the aversive US period (when both presynaptic inputs and postsynaptic neurons may be active) should be necessary for aversive memory formation and learning-related plasticity of auditory input synapses in the LA to occur. Reducing

Significance

The influential Hebbian plasticity hypothesis suggests that an increase in the strength of connections between neurons whose activity is correlated produces memories. Other theories, however, propose that neuromodulatory systems need to be activated together with Hebbian plasticity mechanisms to engage memory formation. The present work provides direct in vivo evidence supporting the idea that a parallel mechanism involving neuromodulation and Hebbian processes is both necessary and sufficient to trigger synaptic strengthening and behavioral associative memory formation. This parallel process may represent a general mechanism used by many learning systems in the brain.

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activity in LA neurons should disrupt the correlation between presynaptic activity induced by the auditory CS and postsynaptic activity induced by the aversive US. In addition, pairing the auditory CS with direct depolarization of LA pyramidal neurons in place of a shock US should be sufficient to produce aversive memories and plasticity of auditory inputs to the LA. This is because direct stimulation of postsynaptic LA neurons as an US would artificially produce coactivity with concurrently active auditory inputs. Previously, we found that this type of training procedure did produce behavioral learning when many training trials were used (35). However, the behavioral memory acquired under these conditions was somewhat weak, suggesting that other factors, such as neuromodulatory receptor activation, might function in a cooperative way to enhance Hebbian neural plasticity in the LA to possibly regulate the gain of aversive memory formation. Here we optogenetically manipulated correlated activity between auditory inputs and LA postsynaptic pyramidal neurons to directly test whether Hebbian mechanisms are both necessary and sufficient to produce changes in auditory processing in the LA and fear memories.

Results

Activation of Amygdala Pyramidal Neurons During Aversive Shock Period Is Necessary for Memory Formation and Associative Plasticity in the Amygdala. We first examined whether coactivity of CS inputs and US-evoked activation of postsynaptic LA pyramidal neurons is necessary for the acquisition of aversive memories and changes in neural processing in the LA. Because the aversive US occurs at the end of a 20-s CS, we examined whether auditory CS inputs were active at the time of the occurrence of the US, a necessary precondition for Hebbian plasticity. Because LA neurons primarily exhibit spikes to CS onset, we first analyzed auditory-evoked local field potential (A-EFP) responses and single-neuron spiking activity to determine whether CS responses were evident during the end of the CS period. Both A-EFP and single-unit activity was measured before threat conditioning when the auditory CS-evoked responses could be isolated from those evoked by the US. We found that before training CS inputs to the LA were active during the final second of the 20-s CS presentation (Fig. 1*A*), a time when the US occurs during threat conditioning, although these responses were reduced compared with the first pip-evoked A-EFP response of the CS period (first pip = 27.09 μV; last pip = 14.54 μV, a 46% reduction in amplitude). Further supporting this idea, from in vivo electrophysiological recordings in awake, behaving animals we found that before conditioning single neurons in the LA responded throughout the CS period and during the final CS pip (Fig. 1*B*), although the responses were larger to the first pip compared with the last (a 62% reduction in pip-evoked spiking activity). This converging evidence demonstrates that CS inputs are active during the end of the CS period. This, along with the knowledge that shock USs strongly activate LA neurons (see below and ref. 36) and that CSs and USs activate at least some of the same cells (29, 30, 36), suggests that correlated CS- and US-evoked activity occurs in LA neurons during threat conditioning.

We then used an optogenetic approach (37–41) to inhibit neural activity specifically during the US presentation period to disrupt this correlated activity of auditory CS inputs and US-evoked postsynaptic firing in LA pyramidal neurons. Specifically, we infected LA neurons with a virus encoding an Archaerhodopsin-T(ArchT)/EYFP fusion protein under the control of the CaMKIIα promoter (to target expression to pyramidal cells). ArchT is a green/yellow light-responsive outward proton pump and has been shown to potently inhibit neural activity when activated (42). Immunohistochemical quantification showed that ArchT was expressed preferentially in LA pyramidal neurons (95 ± 5% of ArchT+ cells were also CaMKIIα+, Fig. 1*C*) and in a large proportion of this cell population (54 ± 12% of LA CaMKIIα+ cells were also ArchT+). We then determined that shock-evoked neural activity could be inhibited with laser light in LA neurons expressing this protein (Fig. 1*D*). Next, using optogenetic/behavioral experiments, we tested whether activation of LA pyramidal neurons during the shock US period was necessary for threat learning to occur. For these experiments, animals were infected with CaMKII-ArchT/EYFP or a control CaMKII-GFP virus in the LA, and the LA was illuminated during ("ArchT/overlap" and "GFP/Overlap" groups), before (during the auditory CS, "ArchT/during CS" group) or after US delivery ("ArchT/offset" group), and their memory was tested 24 h later (Fig. 2 *A* and *B*). We found that in the ArchT-treated animals, inhibition of neural activity specifically during, but not before or after, the US period significantly reduced aversive memory formation compared with GFP-treated animals (Fig. 2*C*). Thus, one-way ANOVA indicated a significant effect of

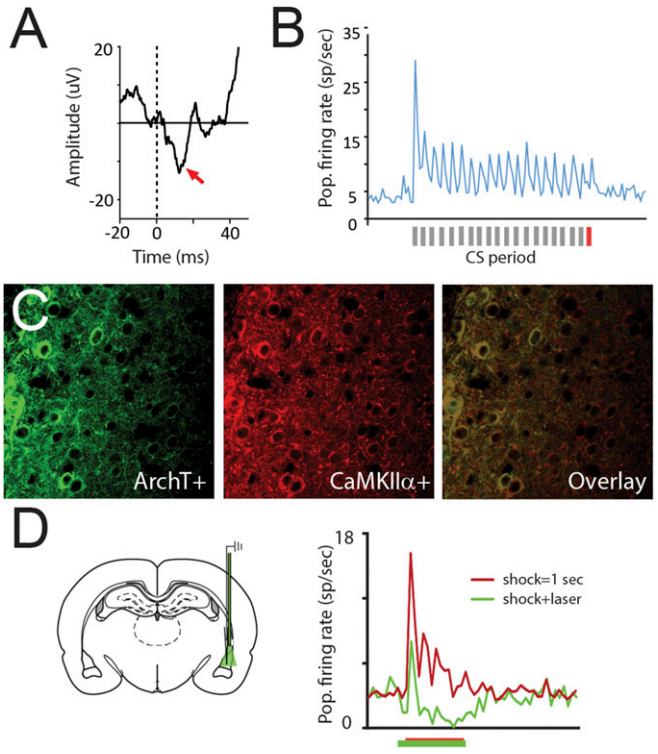

Fig. 1. Auditory CS-evoked responding in LA cells, preferential ArchT expression in LA pyramidal neurons, and optical inhibition of aversive shock-evoked responding. (*A*) Population-averaged auditory-evoked field potential response amplitude (*y* axis) in response to the final auditory CS pip (the time point at which the auditory stimulus will overlap with the aversive shock during subsequent training) before threat conditioning. The *x* axis "0" point represents the onset of the auditory stimulus. Red arrows denote the short latency portion of the response, which is known to be potentiated following fear conditioning and was used for the statistical analyses as in prior work. (*B*) Population-averaged CS-evoked firing rate responses during the preconditioning test session from single tone-responsive LA neurons (n = 11/38 total cells) recorded in awake, behaving animals (−5 − 25 s total time period in PSTH from CS onset at first gray bar, 250-ms bins on *x* axis). Gray bars under the *x* axis denote individual auditory pips during the CS with the final pip denoted by a red bar. (*C*) ArchT (*Left*) and CaMKIIα (*Center*), a marker for LA pyramidal neurons, immunolabeling in LA sections. Overlayed image is shown on *Right*. (*D*, *Left*) Graphical depiction of dual optogenetic illumination and LA neural recording of shock-evoked responses. (*Right*) Population-averaged peri-event time histogram showing footshock-evoked firing rate responses (in spikes per second) in extracellularly recorded LA neurons (n = 7) without (red trace) or with (green trace) overlapping laser illumination. Shock-evoked responses were significantly larger during the shock alone compared with shock + laser trials (Wilcoxon matched-pairs test: $Z = 2.20$, $P = 0.03$).

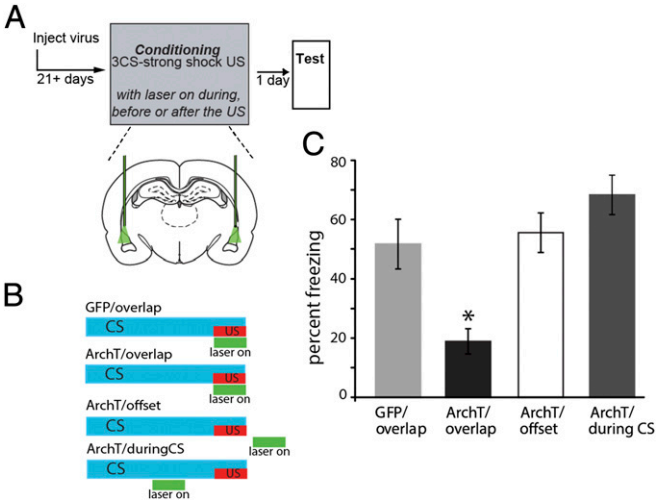

Fig. 2. Hebbian mechanisms are necessary for aversive memory formation. (*A*) Experimental design (*Top*) and graphical depiction of bilateral optogenetic inhibition of LA neurons (*Bottom*) during the training period of threat conditioning. (*B*) Schematic diagram of the experimental groups and temporal aspects of the study. Auditory CS on periods in blue, aversive shock US on periods in red, and 532-nm laser on periods in green. (*C*) Optogenetic inhibition during, but not before or after, the US period reduced threat memory formation. Percentage freezing (percent of total duration of a 20-s CS, *y* axis) at the long-term memory time point in the GFP/overlap group (n = 7), ArchT/Overlap (n = 9), and ArchT/offset and Arch/during CS (n = 7 for both) groups (*x* axis). An asterisk indicates statistically significant differences between the ArchT/overlap group and all other groups. All error bars indicate SEM.

group [$F_{(3, 26)} = 10.05$, $P = 0.0001$], and post-hoc Newman–Keuls test revealed significantly less freezing in the ArchT/overlap group compared with the GFP/overlap ($P = 0.002$), ArchT/offset ($P = 0.002$), and ArchT/during CS ($P = 0.005$) groups. This shows that activation of LA neurons specifically during the shock period, when the activity of CS inputs and postsynaptic pyramidal neurons is correlated, and not after or during the CS period, is necessary for the formation of threat memories.

We next tested whether inhibition of neural activity during the CS–US period also disrupted learning-induced plasticity in the LA in conjunction with threat learning. The A-EFP and auditory synapses in the LA are known to be enhanced by threat conditioning, and this requires recruitment of intracellular molecules and signaling processes in LA neurons (31–34, 43, 44). This allowed us to examine whether inhibiting neural activity during the auditory CS-aversive US overlap period reduced this learning-induced change in neural processing. For these experiments, A-EFP responses were measured before and after threat conditioning. In the training session, neural activity was optogenetically inhibited (as described above and in Fig. 3*A*) during or after the aversive US period. We found that inhibiting neural activity in LA pyramidal neurons during (ArchT/overlap group), but not after (ArchT/offset group), the shock US blocked threat conditioning-induced enhancement of auditory-evoked responses in LA neurons (Fig. 3 *B* and *C* and Table S1 and Fig. S1). Importantly, this blockade of A-EFP went in parallel with a blockade of behavioral memory formation (Fig. 3*D*). Thus, the percentage change measured 24 h after conditioning [long-term memory (LTM) test] from the pretraining baseline in the A-EFP was significantly larger in the ArchT/offset group compared with the ArchT/overlap group ($t_{14} = 2.77$, $P = 0.01$) as measured by two-tailed, unpaired Student's *t*-test (Fig. 3*C*). In addition, freezing behavior during the LTM test was significantly reduced in the ArchT/overlap group compared with the ArchT/offset group ($t_{14} = 5.04$, $P = 0.0002$) as measured by two-tailed unpaired Student's *t*-test (Fig. 3*D*). Together, these data demonstrate that disrupting correlated activity between auditory CS inputs and postsynaptic LA pyramidal neurons reduced learning-induced plasticity in vivo as well as aversive memory formation.

Coactivation of Hebbian and Neuromodulatory Mechanisms Is Sufficient to Produce Aversive Memories and Amygdala Associative Plasticity. The above findings show that Hebbian mechanisms are necessary for neural plasticity in the LA underlying threat

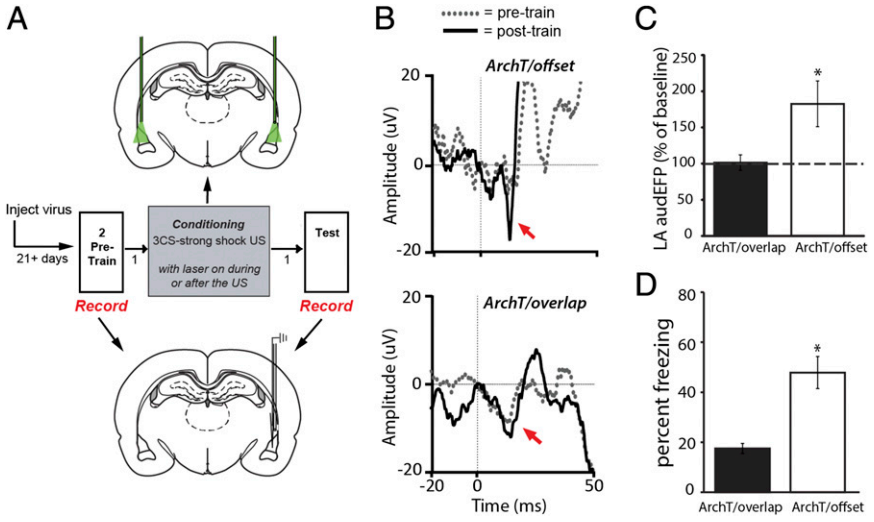

Fig. 3. Hebbian mechanisms are necessary for amygdala neural plasticity and aversive memory formation. (*A*) Experimental design for in vivo physiology experiment (*Middle*). Graphical depiction of laser illumination during threat training (*Top*) and physiological recordings before and after training (*Bottom*). (*B*) Laser inhibition during the US period blocks threat conditioning-induced plasticity. Sample traces of the amplitude of A-EFP responses for ArchT/offset group (*Top*) and ArchT/overlap group (*Bottom*) before (gray, dotted trace) and after (black trace) conditioning. Red arrows denote the short latency portion of the response that was used for the statistical analyses as in prior work. (*C*) Population-averaged A-EFP response pretraining vs. postraining. Percentage of pretraining baseline (*y* axis) in the ArchT/overlap (black bar, n = 9) and ArchT/offset (white bar, n = 7) groups. Dashed black line represents no change from baseline. (*D*) In the same animals as in *C*, optogenetic inhibition during the US period reduced threat memory formation. For *C* and *D*, an asterisk indicates a statistically significant difference between ArchT/offset and overlap groups. All error bars indicate SEM.

conditioning. However, recent work has demonstrated that activation of beta-noradrenergic receptors (β-ARs) also contributes to this form of learning (45). If multiple, codependent processes occur in parallel during threat learning, then inhibiting one of them may reduce memory formation.

To test whether Hebbian alone or a combined Hebbian plus neuromodulatory mechanism in the LA produces these neural and behavioral changes, it is necessary to examine whether correlated auditory CS input and postsynaptic pyramidal cell neural activity is sufficient to produce threat conditioning and LA associative plasticity. In previous work (35), we demonstrated that expressing the blue light-activated channelrhodopsin (ChR2) (46) in LA pyramidal neurons and pairing an auditory CS with direct, optogenetic depolarization of these cells produced threat learning. However, this learning was weak despite the fact that unusually large numbers of CS–US pairings were used. This may have been because activation of β-ARs is necessary to enhance Hebbian processes to produce threat learning.

To begin to test the question of whether Hebbian alone or a combination of Hebbian plus activation of β-ARs is sufficient to produce aversive memories, we used a minimal number of pairings of an auditory CS with a combined US ("ChR2/weak shock" group). The combined US included laser-induced depolarization of LA pyramidal neurons (to engage Hebbian processes) overlapping with a weak footshock (which produces NE release in the amygdala) (47) (Fig. 4*A*). In two control groups, the auditory CS was paired with either laser depolarization alone ("ChR2/no shock") or weak shock alone as a US ("GFP/weak shock"; these animals received a control AAV-GFP virus in the LA and laser illumination during the shock US). We previously showed preferential expression of ChR2 in LA pyramidal neurons (33, 35), and we replicated that here (90.1% of ChR2+ cells were also CaMKII+, a marker of pyramidal neurons). To activate LA neurons in this study, we used a laser stimulation protocol that we have shown robustly activates this cell population (35). Behaviorally, we found that robust aversive memories were only produced in the ChR2/weak shock group (Fig. 4*B*), demonstrating that learning was produced only when the CS was paired with a combination of laser activation of LA neurons and weak shock as a US. Supporting this, a one-way ANOVA indicated a significant effect of group [$F_{(2,\ 26)} = 19.72$, $P = 0.00001$]. Post-hoc Newman–Keuls test revealed significantly more freezing in the ChR2/weak shock group compared with the GFP/weak shock ($P = 0.001$) and ChR2/no shock ($P = 0.0001$) groups. There was no statistically significant learning apparent in either the GFP/weak shock or the ChR2/no shock group when comparing baseline levels of freezing (pre-CS1 freezing) to freezing induced by the first auditory CS (GFP/weak shock: $t_9 = 1.33$, $P = 0.23$; ChR2/no shock: $t_8 = 1.55$, $P = 0.16$), demonstrating that learning did not occur in response to CS-weak US or to the CS-laser stimulation alone pairings. Related to this, shock-responsive LA neurons recorded in vivo from awake, behaving animals (34/70 total neurons were shock responsive) distinguished between high and low shock intensities [comparing population averaged single-unit firing rate responses to high (10.3 ± 1.5 average spikes) and low (6.1 ± 1.5 average spikes) shock intensities] ($t_{33} = 4.03$, $P = 0.0003$). This demonstrates that LA neurons code for shock US intensity and suggests that one factor that may have limited the weak shock learning was reduced shock-evoked activation of LA neurons.

Thus, compared with weak shock or laser depolarization alone, Hebbian mechanisms synergized with weak shock to

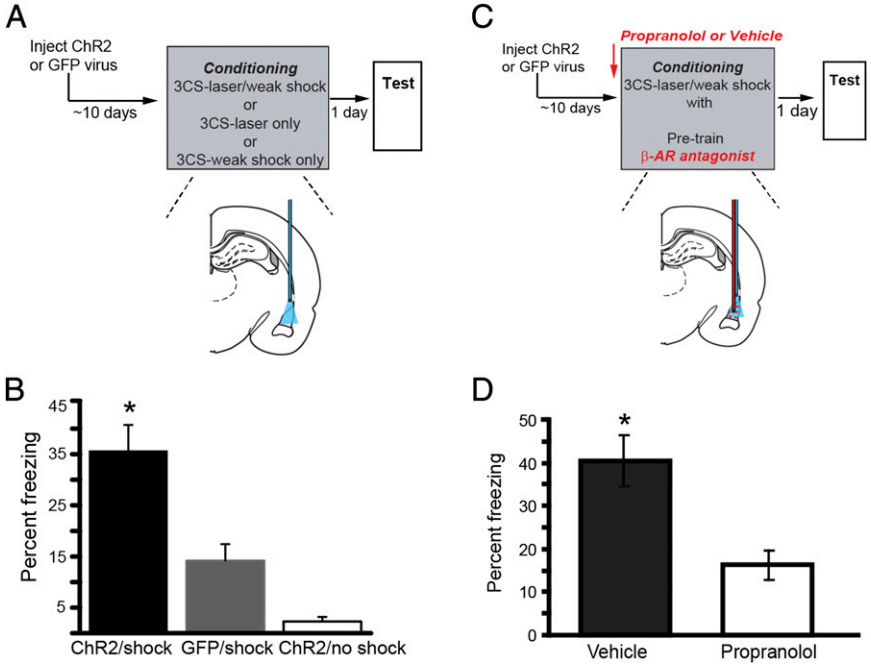

Fig. 4. Hebbian and β-AR–mediated processes are required to trigger aversive memories. (*A*) Experimental design (*Top*) and graphical depiction of optogenetic stimulation of LA neurons (*Bottom*) during the training period of threat conditioning. (*B*) Auditory CS paired with combined optogenetic stimulation of LA pyramidal neurons and weak shock produces supra-additive levels of threat conditioning. Graph shows percentage freezing (percent of total duration of a 20-s CS, *y* axis) at the LTM time point in the ChR2/shock group (black bar, $n = 12$), the GFP/shock group (gray bar, $n = 9$), and the ChR2/no shock group (white bar, $n = 8$) (*x* axis). An asterisk indicates statistically significant differences between the ChR2/weak shock group and all other groups. (*C*) Experimental design (*Top*) and graphical depiction of LA laser and propanolol delivery during threat conditioning (*Bottom*). (*D*) β-AR blockade in the LA reduced the threat conditioning produced by optogenetic stimulation and weak shock. Graph shows that freezing is significantly (denoted by an asterisk) reduced in the propranolol treated group (white bar, $n = 10$) compared with the vehicle-treated animals (black bar, $n = 10$). All error bars indicate SEM.

produce robust aversive memory formation. One explanation for this effect could be that the weak shock activated β-ARs in the LA. This may then have interacted with the Hebbian mechanisms engaged by laser activation of LA neurons at the same time that the auditory CS was present. To test this question, we examined the effect of blocking β-ARs in the LA on threat conditioning induced by pairing a CS with the weak shock/laser depolarization US (Fig. 4*C*). We found that microinjections of the β-AR antagonist propranolol into the LA before auditory CS-weak shock/laser depolarization pairings significantly reduced learning compared with animals that had received intra-LA vehicle injections before training (Fig. 4*D*). Specifically, we found that freezing behavior was significantly reduced in the drug-free long-term memory test in animals that had been treated with propranolol before training compared with vehicle-treated animals ($t_{18} = 3.56$, $P = 0.002$) as measured by two-tailed, unpaired Student's *t*-test. These behavioral findings suggest that, under moderate training conditions, Hebbian plasticity is not sufficient to produce associative threat learning but rather that activation of Hebbian and neuromodulatory mechanisms, involving β-ARs in the LA, interact to produce memory formation. In addition, the fact that CS-laser only pairings (ChR2/no shock group, Fig. 4*B*) were not effective in producing memory formation suggests that the aversive shock itself, not the CS or background levels of β-AR activity, activates β-ARs to produce learning.

We next sought to determine whether optogenetic activation combined with weak shock was also sufficient to produce potentiation of CS processing in the LA (as normal learning does) along with behavioral learning and whether these effects were dependent on β-AR activation in LA. To this end, we recorded LA A-EFP responses before and after pairing the auditory CS with either the weak shock/laser depolarization US ("ChR2/veh" group) or with weak shock US alone ("GFP/veh" group) (Fig. 5*A*). We found that CS-weak shock/laser depolarization pairings did produce training-induced potentiation of the A-EFP and threat conditioning, but that CS-weak shock alone pairings did not (Fig. 5 *B–D*). Furthermore, this potentiation and behavioral learning were dependent on β-AR activation in the LA as the potentiation of the A-EFP and threat conditioning by weak shock/laser depolarization training was abolished when propranolol was microinjected into the LA before training ("ChR2/prop" group, Fig. 5 *B–D*; note that vehicle was injected into the LA before training for the ChR2/veh and GFP/veh as a control for the ChR2/prop group). This was supported by statistical analysis as a one-way ANOVA indicated a significant effect of group [ChR2/veh vs. GFP/veh vs. ChR2/prop, $F_{(2,25)} = 4.05$, $P = 0.03$]. The learning-induced potentiation in the A-EFP was significantly larger in the ChR2/veh group compared with the GFP/veh group ($P = 0.03$) and the ChR2/Prop group ($P = 0.04$) as measured by a post-hoc Newman–Keuls test (Fig. 5 *B* and *C*, Table S2, and Fig. S2). Freezing behavior 24 h after conditioning was also significantly higher in the ChR2/veh group compared with the GFP/veh and ChR2/prop groups as a one-way ANOVA indicated a significant effect of group [$F_{(2,25)} = 104.82$, $P = 0.000001$], and post-hoc Newman–Keuls revealed significantly more freezing in the ChR2/veh group compared with the GFP/veh ($P = 0.0001$) and ChR2/Prop ($P = 0.0002$) groups (Fig. 5*D*). Thus, activation of LA pyramidal cells in combination with shock-

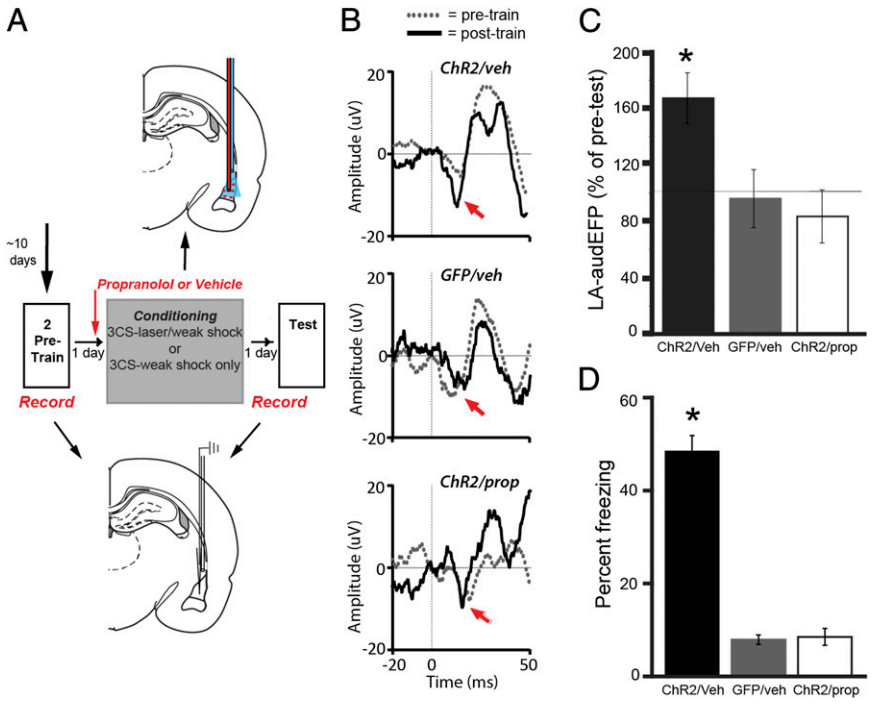

Fig. 5. Hebbian and β-AR–mediated processes are required to trigger amygdala neural plasticity and aversive memories. (*A*) Experimental design for in vivo physiology study (*Middle*). Graphical depiction of LA propanolol/vehicle and laser delivery (*Top*) and physiological recordings before and after threat training (*Bottom*). (*B*) Combining optogenetic stimulation with weak shock potentiates the A-EFP in the LA in a β-AR–dependent manner. Sample traces of the amplitude of A-EFP responses for the ChR2/Veh group (*Top*), the GFP/Veh group (*Middle*) and the ChR2/prop group (*Bottom*) before (gray trace) and after (black trace) conditioning. Red arrows denote the short latency portion of the response that was used for the statistical analyses as in prior work. (*C*) Population-averaged A-EFP response pretraining vs. postraining [percent of pretraining baseline (*y* axis) in the ChR2/Veh group (black bar, $n = 9$), the GFP/Veh group (gray bar, $n = 9$), and the ChR2/prop group (white bar, $n = 10$) . Gray line represents no change from baseline. (*D*) Percentage freezing during the LTM test in the same animals as in *C*. An asterisk indicates a statistically significant difference between the ChR2/Veh-treated group and both the GFP/veh and ChR2/prop groups. All error bars indicate SEM.

evoked β-AR activation was sufficient to produce neural plasticity of auditory processing in the LA and aversive memories.

To more specifically determine whether this β-AR/Hebbian synergy occurs in the LA to produce threat memories, isoproterenol, a β-AR receptor agonist, was microinjected directly into the LA before auditory CS-laser US pairings in the absence of any shock US (Fig. 6*A*). We found that auditory CS-laser US pairings alone were sufficient to produce threat learning only when β-ARs in the LA were concurrently activated by pretraining LA injections of isoproterenol ("ChR2/Iso" group, Fig. 6*B*). In contrast, this was not seen when vehicle was injected into the LA before CS-laser, US-alone pairings (ChR2/veh group) or when isoproterenol was injected before auditory CS alone presentations ("GFP/iso"). Statistical analysis supported this as a one-way ANOVA indicated a significant effect of group [$F_{(2,19)} = 5.21$, $P = 0.02$]. Post-hoc Newman–Keuls test revealed that the ChR2/Iso group froze more to the auditory CS than the ChR2/veh ($P = 0.015$) and GFP/Iso ($P = 0.03$) groups as measured 24 h after conditioning.

Thus, coactivation of Hebbian mechanisms and β-ARs in the LA was sufficient to produce aversive memories. In addition, these data suggest that the auditory CS does not provide the postsynaptic activity in LA neurons that is necessary for learning. If the CS by itself produced the correlated pre- and postsynaptic activity that synergizes with β-AR activation, then pharmacologically activating β-ARs should be sufficient in the presence of CS-alone presentations to produce learning, and this does not occur (GFP/iso group in Fig. 6*B*). However, because auditory CSs produce postsynaptic activity in LA neurons (Fig. 1 *A* and *B*) and optogenetic stimulation of auditory inputs to LA is sufficient as a CS when paired with a shock to produce learning (48), we cannot rule out the possibility that auditory CS-induced postsynaptic activity contributes, along with US-evoked activation of these cells, to Hebbian plasticity mechanisms.

Discussion

To examine whether Hebbian mechanisms alone are necessary and sufficient to produce learning and neural plasticity, we optically and pharmacologically manipulated LA neurons and examined the effects of these manipulations on in vivo learning-induced changes in neural processing in the LA and behavioral threat conditioning. We demonstrate that a temporally specific correlation between presynaptic activity (evoked by the auditory CS) and postsynaptic firing in LA neurons (by the aversive US) is necessary for neural plasticity in the threat learning circuit and aversive memory formation, providing support for the involvement of Hebbian mechanisms in associative neural plasticity mediating memory formation and consistent with previous findings (7, 49). However, when a small number of training trials was used, Hebbian mechanisms alone were not sufficient to produce neural plasticity in the LA and behavioral learning unless neuromodulatory β-ARs were coactivated. Together, these data suggest that with limited training a combination of Hebbian and neuromodulatory mechanisms triggers plasticity in the LA and behavioral associative learning.

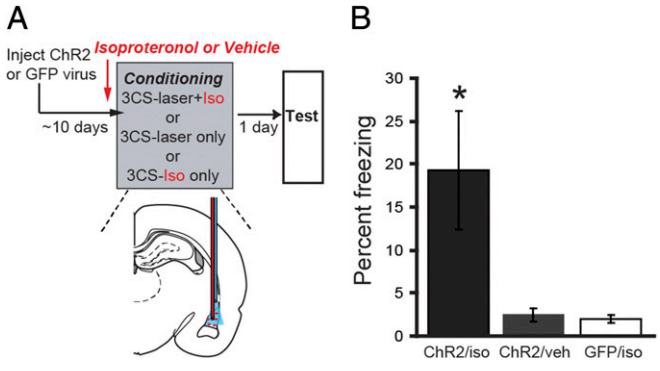

Fig. 6. Activation of Hebbian and β-AR–mediated processes is sufficient to produce aversive memory formation. (*A*) Experimental design (*Top*) and graphical depiction of LA laser and isoproterenol delivery during threat conditioning (*Bottom*). (*B*) Laser activation of LA pyramidal cells combined with β-AR stimulation produces threat learning. Graph shows percentage freezing at the LTM test in the ChR2/Iso group (black bar, $n = 8$), the ChR2/veh group (gray bar, $n = 8$) and the GFP/Iso group (white bar, $n = 6$). An asterisk indicates statistically significant differences between the ChR2/Iso group and all other groups. All error bars indicate SEM.

It is possible that the optogenetic manipulations used in this study affected plasticity in other parts of the threat circuit, as is known to occur with learning (50). For example, optogenetic inhibition of LA neurons during the US period may have reduced threat learning by attenuating learning-related plasticity in other brain regions to which the stimulated LA neurons project. However, optogenetic inhibition reduced the learning-dependent increase in the CS-evoked local field potential response in the LA. These responses are known to be dependent on intracellular signaling pathways within LA neurons (such as the mitogen-activated protein kinase pathway) and can occur independently of plasticity in areas that convey auditory information to the LA such as the auditory thalamus (44). Although effects on other brain regions cannot be completely ruled out, this suggests that the observed results are due to changes in local synaptic plasticity within the LA. Relatedly, the fact that local LA manipulations of β-ARs affect LA neural plasticity and threat learning further suggests that the measured behavioral and physiological effects were due to regulation of local LA processes and not solely to effects on other brain structures.

Previous work has shown that β-ARs modulate the initial acquisition, and not the consolidation (in contrast to hippocampal-dependent memories), of threat conditioning (45, 51). Thus, our results along with this previous work provide strong evidence that Hebbian and neuromodulatory processes function together during learning to synergistically engage LA neural plasticity mediating this form of learning. Furthermore, the fact that Hebbian processes appear to be sufficient when many training trials are used (33) suggests that weakly aversive, iterative learning may use purely Hebbian plasticity mechanisms and/or that noradrenergic signaling may regulate the gain or strength of aversive memory formation. It will be important in future studies to determine whether neural plasticity mediating behavioral learning in other neural circuits or under different learning conditions is mediated by similar mechanisms.

Although we propose that Hebbian mechanisms are not sufficient to produce LA plasticity and aversive learning, important alternate interpretations are possible. It is known that the LA is involved in both appetitively and aversively motivated learning and that distinct populations of aversive and reward-responsive cells exist in the amygdala (52–54). One alternate interpretation of our results is that Hebbian mechanisms alone might be sufficient to produce LA plasticity without β-AR activation, but that CS-laser US pairings in the absence of β-AR stimulation produced plasticity in an indiscriminate population of neurons (both aversive and reward-responsive neurons, for example) during learning, some of which generate competing behavioral responses to threat. In this scenario, this other cell population (possibly reward-responsive cells), which does not output to produce defense responses but instead produces competing behaviors, could have acted to oppose the expression of defensive behaviors during memory testing. β-ARs in this case would not be involved in producing plasticity, but rather in directing it to the threat-specific neurons in the LA. This is unlikely, however, because if this were true, then auditory CS-laser stimulation pairings when

β-ARs in the LA are blocked should produce enhancement of auditory inputs to the LA without producing learning, and this did not occur (Fig. 5 *B* and *C*). In fact, these data show that the plasticity in the LA could be induced only when both shock-evoked activation of β-ARs *and* depolarization of the LA pyramidal neurons occurred. Another possibility is that not enough cells were activated by the ChR2 stimulation to produce aversive learning through Hebbian mechanisms. This is also implausible as we have shown previously that the same optogenetic stimulation in ChR2-treated animals as is used in these experiments activated a large population of LA neurons (~63% of pyramidal neurons) and, like a shock US, caused robust action potential firing in LA cells (35, 36). Furthermore, pairing an auditory CS with optical activation of LA pyramidal neurons produced aversive memories and neural plasticity when β-ARs were activated (Figs. 4–6). This further supports the idea that the optogenetic stimulation activated enough cells and with enough strength to produce behavioral and physiological changes that simply required another cofactor (β-AR activation) to occur.

The magnitude of the changes in neural processing in the LA observed using optogenetic stimulation (Fig. 5) were comparable to changes produced by actual learning (Fig. 3) (55). Because a relatively small population of LA cells participate in the fear memory trace (26, 56, 57) and the plasticity produced by our manipulations is comparable to normal learning, it is likely that plasticity was produced in only a small fraction of LA neurons in our experiments despite the fact that large numbers of cells were light-stimulated. Thus, other factors such as the availability or levels of intracellular signaling molecules, local competition between cell assemblies, or the availability of auditory CS inputs to a given cell may have played a role in allocation of plasticity to a small proportion of the stimulated neurons (57–59). An intriguing question raised by these results is how β-AR activation in the LA modulates Hebbian processes and synaptic plasticity in this small cell population to produce threat learning. One possibility is that activation of β-ARs, which are Gs protein-coupled receptors, modulates Hebbian, calcium-dependent processes through direct interactions in intracellular signaling networks (17). This type of mechanism has been elegantly identified in invertebrates (3, 11, 16) and in studies of mammalian synaptic plasticity (7, 13, 14, 18, 19) and could serve both to facilitate plasticity induction and learning and to enhance long-term memory consolidation through synergistic action occurring within LA glutamatergic projection neurons (for reviews see refs. 4, 11, 12, 17). However, in addition to being expressed in glutamatergic neurons in LA, β-ARs are also expressed in presynaptic inputs to these neurons and in GABAergic and astrocytic cells (60). Thus, the action of β-ARs could be mediated through a variety of mechanisms. One mechanism through which β-AR activation could modulate Hebbian processes during threat learning could be through actions on spike timing-dependent plasticity (STDP) (6). By regulating local LA networks and/or intracellular signaling cascades, β-AR activation could modulate the timing, directionality, and/or size of STDP in the LA to produce aversive learning. Although there has been a great deal of work in mammalian systems using slice physiology approaches to identify different mechanisms through which neuromodulators regulate synaptic plasticity and long-term potentiation, it will be important in future work to determine how β-ARs act in vivo during learning to modulate neural plasticity mediating actual memory formation.

Other neuromodulatory systems in the LA (17, 61) and plasticity in other parts of the defense circuit (50, 62) appear to also be important for threat conditioning. These mechanisms may work together with Hebbian and β-AR–mediated plasticity processes in the LA to produce learning and the subsequent full and appropriate expression of threat memories. For example, the neuromodulator dopamine is known to suppress feed-forward inhibition in the LA and enhance long-term potentiation, and this may serve to directly regulate Hebbian plasticity mechanisms (63, 64). However, it is not clear at present what sorts of stimuli activate these other neuromodulatory inputs to the LA or when activation of these systems is important during aversive memory formation and/or consolidation. In addition, plasticity in other parts of the threat-processing circuit may function on top of the contribution of LA plasticity (which we have targeted here) to reduce generalization and increase the robustness and duration of aversive memories. An intriguing future direction will be to define how other neuromodulators in the LA and plasticity in other parts of the circuit work in concert with the β-AR and Hebbian plasticity mechanisms in the LA to produce threat conditioning.

Intensely arousing experiences (such as those that are rewarding or aversive) can produce learning by activating "teaching signal" neural circuits that trigger neural plasticity and memory formation. Although a number of putative teaching signal circuits have been studied (65–69), the identity of the signals that are activated by arousing experiences in brain regions that store behavioral memories to trigger neural plasticity and learning is not entirely clear. In addition to shedding light on mechanisms of plasticity, the discovery that both neuronal depolarization and noradrenergic signals activated by aversive USs are necessary to engage memory formation also informs our understanding of the identity of the teaching signal for amygdala plasticity mediating threat learning. This parallel instructive signaling at the level of neural circuits and intracellular signaling pathways could enhance the flexibility and computational power of the learning system. This type of teaching signal mechanism may confer an evolutionarily adaptive benefit on the organism and be conserved across neural circuits and species (3, 11, 16, 70). It will be important to determine how these signals are computed in these parallel neural pathways to the LA and how they interact in LA neuronal intracellular signaling networks. Dysfunction in these aversive instructive circuits could be an important factor in chronic pain and anxiety disorders that are typified by exaggerated aversively motivated learning. A deeper understanding of these circuits may provide new treatment avenues for these debilitating disorders.

Materials and Methods

Subjects. 275–300 g male Sprague-Dawley rats were housed individually on a 12-h light/dark cycle with ad libitum food and water. All experimental procedures were approved by the New York University Animal Care and Use Committee or the Animal Care and Use Committees of the RIKEN Brain Science Institute, and conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (71).

Viral Vectors. Vector construction, production, and purification for ChR2 are as described previously (35). Lentiviral vectors containing ArchT were produced by and purchased from the University of North Carolina Vector Core.

Stereotaxic Cannula Implantation and Virus Injection. For behavioral, anesthetized electrophysiological, and double-labeling immunocytochemistry experiments, animals were anesthetized, surgerized, and injected either directly with virus or implanted with a chronic guide cannula (bilaterally for ArchT experiments, unilaterally for ChR2 experiments) and then injected with virus into the LA. These procedures were as described previously (35). For awake, behaving electrophysiological experiments, an insulated stainless steel recording wire (1–2 MΩ) (FHC, Inc.) that extended 1.4 mm from the base of the infusion cannula was attached to one guide cannula. The tip of the electrode was targeted to the LA (stereotaxic coordinates from Bregma were anterior–posterior: −3.0 mm; dorsal–ventral: −8.0 mm; and medial–lateral: 5.4 mm) In addition, two silver balls, placed contralaterally above the neocortex served as a reference and ground. For all experiments, guides were affixed to the skull using surgical screws and dental cement.

Immunocytochemistry. To determine the specificity of opsin (ArchT and ChR2) targeting in CaMKIIα+ cells, rats were overdosed and perfused and sections were cut and underwent immunohistochemistry as described previously (35).

Following immunohistochemistry, confocal analysis was applied to three to four amygdala sections (at the same rostro-caudal position; $n = 2$). The proportion of double-labeled cells was calculated as the number of opsin+/CaMKIIα+ double-labeled cells divided by the total number of opsin+ cells. To quantify the infection efficacy of ArchT, the total number of ArchT+/CaMKII+ cells was calculated and divided by the total number of CaMKII+ cells in the counting window (three sections/animal, matched for rostro-caudal position; $n = 4$). For behavioral experiments, sections were processed as described above, and an experimenter blind as to animal and treatment group assessed whether ArchT was specifically expressed in LA neurons and whether the tip of the guide cannula was dorsal and proximal to the LA. If these criterions were not met, animals were not included in the analysis.

In Vivo-Anesthetized Electrophysiological Recording and Laser Stimulation. Three to four weeks after virus injection, animals were anesthetized as described in *Stereotaxic cannula implantation and virus injection* and placed in a stereotaxic apparatus. A tungsten electrode (5 MΩ impedance; AM Systems) was mounted alongside a fiber optic cable (200-μm core diameter, 0.37 numerical aperture). The tip of the electrode extended ∼0.3 mm beyond the tip of the electrode. The fiber optic cable was attached to a 532-nm diode pumped solid-state laser (Shanghai Laser and Optics Century Co.), which output 15–20 mW from the tip of the fiber optic cable. The electrode/cable apparatus was targeted to the dorsal tip of the LA. During surgery, electrodes were placed into the contralateral hindpaw for electrical shock delivery. Once recording began, periodic footshocks were given as the electrode was advanced in 1-μm steps until single shock-responsive cells were isolated. Signal acquisition and analysis were as described previously (35). Once single LA neurons were isolated, footshock alone trials (2-ms footshock pulse duration at 7 Hz for 1 s) were intermixed with laser illumination overlapping with footshock (laser onset occurred 250 ms before and terminated 50 ms after footshock).

Stimulus-evoked responses were analyzed by plotting peristimulus time histograms (PSTHs) triggered by the footshock onset using Neuroexplorer data analysis software. For each cell, raw spike counts in each bin of the PSTH were converted to firing rates using the equation $R_i = S_i/N(\Delta t)$, where R_i is the firing rate for the ith bin of the PSTH (in Hz), S_i is the raw spike count in the bin, N is the number of trigger events for the PSTH, and Δt is the PSTH bin size in seconds. Each cell's PSTH was averaged to provide a population-averaged neural response. PSTH bin size was 100 ms. The average number of spikes during each shock period for each cell was calculated and compared between footshock alone and footshock + laser trials using a nonparametric Wilcoxon matched-pairs test.

Behavioral Conditioning Experiments. In vivo optical stimulation was as described previously (35). For the ArchT experiments, the animals were placed into a sound isolating chamber and underwent one of three conditioning protocols. The "overlap" groups (which had received ArchT or GFP control virus) received three auditory CS–US (1.0 mA footshock for 1 s coterminating with the CS) pairing with laser illumination occurring 250 ms before US onset and lasting until 50 ms after US offset. The CS for all experiments was a series of 5-kHz tone pips (at 1 Hz with 250 ms on and 750 ms off) for 20 s, and the US onset occurred and coterminated with the final CS. The "offset" and "during CS" groups were identical except that laser illumination occurred either after (randomly 30–40 s) for the offset group or before (overlapping with the 10th CS pip) the US period for the during CS group. For the ChR2 experiments, animals experienced one of two different conditioning protocols. The ChR2/weak shock and GFP/weak shock groups received three CS–US (1 s, 0.32-mA footshock) pairings and 473 nm of laser illumination during the US period [20 Hz stimulation for 1 s, as described previously (35)]. Stimulation (20 Hz) was chosen based on a previous study from our laboratory showing that this protocol produced robust action potential firing in LA neurons similar to actual shocks (35). Another group of rats received identical treatment except that no footshock was used (i.e., they received three CS–laser stimulation pairings). In subsequent experiments, 0.3 μL of the β-AR antagonist (propranolol, 0.4 μg/μL), agonist (isoproterenol, 5 μg/μL), or vehicle (artificial cerebrospinal fluid) were microinjected into the LA before the conditioning phase in experiments described in *Results*. Twenty-four hours after conditioning, animals received a memory test in which behavioral freezing responses were recorded in response to five CS-alone presentations (random around 2-min intertrial intervals) in a novel context.

Data acquisition and rating were as described previously (35). Freezing scores averaged across all five CS presentations during the memory test were statistically analyzed and compared using ANOVA statistical tests followed by post hoc analysis using the Newman–Keuls test. For all reported data, variance is expressed as SEM, and $P < 0.05$ was considered significant for all experiments.

In Vivo Awake, Behaving Local Field Potential Physiology. Rats were habituated to the memory test context (sound-isolated chamber with peppermint odor and without electrified grid bars) and to the conditioned stimulus (5-kHz tone pips at 1 Hz with 250 ms on and 750 ms off for 20 s) during 2 consecutive days. After 5 min of an acclimation period, rats received three pre-exposures of the CS alone during each of the 2 habituation days, with a variable intertrial interval (130 s on average). LA local field potentials were recorded during these two sessions. On the third day all rats were conditioned as previously described in *Behavioral conditioning experiments*. Twenty-four hours after conditioning, rats were placed back in the memory test chamber; after 5 min of acclimation, four CSs were delivered, and LA local field potentials and freezing behavior were recorded. Stimulus presentation in the testing context was automated using Spike2 software (CED). Electrical signal was enhanced and filtered (gain, X10,000; low pass, 0.1 Hz; high pass, 1,000 Hz) using a model 1700 differential AC amplifier (A-M Systems) and transformed into a digital signal through a power 1401 CED interface (55). Duration of freezing during CS (in seconds) and local field potential waveform was averaged from every pip from three or four CS presentations within every session (some CS-evoked field potentials were discarded if contaminated by noise during the CS period). The waveform was averaged using Spike2 version 6.16 software. Waveforms were normalized to 0 μV at the onset of the CS for each rat and for each stage of memory (habituation and long-term memory) to be able to make comparisons among days and rats. The LA auditory-evoked field potential (LA-AEFP) amplitude (μV) was defined as the amplitude of the waveform from the onset of the CS (time 0) to the minimum of the first negative peak occurring within the 12- to 16-ms time window in which auditory-evoked responses are known to occur in the LA (72). For histological verification of recording location, electrolytic marking lesions were made before animals were perfused. Latencies of the LA-AEFP, average waveform amplitudes, and average freezing were statistically analyzed and compared using two-tailed unpaired, or paired, t-test or ANOVA followed by Newman–Keuls post hoc tests. For all reported data, variance is expressed as SEM, and $P < 0.05$ was considered significant for all experiments.

In Vivo, Single-Unit Electrophysiological Recordings in Awake, Behaving Animals. For all physiological experiments done in awake, behaving animals, rats received surgical implantation of bundles of 16 tungsten stereotrodes. For recording auditory CS responses before conditioning, animals were presented with five CSs (identical to CSs used for behavioral experiments), and single-unit spiking responses were recorded. For analysis purposes, firing rate of individual cells was calculated and then averaged across all tone-responsive cells to generate a population-averaged peri-event time histogram. For recording shock-evoked responses, rats were presented with larger (2 mA, 1 ms, and 14 Hz for 1 s) and smaller (1 mA, 1 ms, and 7 Hz for 1 s) contralateral (from recording side) eyelid shocks, and single-unit spiking responses were recorded from LA neurons. Larger and smaller shock-evoked responses in shock-responsive cells were compared using paired t-tests of spikes over the 99% confidence interval under the two conditions. Shock-response experiments were performed in well-trained animals, and both shock intensities were preceded by auditory CSs. To classify a cell as shock- or tone-responsive, z-score averaged peri-event time histograms were generated and individual cells had to exhibit two consecutive bins with a z-score >1 and one bin with a z-score >2. For both experiments, data were acquired through a Neuralynx data acquisition system, and spike clustering was done offline using Neuralynx SpikeSort 3D software. Single-unit isolation was achieved by ensuring that clusters remained stable throughout a recording session and that spike trains had a refractory period greater than 1 ms.

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Supporting Information

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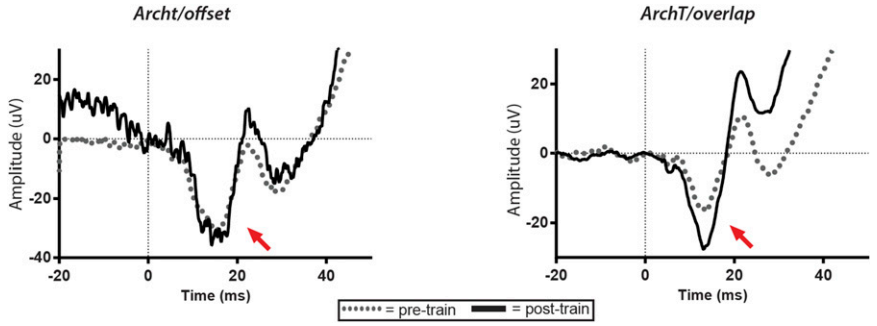

Fig. S1. Laser inhibition during the US period blocks threat conditioning induced plasticity in the lateral amygdala. Population averaged (across all rats in each group) traces of A-EFP responses for ArchT/offset group (*Left*) and ArchT/overlap group (*Right*) before (gray, dotted trace) and after (black trace) conditioning. Red arrows denote the short latency portion of the trace which was used for the statistical analyses as in prior work.

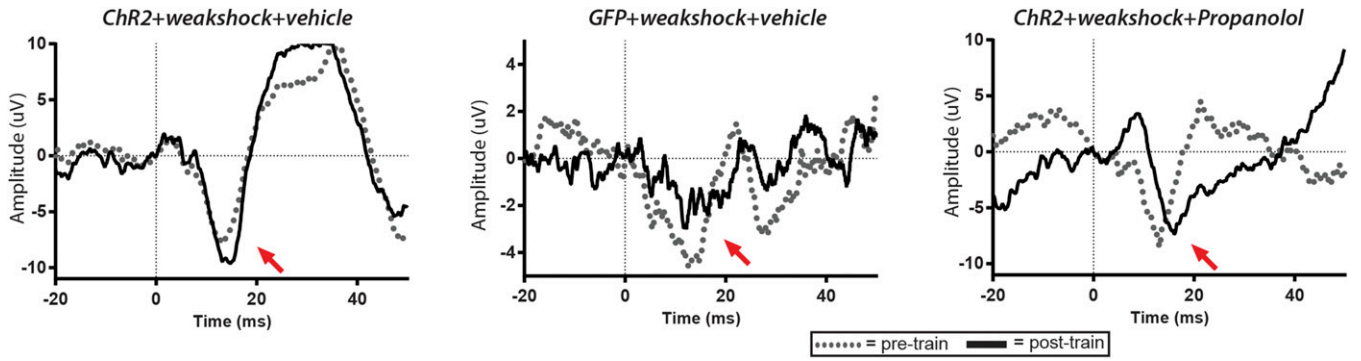

Fig. S2. Activation of Hebbian and β-AR mediated processes are required to trigger threat conditioning induced plasticity in the amygdala. Population averaged traces of A-EFP responses for ChR2 & weak shock & vehicle group *(Left),* GFP and weak shock and vehicle group (*Center*) and ChR2 & weak shock & propanolol group (*Right*) before (gray, dotted trace) and after (black trace) conditioning. Red arrows denote the short latency portion of the response which was used for the statistical analyses as in prior work.

Table S1. Latency and amplitude data for the ArchT in vivo physiology study

	LA-AEFP				
Group	Latency (ms) HAB	Amplitude (μV) HAB	Latency (ms) LTM	Amplitude (μV) LTM	(% from HAB) LTM
ArchT/offset (n = 7)	13.0 ± 0.7	19.5 ± 9.7	13.1 ± 0.6	28.9 ± 12.2*	191.9 ± 21.3†
ArchT/overlap (n = 9)	14.1 ± 0.7	32.8 ± 6.1	14.1 ± 0.7	33.12 ± 7.3	102.4 ± 17.7

LA-AEFP latencies were not statistically different during habituation to the context (t_{14} = 1.17, not significant) or during the LTM test (t_{14} = 0.93, not significant). No difference between groups was observed in the LA-AEFP amplitude during habituation (t_{14} = 1.21, not significant), showing no effect of group in the baseline amplitude. However, in the ArchT/offset group, amplitude during LTM was significantly different from habituation (t_6 = 3.46, P < 0.01), demonstrating potentiation of the LA-AEFP. The LA-AEFP amplitude comparison among groups during LTM revealed no difference (t_{14} = 0.32, not significant), but the comparison of the LA-AEFP amplitude represented as a percentage of baseline showed a significant difference among groups (t_{14} = 3.26, P < 0.01); the ChR2/Veh group differed from the GFP/Veh and ChR2/Prop groups. HAB is the habituation session prior to training in which the CSs are presented alone.

*Different from its own habituation value, P < 0.05.

†Statistically significant difference from the other group.

Table S2. Latency and amplitude data for the ChR2 in vivo physiology study

	LA-AEFP				
Group	Latency (ms) HAB	Amplitude (μV) HAB	Latency (ms) LTM	Amplitude (μV) LTM	(% from HAB) LTM
ChR2/Veh ($n = 9$)	13.3 ± 0.5	7.0 ± 1.6	14.9 ± 0.5	10.1 ± 1.6*	167.3 ± 18.0†
GFP/Veh ($n = 9$)	12.9 ± 0.9	4.9 ± 1.4	12.7 ± 0.8†	3.9 ± 1.3	96.1 ± 20.6
ChR2/Prop ($n = 10$)	13.3 ± 1.1	8.5 ± 3.1	15.3 ± 0.6	7.4 ± 3.7	83.5 ± 18.8

LA-AEFP latencies were not statistically different during habituation to the context [$F_{(2,25)} = 0.08$, not significant]. A one-way ANOVA test revealed a group effect in the LA-AEFP latency during the LTM test [$F_{(2,25)} = 4.49$, $P < 0.05$]; a post hoc test shows that GFP/vehicle latency differs from the other two groups ($P < 0.05$). No difference was observed in the LA-AEFP amplitude during habituation [$F_{(2,25)}$ 0.66, n.s.], showing no effect of group in the baseline amplitude. The LA-AEFP amplitude in the ChR2/Veh group during LTM was different from habituation ($t_8 = 3.88$, $P < 0.01$), revealing an LA-AEFP potentiation. Relatedly, the comparison of the LA-AEFP amplitude represented as a percentage of baseline showed a significant difference among groups [$F_{(2,25)} = 5.49$, $P < 0.01$]; the ChR2/Veh group differed from the GFP/Veh and ChR2/Prop groups. HAB, habituation to tone.

*Differs from its own habituation value, $P < 0.05$.

†Statistically significant difference from other groups.