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Authors

Yang, Yang
Liu, Dan-qian
Huang, Wei
et al.

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Selective synaptic remodeling of amygdalocortical connections associated with fear memory

Yang Yang[1,5,6], Dan-qian Liu[1,2,5], Wei Huang[1 3], Juan Deng[1,2], Yangang Sun[1], Yi Zuo[43], and Mu-ming Poo[1]

1. Institute of Neuroscience, State Key Laboratory of Neuroscience, CAS Center for Excellence in Brain Science and Intelligence Technology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China.

2. Institute of Neuroscience, Graduate School, University of Chinese Academy of Sciences, Beijing, 100049, China.

3. Current address: Eli and Edythe Broad Center of Regeneration Medicine and Stem Cell Research, University of California San Francisco, San Francisco, CA 94143, USA

4. Department of Molecular, Cell and Developmental Biology, University of California Santa Cruz, Santa Cruz, California 95064, USA.

45. These authors contributed equally to this work.

56. To whom the correspondence should be addressed (E-mail: yangyang@ion.ac.cn).

Neural circuits underlying auditory fear conditioning have been extensively studied. Here we identified a previously unexplored pathway from the lateral amygdala (LA) to the auditory cortex (ACx), and found that selective silencing of this pathway using chemo- and optogenetic approaches impairs fear memory retrieval. Dual-color *in vivo* two-photon imaging of mouse ACx showed pathway-specific increases in the formation of LA axon boutons, dendritic spines of ACx layer-5 pyramidal cells, and putative LA-ACx synaptic pairs after auditory fear conditioning. Furthermore, co-imaging of pre- and postsynaptic structures showed that essentially all new synaptic contacts were made by adding new partners to existing synaptic elements. Together, these findings identify an amygdalocortical projection that plays an important role in fear memory expression and is selectively modified by associative fear learning, and unravel a distinct architectural rule for synapse formation in the adult brain.

Introduction

Associative learning enables an animal to adapt to and survive in a complex environment. In classical auditory fear conditioning, animals learn to associate a neutral stimulus (a sound) with a foot-shock, and exhibit fear responses to the sound presentation. Amygdala is critical for the formation of auditory fear memory[1]. Previous studies have also shown that the auditory cortex (ACx) is required for auditory fear learning [2-4], and that fear conditioning could induce rapid and long-term changes in neuronal responses and spine dynamics in ACx. However, which synapses in ACx underwent modification remain unclear.

Long-term *in vivo* two-photon imaging has been used to monitor structural remodeling of synaptic connectivity, as was shown by changes in presynaptic boutons or postsynaptic spines that represent the formation or elimination of synapses. Previous studies have shown that sensory experience and learning can induce changes in the turnover of presynaptic axon boutons[9] and postsynaptic dendritic spines. To further explore synaptic dynamics in specific pathways, concurrent imaging of pre- and post-synaptic structures in identified connections is required. This approach, although successfully applied in studying synaptic dynamics in hippocampal slices[16], has not been used for *in vivo* imaging of the neocortex.

In this study, combining tracing methods with electron microscopy (EM), we identified a projection to ACx that originates from lateral amygdala (LA), the major

input region of the amygdala. Chemo- and optogenetic silencing of LA axons in ACx during fear recall test greatly reduced animals' fear responses, suggesting that the LA-ACx pathway plays an important role in the expression of fear memory. By co-imaging labeled axons originating from brain regions projecting to ACx and apical dendrites of pyramidal neurons in ACx, we were able to monitor the dynamics of putative synaptic pairs in specific pathways *in vivo*. We observed a selective increase in bouton and spine formation at LA-ACx connections after fear conditioning, resulting in a gradual increase of LA-ACx synapses. By contrast, no fear memory-related synaptic rewiring was observed in connections from medial geniculate body or anterior cingulate cortex to ACx. Furthermore, concurrent imaging of pre- and postsynaptic structures showed that nearly all newly formed synaptic contacts were made by adding new boutons to existing spines, or new spines to existing boutons. These findings reveal an amygdalocortical projection involved in fear memory expression and suggest an architectural rule for synapse formation in the adult brain.

Results

Fear learning with complex sound requires auditory cortex

Previous work has suggested that auditory cortex (ACx) is not required for auditory fear learning when pure tones are used as conditioned stimulus (CS)[17]. We thus used a train of short tone beeps, which co-terminated with a foot shock, for fear conditioning (Fig. 1a). Conditioned mice received 5 repeats of paired CS-footshock, whereas control mice received 5 repeats of explicitly unpaired CS and footshock. Animals

were tested with CS 1 d after conditioning. Conditioned mice showed significantly higher freezing responses than control mice (Fig. 1a, Supplementary Fig. 1a).

We found that bilateral infusion of $GABA_A$ receptor agonist muscimol or N-methyl-D-aspartic acid (NMDA) receptor antagonist (*2R*)-amino -5-phosphonopentanoate (APV)[18] in ACx prior to fear conditioning largely prevented the mice from developing post-conditioning freezing responses, suggesting that the activity and NMDA receptor-dependent plasticity in ACx were necessary for auditory fear conditioning (Fig. 1b, Supplementary Fig. 1b, c, Supplementary Fig. 2). Infusion of both drugs immediately after conditioning also impaired fear responses, indicating that activity and plasticity in ACx in the post-conditioning period were also required for post-conditioning fear memory consolidation (Supplementary Fig. 1d). Furthermore, inactivation of ACx by muscimol injection immediately before the recall test also reduced freezing responses (Fig. 1b). Thus, ACx is required for both acquisition and expression of auditory fear memory.

~~Direct projections from~~ LA ~~to~~ ACx pathway plays a key role in fear memory expression

Layer 1 (L1) of sensory cortices receives long-range inputs from higher-order brain regions that are involved in different behaviors. To examine the origins of the long-range projections to ACx, we injected retrograde fluorescent microspheres (Retrobeads) into the superficial layers of ACx (Fig. 1c). We observed densely labeled

neurons in the medial geniculate body (MG, Fig. 1c), including the medial division that showed fear conditioning-induced changes[21]. Unexpectedly, we also found retrograde labeling in the lateral amygdala (LA, Fig. 1c), which was previously considered to be an input region of the amygdala[22]. We injected AAV-EGFP into LA, and found LA axons terminating in ACx, thus confirming that LA sent direct projections to ACx (Fig. 1d).

To ~~examine~~ explore whether this pathway is involved in fear learning and memory, we used the Designer Receptors Exclusively Activated by Designer Drugs (DREADD) system, *i.e.*, Gi-protein-coupled receptorhM4D and its ligand clozapine-N-oxide (CNO), to selectively silence the LA-ACx pathway during fear acquisition or expression (Fig. 1e). We first validated that local CNO application in ACx could inhibit synaptic transmission of hM4D-expressing LA axons using slice recording[25]. We co-injected AAV-ChR2 and AAV-hM4D into LA (Supplementary Fig. 3), and performed whole-cell recordings on ACx Layer 5 neurons in coronal brain slices containing ACx neurons and axons from LA. We recorded light-evoked excitatory postsynaptic currents (EPSCs) with blue light on the superficial layers of ACx, where LA axons terminate. We found that addition of CNO to the recording solution completely abolished the light-evoked responses (5 cells from 3 animals, p = 0.003, paired t-test, Fig. 1f). To examine how selective silencing of LA-ACx pathway affects behavior, we ~~Wetheninjected only AAV- hM4Dinto LA, and~~ inhibited the synaptic transmission of the LA-ACx pathway by expressing hM4D in LA and locally

infusinged CNO into ACx ~~to inhibit the synaptic transmission of LA axons in ACx,~~ during either fear conditioning or fear recall test (Fig. 1e, Supplementary Fig. 4a). Because of the low success rate in targeting LA for virus injection, we inhibited the LA-ACx pathway unilaterally, and infused muscimol to silence the contralateral ACx. In control experiments, we silenced the right side of ACx with muscimol, and either infused CNO into left ACx of mice injected with AAV-EGFP in left LA, or infused saline into left ACx of mice injected with AAV-hM4D in left LA, during fear conditioning or recall. In all cases, mice exhibited strong freezing responses (Fig.1g). In chemogenetic inactivation experiments, we also silenced the right side of ACx with muscimol, and infused CNO into left ACx of mice injected with AAV-hM4D in left LA. We found that the chemogenetic inactivation of LA-ACx pathway during fear recall test but not fear learning significantly impaired animals' fear responses (Fig. 1~~e,~~ g, Supplementary Fig. 4b), indicating that this pathway is involved in the expression of fear memory.

Similarly, we ~~further~~ performed pathway-specific optogenetic experiments ~~and found that optogenetic inhibition of LA-ACx pathway could also impair fear memory recall (Fig. 1h). This was accomplished by injecting AAV-eArch3.0 into LA, and~~ by photo-inhibiting eArch3.0-expressing LA axons in ACx during fear recall test (Fig. 1h, Supplementary Fig. 4c). We found that optogenetic inhibition of LA-ACx pathway could also impair fear memory recall (Fig. 1h). Together, these results showed that LA projects directly to ACx, and this projection plays an important role in the expression

of fear memory.

~~Conditioning-induced increase in B~~bouton formation in LA axons increases after conditioning

To examine the structural plasticity of LA projections in ACx associated with fear memory, we injected AAV-hSYN-EGFP into LA, and imaged the labeled LA axons in L1 of ACx (Fig. 2a, b). Boutons on these LA axons were mostly *en passant* boutons, in contrast to *terminaux* boutons along the axons of L6 pyramidal cells[26]. To search for conditioning-induced presynaptic structural changes, we imaged the same set of boutons (Fig. 2c, see Supplementary Fig. 5 for the criterion; see also ref [26]) at 1d before as well as 2h and 3d after conditioning. We calculated the percentages of bouton formation and elimination by comparing the images obtained at 2h and 3d after conditioning with those obtained at 1d before conditioning. Consistent with previous findings, only small percentages of boutons underwent turnover with time in control adult mice (Fig. 2d). Compared with control mice which received unpaired CS and footshock, the percentages of newly formed boutons in conditioned mice were slightly higher at 2h (8.0 ± 0.6% vs. 6.5 ± 0.6%, p = 0.07, Fig. 2d), and became significantly higher at 3d (14.4 ± 0.9% vs. 9.9 ± 0.9%; p = 0.003, Fig. 2d). By contrast, no difference was found for the percentages of eliminated boutons between conditioned and control groups (p > 0.1, Fig. 2d). Therefore, remodeling of LA axons in ACx is associated with fear learning.

No changes in bouton dynamics in MG and ACC axons

Layer 1 of ACx also receives inputs from the medial division of MG (Fig. 1c, see also ref [27]), which is known to exhibit auditory fear conditioning-induced plasticity[21]. To explore whether MG projections in ACx undergo conditioning-induced structural modification, we injected AAV-hSYN-EGFP into mouse MG (Fig. 3a, b) and imaged the MG axons in L1 of ACx before and after fear conditioning (Fig. 3c). We found no difference in the rate of bouton formation or elimination between conditioned and control mice ($p > 0.3$, Fig. 3d). In addition to LA and MG, ACx receives long-range feedback projections from the anterior cingulate cortex (ACC, Supplementary Fig. 6, see also ref [28]), which is also known to be involved in fear conditioning[29]. Similar imaging experiments of ACC axons in L1 of ACx showed no conditioning-induced change in bouton formation or elimination (Fig. 3e-h). Taken together, fear conditioning induces a behavior-correlated increase of axon boutons only in LA axon inputs, by increasing the rate of bouton formation without affecting that of bouton elimination.

LA axon boutons form synapses with L5 neurons in L1 of ACx

Long-range connections could serve as a substrate for long-term memory storage, via behavior-induced plasticity. Having found that formation of LA boutons in ACx is selectively elevated after fear conditioning, we then set out to explore the postsynaptic partners of these boutons. Previous studies have shown that long-range projections from subcortical areas innervate distal apical dendrites of L5 cortical pyramidal

neurons. To examine whether LA axon boutons made direct synaptic contact with L5 neurons in L1 of ACx, we performed immuno-gold electron microscopy (EM) experiments. We first injected AAV-Cre into LA of YFP-H mice[34], in which a small population of L5 cortical neurons expresses YFP, and immuno-stained Cre and YFP with different gold particles. We found that Cre-labeled LA boutons formed asymmetric synapses with YFP-labeled spines in L1 of ACx (Fig. 4a), confirming that the L1 apical dendrites originating from L5 neurons in ACx receive direct synaptic inputs from LA neurons.

~~Conditioning-induced increase in spine formation in L1~~ Spine formation ~~dendrites~~ of ACx L5 cells increases after conditioning

We next investigated whether there are conditioning-induced changes in L1 spine of L5 neurons in ACx, by measuring spine turnover rate in apical dendrites with *in vivo* two-photon imaging. Using YFP-H transgenic mice, we found that the percentages of newly formed spines on apical dendrites of L5 neurons were significantly higher in fear-conditioned mice than those in control mice at 3d after conditioning (10.0 ± 0.7% vs. 6.7 ± 0.7%; $p = 0.002$; Fig. 4b, c), but not at 2h (5.0 ± 0.6% vs. 4.8 ± 0.5%; $p = 1.0$; Fig. 4b, c). Further analysis showed a tendency for correlation between spine formation rate and freezing responses of fear conditioned animals (Supplementary Fig. 7). No change in spine elimination was observed ($p > 0.2$, Fig. 4b, c). These results were consistent with the previous finding that spines formed in ACx during auditory fear conditioning could persist for a long time[6]. Our failure to see a transient

increase at 2h[6] could be due to the difference in the imaged population of labeled neurons in different transgenic lines[34].

To examine whether the increase in spine formation after fear conditioning is specific to apical dendrites of L5 neurons in ACx, we labeled L2/3 neurons with tdTomato via *in utero* electroporation (Supplementary Fig. 8a, b) and performed similar imaging experiments. Neither spine formation nor elimination was changed in the apical dendrites of L2/3 neurons (Supplementary Fig. 8c, d). Thus, fear conditioning-induced changes in spine formation of the apical dendrites in L1 were not part of a general spine dynamics change in ACx, but rather specific to L5 pyramidal neurons.

Blocking fear memory prevents ~~could eliminate conditioning-induced~~ increase in spine formation ~~increase~~

To determine whether the changes in spine dynamics were linked to fear learning, we bilaterally infused muscimol into ACx of YFPH mice, which were implanted with cranial chronic windows for two-photon imaging (Fig. 4d). We found that as muscimol infusion impaired fear memory, it also prevented any fear conditioning-induced changes in the dynamics of spines on apical dendrites of L5 neurons (p = 0.03, Fig. 4d, e). This result further supports the notion that synaptic changes in ACx were associated with fear memory.

Pathway-specific changes in bouton and spine dynamics

Spines on the same dendrite could respond differently during a certain behavioral task[35], possibly due to different inputs[36]. It is thus of interest to determine whether spines on the same dendrite could exhibit different plasticity, and if so, whether different origins of the inputs could account for this difference. Similarly, boutons along the same axon show highly correlated and yet variable activity patterns[37]. Postsynaptic signals also could affect the presynaptic functional and structural plasticity[38]. Whether boutons on the same axons exhibit differential structural plasticity due to different postsynaptic connections thus remains an open question.

To examine whether the turnover dynamics of boutons and spines in ACx were differentially modulated in a pathway-specific manner, we performed concurrent two-photon imaging on long-range projecting axons and ACx dendrites. We injected AAV-EGFP into LA, MG or ACC of YFP-H mice, and simultaneously imaged pre-synaptic GFP and post-synaptic YFP signals in ACx. GFP and YFP signals were separated using bandpass filters (Methods, Supplementary Fig. 9). We identified putative synaptic contacts using a defined morphological criterion (Methods, see also ref [16]). We compared the turnover of boutons/spines that contacted with labeled identified synaptic partners (Fig. 5a, b) with that of randomly selected boutons/spines in the same images. We found that formation rates of boutons and spines in LA-ACx connections were higher than those in unidentified connections at 3d in fear-conditioned mice but not in control mice (Fig. 5c, Supplementary Fig. 10a), indicating that fear learning induced an increase in bouton and spine formation in this pathway.

By contrast, the formation rates of spines in MG-ACx and ACC-ACx connections were significantly lower than those of randomly selected spines in both conditioned and control mice, suggesting that the L1 spines of L5 ACx neurons that received MG and ACC inputs were relatively more stable (Figure 5d, e, Supplementary Fig. 10b, c). Thus, fear conditioning-induced increases in bouton and spine formation were specific to the LA-ACx connections, indicating a pathway-specific modulation of structural changes among LA axons and ACx dendrites by fear learning.

Rapid and gradual increase in LA-ACx synapses over days

To examine the time course of synaptic modification of LA-ACx connections after fear learning, we performed daily imaging of dual-labeled LA-ACx synapses after fear conditioning, and compared images taken at 2h, 1d, 2d and 3d to those taken at 1d before for quantification of “stable”, “newly formed”, and “eliminated” synaptic pairs (Fig. 6a, b). Fear memory was tested at 1d after conditioning. We found that the percentage of newly formed LA-ACx synaptic pairs started to show a significant increase in conditioned mice (15.2 ± 1.1%) compared control mice (7.4 ± 1.8%; p = 0.02, Fig. 6c) as early as 2h after conditioning. This rapid change was not observed in either bouton or spine dynamics when their synaptic partners were unidentified. Daily imaging over the next 3d after conditioning showed a gradual increase in the percentage of newly formed synaptic pairs (p = 0.01, Kolmogorov-Smirnov test, Fig. 6c), while that of eliminated pairs maintained similar between the two groups ($p > 0.5$, Fig. 6c). Imaging of MG-ACx and ACC-ACx connections showed no changes in the

percentages of formation or elimination of putative synaptic pairs between conditioned and control groups ($p > 0.5$, Fig. 6d, e). Thus, fear conditioning induced a rapid (within 2h) elevation in the formation of new LA-ACx connections that continued over a period of days, consistent with the rapid formation and post-conditioning consolidation of fear memory[39].

"Additive" form of new synapse formation in adult brain

~~Synapses are formed by adding new partners to existing synaptic elements~~

During data analysis of dual-color imaging studies, we discovered unexpectedly that new LA-ACx synaptic pairs were~~as~~ essentially all in "additive form", *i.e.*, due to either the appearance of a new spine on an existing bouton ("*Type I*"; Fig. 7a; and Supplementary Fig. 11a; ~43.5%) or a new bouton on an existing spine ("*Type II*"; Fig. 7c; and Supplementary Fig. 11b; ~55%), with extremely rare occurrence (~1.5%, 4 out of 262 new pairs) of *de novo* synapse formation (Supplementary Fig. 11c). These existing boutons and spines are likely to have an unlabeled existing synaptic partner due to sparse labeling of pre- and postsynaptic cells. Thus "*Type I*" and "*Type II*" formation may result in multi-synapse boutons and multi-synapse spines, respectively, which were indeed observed occasionally when both pre- and postsynaptic cells happened to be labeled (Fig. 7b, d; and Supplementary Fig. 11d, e, 31/262 cases). By contrast, replacement of an existing pre- or postsynaptic element with a new one was rarely observed (2/262, Supplementary Fig. 11f). Using EM, we confirmed the existence of both multiple-synapse boutons and multiple-synapse

spines in L1 of ACx of conditioned animals (Fig. 7e, f).

Similar percentages of *Type I* and *Type II* new synaptic pair formation were found for LA-ACx connections in control mice, as well as for MG-ACx and ACC-ACx connections in conditioned and control mice (Fig. 7g-i). Furthermore, the percentages of *Type II* formation were in general higher than that of *Type I* formation, in both control and conditioned mice (Fig. 7g-i). Given that this mode of new synapse formation was found for all connections in ACx, addition of new partners to existing synaptic elements may represent a general architecture rule for making new synaptic connections in the adult cortex. Finally, we observed that in some cases (n=31), a new bouton (or spine) added to an existing synapse was initiated by the same axon (or dendrite) that harbored the existing synapse (Fig. 7b, d, Supplementary Fig. 11d, e), thus strengthening the connections between existing neuronal partners. This result is consistent with previous findings that after LTP induction, multiple spines from the same dendrite made contacts with the same presynaptic axon bouton[40]. Such synapse addition could also lead to spatially clustered spines that are more likely to be coactive[41], providing a potential explanation for spatial clustering of learning-induced new spine formation.

Discussion

The neural circuits underlying auditory fear conditioning have been extensively characterized. Auditory information flows from the auditory thalamus and ACx to the

LA, then to the basolateral amygdala and the central amygdala, which projects to the brainstem that controls fear responses. In this study, we identified a feedback pathway from LA to ACx, and showed that this pathway plays an important role in the expression of fear memory. Specifically inhibiting this pathway during fear recall test using the DREADD system and optogenetics significantly reduced fear responses. By contrast, we found no effect when the pathway was inhibited before fear learning. Nevertheless, we cannot exclude the possibility that LA-ACx pathway also contributes to fear learning, because we could at best partially block LA-ACx connections using the current method, due to limited virus expression in LA.

Synaptic connections were modified by experience, providing a substrate for learning and memory. Sensory experience, motor learning and aging were all found to induce changes in the dynamics of presynaptic axon boutons or postsynaptic dendritic spines. Whereas both boutons and spines could represent synaptic connections on their own[7,8,10-14], concurrent imaging of pre- and postsynaptic structures provides additional information on pathway specificity. In this study, we found that fear learning induced structural remodeling specifically at LA-ACx connections. The pathway-specific synapse formation may depend on coordinated activation of LA and ACx neurons, via intercellular anterograde and retrograde signaling[43]. Furthermore, the remodeling was detectable within hours and continued over days after learning and correlated with freezing responses at both 2h and 3d, suggesting that the same pathway is involved in both short-term and long-term memory. These results also

support the notion that memory consolidation involves gradual structural reorganization outlasting the learning process[44] and that long-term memory becomes increasingly dependent on the cortex with time .

Fear memory-related synapse formation specifically at LA-ACx connections could enhance L5 neuronal excitation in ACx by increasing coincident inputs, leading to potential modulation of behaviorally relevant sensory processing. This direct LA-ACx modulation may add to the known indirect cortical modulation by amygdala through its subcortical projections, and work in concert with cholinergic inputs to L1 during fear memory formation[2]. Although MG-ACx and ACC-ACx connections were not modified by fear learning, they may be involved in other auditory behaviors.

We found that new synapses are formed in an “additive” manner by either adding a new spine to an existing bouton or a new bouton to an existing spine. Previous EM studies have shown preferential contact of new spines on existing boutons. Our studies further revealed that new bouton formation on existing spines represents a more frequent event in such “additive” form of synapse formation. As compared to *de novo* formation of new synapses, this “additive” synapse formation is less demanding for cellular energy, synaptic resources, and synaptic space, thus more suited for circuit remodeling in the adult brain[50]. Finally, although we have observed only few examples of a new spine replacing an old one on the existing bouton, possibility remains that new synaptic elements could also compete out existing ones of other pathways.

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Author Contributions

Y.Y., D-q.L. and M-m.P. designed the experiments. Y.Y. and D-q.L. performed the experiments and analyzed the data. W.H. performed *in utero* electroporation experiments. Y.S. and J.D. performed the electrophysiology experiments. Y.Z. guided spine data analysis. Y.Y., D-q.L. Y.Z. and M-m.P. wrote the paper.

Competing financial interests

The authors declare no competing financial interests.

References

1 Goosens, K. A. & Maren, S. Contextual and auditory fear conditioning are mediated by the lateral, basal, and central amygdaloid nuclei in rats. *Learning & memory* **8**, 148-155, doi:10.1101/lm.37601 (2001).

2 Letzkus, J. J. *et al.* A disinhibitory microcircuit for associative fear learning in the auditory cortex. *Nature* **480**, 331-335, doi:10.1038/nature10674 (2011).

3 Peter, M. *et al.* Induction of immediate early genes in the mouse auditory cortex after auditory cued fear conditioning to complex sounds. *Genes, brain, and behavior* **11**, 314-324, doi:10.1111 (2012).

4 Boatman, J. A. & Kim, J. J. A thalamo-cortico-amygdala pathway mediates auditory fear conditioning in the intact brain. *Eur J Neurosci* **24**, 894-900, doi:EJN4965 (2006).

5 Weinberger, N. M. Specific long-term memory traces in primary auditory cortex. *Nat Rev Neurosci***5**, 279-290, doi:10.1038/nrn1366 (2004).

6 Moczulska, K. E. *et al.* Dynamics of dendritic spines in the mouse auditory cortex during memory formation and memory recall. *Proc Natl Acad Sci U S A***110**, 18315-18320, doi:10.1073 (2013).

7 Denk, W. & Svoboda, K. Photon upmanship: why multiphoton imaging is more than a gimmick. *Neuron* **18**, 351-357 (1997).

8 Harris, K. M. & Kater, S. B. Dendritic spines: cellular specializations imparting both stability and flexibility to synaptic function. *Annual review of neuroscience* **17**, 341-371, doi:10.1146/annurev.ne.17.030194.002013 (1994).

9 Grillo, F. W. *et al.* Increased axonal bouton dynamics in the aging mouse

cortex. *Proceedings of the National Academy of Sciences of the United States of America* **110**, E1514-1523, doi:10.1073 (2013).

10 Holtmaat, A., Wilbrecht, L., Knott, G. W., Welker, E. & Svoboda, K. Experience-dependent and cell-type-specific spine growth in the neocortex. *Nature* **441**, 979-983, doi:10.1038/nature04783 (2006).

11 Knott, G. W., Holtmaat, A., Wilbrecht, L., Welker, E. & Svoboda, K. Spine growth precedes synapse formation in the adult neocortex in vivo. *Nature neuroscience* **9**, 1117-1124, doi:10.1038/nn1747 (2006).

12 Lai, C. S., Franke, T. F. & Gan, W. B. Opposite effects of fear conditioning and extinction on dendritic spine remodelling. *Nature* **483**, 87-91, doi:10.1038/nature10792 (2012).

13 Xu, T. *et al.* Rapid formation and selective stabilization of synapses for enduring motor memories. *Nature* **462**, 915-919, doi:10.1038/nature08389 (2009).

14 Hofer, S. B., Mrsic-Flogel, T. D., Bonhoeffer, T. & Hubener, M. Experience leaves a lasting structural trace in cortical circuits. *Nature* **457**, 313-317, doi:10.1038/nature07487 (2009).

15 Zuo, Y., Yang, G., Kwon, E. & Gan, W. B. Long-term sensory deprivation prevents dendritic spine loss in primary somatosensory cortex. *Nature* **436**, 261-265, doi:10.1038/nature03715 (2005).

16 Becker, N., Wierenga, C. J., Fonseca, R., Bonhoeffer, T. & Nagerl, U. V. LTD induction causes morphological changes of presynaptic boutons and reduces their contacts with spines. *Neuron* **60**, 590-597,

doi:10.1016/j.neuron.2008.09.018 (2008).

17 Romanski, L. M. & LeDoux, J. E. Equipotentiality of thalamo-amygdala and thalamo-cortico-amygdala circuits in auditory fear conditioning. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **12**, 4501-4509 (1992).

18 Malenka, R. C. & Bear, M. F. LTP and LTD: an embarrassment of riches. *Neuron* **44**, 5-21, doi:10.1016/j.neuron.2004.09.012 (2004).

19 Cauller, L. Layer I of primary sensory neocortex: where top-down converges upon bottom-up. *Behavioural brain research* **71**, 163-170 (1995).

20 Zhang, S. *et al.* Selective attention. Long-range and local circuits for top-down modulation of visual cortex processing. *Science* **345**, 660-665, doi:10.1126/science.1254126 (2014).

21 Weinberger, N. M. Physiological memory in primary auditory cortex: characteristics and mechanisms. *Neurobiology of Learning and Memory* **70**, 226-251, doi:S1074-7427(98)93850-3 (1998).

22 LeDoux, J. E. Emotion circuits in the brain. *Annual review of neuroscience* **23**, 155-184, doi:10.1146/annurev.neuro.23.1.155 (2000).

23 Armbruster, B. N., Li, X., Pausch, M. H., Herlitze, S. & Roth, B. L. Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 5163-5168, doi:0700293104 (2007).

24 Stachniak, T. J., Ghosh, A. & Sternson, S. M. Chemogenetic synaptic silencing

of neural circuits localizes a hypothalamus-->midbrain pathway for feeding behavior. *Neuron* **82**, 797-808, doi:10.1016/j.neuron.2014.04.008 (2014).

25 Zhu, Y., Wienecke, C. F., Nachtrab, G. & Chen, X. A thalamic input to the nucleus accumbens mediates opiate dependence. *Nature* **530**, 219-222, doi:10.1038/nature16954 (2016).

26 De Paola, V. *et al.* Cell type-specific structural plasticity of axonal branches and boutons in the adult neocortex. *Neuron* **49**, 861-875, doi:S0896-6273(06)00134-6 (2006).

27 Rubio-Garrido, P., Perez-de-Manzo, F., Porrero, C., Galazo, M. J. & Clasca, F. Thalamic input to distal apical dendrites in neocortical layer 1 is massive and highly convergent. *Cereb Cortex* **19**, 2380-2395, doi:10.1093/cercor/bhn259 (2009).

28 Schneider, D. M., Nelson, A. & Mooney, R. A synaptic and circuit basis for corollary discharge in the auditory cortex. *Nature* **513**, 189-194, doi:10.1038/nature13724 (2014).

29 Gilmartin, M. R., Balderston, N. L. & Helmstetter, F. J. Prefrontal cortical regulation of fear learning. *Trends Neurosci* **37**, 455-464, doi:10.1016/j.tins.2014.05.004 (2014).

30 Senn, V. *et al.* Long-range connectivity defines behavioral specificity of amygdala neurons. *Neuron* **81**, 428-437, doi:10.1016/j.neuron.2013.11.006 (2014).

31 Xiong, Q., Znamenskiy, P. & Zador, A. M. Selective corticostriatal plasticity during acquisition of an auditory discrimination task. *Nature* **521**, 348-351,

doi:10.1038/nature14225 (2015).

32 Harris, K. D. & Mrsic-Flogel, T. D. Cortical connectivity and sensory coding. *Nature* **503**, 51-58, doi:10.1038/nature12654 (2013).

33 Douglas, R. J. & Martin, K. A. Neuronal circuits of the neocortex. *Annual review of neuroscience* **27**, 419-451, doi:10.1146/annurev.neuro.27.070203.144152 (2004).

34 Feng, G. *et al.* Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. *Neuron* **28**, 41-51, doi:S0896-6273(00)00084-2 (2000).

35 Cichon, J. & Gan, W. B. Branch-specific dendritic Ca(2+) spikes cause persistent synaptic plasticity. *Nature* **520**, 180-185, doi:10.1038/nature14251 (2015).

36 Stuart, G. J. & Spruston, N. Dendritic integration: 60 years of progress. *Nature neuroscience* **18**, 1713-1721, doi:10.1038/nn.4157 (2015).

37 Otazu, G. H., Chae, H., Davis, M. B. & Albeanu, D. F. Cortical Feedback Decorrelates Olfactory Bulb Output in Awake Mice. *Neuron* **86**, 1461-1477, doi:10.1016/j.neuron.2015.05.023 (2015).

38 Wilson, R. I. & Nicoll, R. A. Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses. *Nature* **410**, 588-592, doi:10.1038/35069076 (2001).

39 Schafe, G. E., Nader, K., Blair, H. T. & LeDoux, J. E. Memory consolidation of Pavlovian fear conditioning: a cellular and molecular perspective. *Trends Neurosci* **24**, 540-546 (2001).

40 Toni, N., Buchs, P. A., Nikonenko, I., Bron, C. R. & Muller, D. LTP promotes formation of multiple spine synapses between a single axon terminal and a dendrite. *Nature* **402**, 421-425, doi:10.1038/46574 (1999).

41 Kleindienst, T., Winnubst, J., Roth-Alpermann, C., Bonhoeffer, T. & Lohmann, C. Activity-dependent clustering of functional synaptic inputs on developing hippocampal dendrites. *Neuron***7 2**, 1012-1024, doi:10.1016/j.neuron.2011.10.015 (2011).

42 Fu, M., Yu, X., Lu, J. & Zuo, Y. Repetitive motor learning induces coordinated formation of clustered dendritic spines in vivo. *Nature* **483**, 92-95, doi:10.1038/nature10844 (2012).

43 Park, H. & Poo, M. M. Neurotrophin regulation of neural circuit development and function. *Nat Rev Neurosci* **14**, 7-23, doi:10.1038/nrn3379 (2013).

44 Caroni, P., Donato, F. & Muller, D. Structural plasticity upon learning: regulation and functions. *Nat Rev Neurosci* **13**, 478-490, doi:10.1038/nrn3258 (2012).

45 Wiltgen, B. J., Brown, R. A., Talton, L. E. & Silva, A. J. New circuits for old memories: the role of the neocortex in consolidation. *Neuron* **44**, 101-108, doi:10.1016/j.neuron.2004.09.015 (2004).

46 Frankland, P. W. & Bontempi, B. The organization of recent and remote memories. *Nat Rev Neurosci* **6**, 119-130, doi:nrn1607 (2005).

47 Krettek, J. & Price, J. Amygdaloid projections to subcortical structures within the basal forebrain and brainstem in the rat and cat. *Journal of Comparative Neurology* **178**, 225–253, doi:10.1002/cne.901780204 (1978).

48 Gray, T. S., Carney, M. E. & Magnuson, D. J. Direct projections from the central amygdaloid nucleus to the hypothalamic paraventricular nucleus: possible role in stress-induced adrenocorticotropin release. *Neuroendocrinology* **50**, 433-446 (1989).

49 Kirov, S. A., Sorra, K. E. & Harris, K. M. Slices have more synapses than perfusion-fixed hippocampus from both young and mature rats. *J Neurosci* **19**, 2876-2886 (1999).

50 Chklovskii, D. B., Mel, B. W. & Svoboda, K. Cortical rewiring and information storage. *Nature* **431**, 782-788, doi:10.1038/nature03012 (2004).

Figure 1: Projections from lateral amygdala (LA) to auditory cortex (ACx) were important for fear memory expression.

(**a**) Left: Fear conditioning protocols. Conditioned mice received 5 repeats of paired sound and footshock. Control mice received 5 repeats of explicitly unpaired sound and footshock. Right: CS sound-triggered freezing responses of control (n = 9) and conditioned (n = 10) mice in a new context. Student's t-test, t (17) = 7, p = 3e^-6. (**b**) Freezing responses in recall test for mice bilaterally infused with muscimol at 30 min before fear conditioning (saline, n = 4; muscimol, n = 3; t (5) = 4, p = 0.01) or recall test (saline, n = 5; muscimol, n = 6; t (9) = 3, p = 0.01). Student's t-test. (**c**) Retrograde labeling of neurons projecting to ACx. I, retrobeads injection site in ACx; II, III, retrogradely labeled neurons in medial geniculate body (MG) and LA. Scale bar: 100□□m. (**d**) Image showing anterogradely labeled LA axons in ACx. Red box: virus injection site in LA. Scale bar: 500□□m. (**e**) Diagram showing selective silencing of LA-ACx pathway using DRREAD system. AAV-hM4D (or AAV-EGFP, as a control) was injected into LA, and CNO (or saline, as a control) was infused locally into ACx. (**f**) Light-evoked excitatory synaptic current (EPSC) recorded from postsynaptic ACx neurons before (black) and after (red) CNO perfusion (n = 5). Upper inset shows a recorded neuron in layer 5. Scale bar, 100 □m. Lower inset shows an example of light-evoked EPSC trace. Scale bar, 10 pA, 25 ms. Paired t-test, t (4) = 7, p = 0.002. (**g**) Freezing responses of mice expressing hM4D (or EGFP) in LA infused with CNO (or saline) in ACx before fear acquisition (EGFP/CNO, n = 4;

hM4D/saline, n = 3; hM4D/CNO, n = 5; EGFP/CNO vs hM4D/CNO, t (7) = 0.02, p = 1; hM4D/saline vs hM4D/CNO, t (6) = 0.2, p = 0.8) or expression (EGFP/CNO, n = 5; hM4D/saline, n = 5; hM4D/CNO, n = 4; EGFP/CNO vs hM4D/CNO, t (7) = 3, p = 0.02; hM4D/saline vs hM4D/CNO, t (7) = 4, p = 0.004). Student's *t*-test. Note that silencing LA-ACx projections during memory recall reduced freezing responses. (**h**) Left: Diagram showing selective silencing of LA-ACx pathway using optogenetics. AAV-eArch3.0 or AAV-EGFP was injected into LA, and superficial layers of ACx was illuminated by yellow light. Right: Freezing responses of mice expressing eArch3.0 (n = 6) or EGFP (n = 4) in LA with yellow on in ACx during expression. Student's *t*-test, t (8) = 3, p = 0.02. Error bar, s.e.m.

Figure 2: Fear conditioning induces increase in bouton formation in LA axons that terminated in Layer 1 of ACx.

(**a**) Images showing AAV-EGFP injection site in LA. Scale bar, 500 μm. (**b**) top: EGFP-expressing LA axons in ACx. Scale bar, 100 μm. bottom: Intensity profile of DAPI and EGFP signals in ACx, along the red rectangle (top). Note that EGFP signals are detected mostly in Layer 1 (L1). (**c**) Example images obtained by repeated imaging of the same LA axons in L1 of ACx in control and conditioned animals at 1d before (-1d) and 2h or 3d after conditioning. Green arrows, newly formed boutons, as compared to -1d; red arrows, eliminated boutons. Scale bar, 4 μm. (**d**) Percentages of newly formed and eliminated boutons in control and conditioned mice for LA axons in L1 of ACx at 2h and 3d, relative to the bouton number at -1d. Circles, results from individual mice (conditioned, n = 14; control, n = 12). Mann-Whitney U test, 2h, formation, U = 126, p = 0.07; 2h, elimination, U = 147, p = 0.5; 3d, formation, U = 105, p = 0.003; 3d, elimination, U = 129, p = 0.1. For each mouse, over 200 boutons were included in the analysis. Error bar, s.e.m.

Figure 3: No changes in the bouton dynamics of MG or ACC axons in L1 of ACx.

(**a**) Image showing AAV-hSYN-EGFP injection site in MG, including MGm and MGd. Scale bar, 500 μm. (**b**) Top: EGFP-expressing MG axons in ACx. Scale bar, 100 μm. Bottom: Intensity profile of DAPI and EGFP signals in ACx along the red rectangle. EGFP signals are detected in L1, L4 and L6.(**c**) Examples of repeated imaging of the same MG axons in L1 of ACx in control and conditioned animals at -1 d, 2 h and 3 d. Green arrows, newly formed boutons, as compared to -1 d; red arrows, eliminated boutons. Scale bar, 4 μm. (**d**) Percentages of newly formed and eliminated MG boutons in control and conditioned mice at 2h and 3d, as compared to -1d. (conditioned, n = 5; control, n = 6). Mann-Whitney U test,2h, formation, U = 30, p = 1; 2h, elimination, U = 23, p = 0.3; 3d, formation, U = 27, p = 0.7; 3d, elimination, U = 25, p = 0.4. (**e-h**) Similar to (**a-d**), except that the labeled neurons were in ACC and the imaged axon boutons in L1 of ACx were ACC axons (conditioned, n = 9; control, n = 11). Mann-Whitney U test, 2h, formation, U = 78, p = 0.2; 2h, elimination, U = 106, p = 0.4; 3d, formation, U = 74, p = 0.1; 3d, elimination, U = 97, p = 0.9. Error bar: s.e.m.

Figure 4: Significant increases in spine formation at 3d after conditioning in the apical dendrites of L5 neurons in ACx.

(a) EM images of dual immunogold-labeling of ACx slices, showing that LA axons (green) formed an asymmetric synapse (yellow arrow) with ACx L5 neurons (red) in L1. Red arrows, 15-nm Nanogold particles that immuno-labeled YFP expressed in ACx L5 neurons; green arrows, 5-nm Nanogold particles that immuno-labeled Cre-expressing LA neurons. Scale bars: 200 nm (left); 20 nm (right). (**b**) Example images obtained by repeated imaging of the same apical dendrites of ACx L5 neurons in control and conditioned mice. Green and red arrows, newly formed and eliminated spines, respectively, as compared to -1d. Scale bar, 2 □m.(**c**) Percentages of spine formation and elimination at apical dendrites of L5 neurons in control and conditioned mice at 2h (conditioned, n = 14; control, n = 11; formation, U = 142, p = 1; elimination, U = 118.5, p = 0.2) and 3d(conditioned, n = 18; control, n = 15; formation, U = 169, p = 0.002; elimination, U = 268, p = 0.7). Mann-Whitney U test. (**d**) Freezing responses of animals with chronic windows, infused bilaterally with muscimol or saline in ACx at 30 min before fear conditioning (muscimol, n = 4; saline, n = 5). Students' t-test, t (7) = 4, p = 0.01. (**e**) Percentages of spine formation and elimination in the same group of conditioned mice as in (**d**) at 3d (muscimol, n = 4; saline, n = 5). U = 11, p = 0.03 for formation, U = 23, p = 0.6 for elimination. Mann-Whitney U test. For each mouse, over 200 spines were included for analysis. Error bar: s.e.m.

Figure 5: Fear conditioning-induced increases in bouton and spine formation were specific to LA-ACx connections.

(a, b) Images obtained using dual-color *in vivo* two-photon imaging of LA axons (green) and ACx L5 neurons (red) at -1d, 2h and 3d.**a**, Triangles point to LA boutons with labeled postsynaptic partners (L1 spines of ACx L5 neurons). **b,** Arrows point to spines on apical dendrites of L5 neurons with labeled presynaptic partners (LA boutons). Scale bar: 2 □m. (**c**) Percentages of newly formed boutons/spines in labeled LA-ACx synaptic pairs (red circles), as compared to those in randomly selected boutons/spines in the same animal (black circles), in conditioned (n = 6) and control (n = 4) mice at 3d after conditioning. Bouton, t (5) = 5, p = 0.004 in conditioned, t (3) = 0.3, p = 0.8 in control; spine, t (5) = 8, p = 0.0004 in conditioned, t (3) = 2, p = 0.1 in control. Paired *t*-test. (**d, e**) Similar to (**c**), except that labeled synaptic pairs were in MG-ACx (conditioned, n = 4; control, n = 5; bouton, t (3) = 1, p = 0.4 in conditioned, t (4) = -0.2, p = 0.8 in control; spine, t (3) = 4, p = 0.04 in conditioned, t (4) = 3, p = 0.04 in control. Paired *t*-test.) or ACC-ACx connections (conditioned, n = 4; control, n = 4; bouton, t (3) = 0.3, p = 0.8 in conditioned, t (3) = 0.9, p = 0.4 in control; spine, t (3) = 3, p = 0.04 in conditioned, t (3) = 3, p = 0.049 in control. Paired *t*-test).

Figure 6: Conditioning-induced increase in the formation of putative synapses in LA-ACx connections.

(**a**) Image of a fixed slice showing co-localization of LA axons (GFP, green) and apical dendrites of L5 neurons (YFP, red) in superficial layers of ACx. Dashed lines marked 100 μm from pial surface. Scale bar, 100μm. (**b**) Images obtained using dual-color *in vivo* two-photon imaging of LA axons (green) and ACx L5 neurons (red) at -1d, 2h and 3d. White arrows, triangles and diamonds point to "stable", "newly formed" and "eliminated" putative synaptic pairs, respectively. Scale bar, 2 μm. (**c**) Percentages of newly formed and eliminated LA-ACx synaptic pairs in control (n = 4) and conditioned mice (n = 6) at 2h, 1d, 2d and 3d, as compared to -1d. Mann-Whitney U-test, 2h, formation, U = 11, p = 0.02; 2h, elimination, U = 15, p = 0.2; 3d, formation, U = 10, p = 0.001; 3d, elimination, U = 20, p = 0.8. Kolmogorov-Smirnov test was used for group comparison of all time between control and conditioned mice. p = 0.01 for formation, p= 0.5 for elimination. (**d-e**) Percentages of newly formed and eliminated MG-ACx pairs (conditioned, n = 4; control, n = 5; 2h, formation, U = 18, p = 0.7; 2h, elimination, U = 17, p = 0.6; 3d, formation, U = 19, p = 0.9; 3d, elimination, U = 18, p = 0.7) and ACC-ACx pairs (conditioned, n = 4; control, n = 4; 2h, formation, U = 22, p = 0.3; 2h, elimination, U = 20, p = 0.7; 3d, formation, U = 19, p = 0.8; 3d, elimination, U = 20, p = 0.7) in control and conditioned mice after conditioning. Mann-Whitney U-test. For each mouse, around 100 synaptic pairs were included for analysis. Error bar: s.e.m.

Figure7: New synapses were made by adding new partners to existing synaptic elements.

(**a, b**) Example images showing *Type I* formation of synaptic pairs: new spines on existing boutons, with the postsynaptic partners unlabeled (a) or labeled (b). Scale bar: 2 m. (**c, d**) Example images showing *Type II* formation of synaptic pairs: new boutons on existing spines, with the presynaptic partners unlabeled (c) or labeled (d). Scale bar: 2 m. (**e**) EM images from L1 of ACx showing that two spines (red) of ACx neurons made synaptic contacts with the same bouton (green) to form a multi-synapse bouton. Yellow arrows, asymmetric synaptic contacts. Scale bar: 200 nm. (**f**) EM images from L1 of ACx showing that three boutons (green) made synaptic contacts with the same spine to form a multi-synapse spine. Scale bar: 200 nm. (**g-i**) The proportion of *Type I*, *Type II*, or *de novo* formation in newly formed LA-ACx pairs (g, conditioned, $n = 6$; control, $n = 4$), MG-ACx pairs (h, conditioned, $n = 4$; control, $n = 5$), or ACC-ACx pairs (i, conditioned, $n = 4$; control, $n = 4$) in control and conditioned (cond) mice at 2h and 3d. Error bar, s.e.m.

Methods

Animals

All procedures were approved by the Animal Committee of the Institute of Neuroscience, Chinese academy of sciences. Mice were housed and bred in a 12 h light-dark cycle (7 am-7 pm light) in the animal facility of the institute of Neuroscience. Experiments were performed during the light cycle. C57BL/6 mice were purchased from Slac Laboratory Animals (Chinese Academy of Sciences). YFP-H line mice were obtained from the Jackson Laboratory. Male mice were used for behavioral and imaging experiments. Female mice were used for *in utero* electroporation. Mice used for viral expression were 4 weeks old. Mice used for behavioral experiments and imaging experiments were 7-10 weeks old.

Virus and tracer injection

For virus and retrobeads injection, mice were anaesthetized with sodium pentobarbital (7 mg/kg) and positioned in a stereotaxic frame (Reward Co.). Body temperature was maintained at 37℃ using a heat pad. Viruses and retrobeads (Lumafluor) were injected using a glass micropipette with a tip diameter of 15-20 μm, through a small skull opening (< 0.5 mm^2), with a micro-injector (QSI). Stereotaxic coordinates for LA: 1.0 mm posterior from Bregma, 3.25 mm lateral from the midline, and 3.55 mm vertical from the cortical surface; for MG: 3.15 mm posterior from Bregma, 1.8 mm lateral from the midline, and 2.9 mm vertical from the cortical surface; for ACC: 1.00 mm anterior from Bregma, 0.5 mm lateral from the midline, and 1.5 mm vertical from the cortical surface; for auditory cortex: 2.5 mm from Bregma, 4.5 mm lateral from the midline, and 1.2 mm vertical from the cortical surface.

For AAV-SYN-EGFP, we injected 0.1-0.2 μl (~10^{13} virus particles per ml) into LA, 0.2-

0.6 µl into MG, or 1µl into ACC, and waited 4 weeks for maximal expression before using the mice for *in vivo* imaging. For AAV-SYN-HA-hM4D-ires-mCitrine, AAV-CamKIIa-eArch3.0-2A-EYFP and AAV-CamkIIa-ChR2-mCherry, we injected 0.8-1 µl (~$5*10^{12}$ virus particles per ml) into left LA. For retrobeads, we injected 0.2 µl into the superficial layers ACx (depth from cortical surface: 0.1 mm) and waited 5-7 d to allow the retrograde labelling. For cholera toxin subunit B (CTB) that was conjugated to Alexa 488, we applied 0.5 µl (0.5 mg/ml) directly above the ACx to make sure that the CTB was infused into the superficial layers.

***In utero* electroporation**

Pregnant mice at E15.5 were anesthetized with sodium pentobarbital (7 mg/kg), and subjected to abdominal incision to expose the uterus. The plasmid CAG-tdTomato (3 µg/µl) was injected through the uterine wall into the posterior lateral region of the lateral ventricle of embryonic brains with a glass micropipette. Electrical pulses were then delivered to embryos by gently clasping their heads with forceps-shaped electrodes connected to a square-pulse generator CUY21 (Bex Co., Ltd). For each electroporation, five 30 V pulses of 50 ms were applied at 1 s intervals. After the electroporation, the uterine horns were repositioned into the abdominal cavity, followed by suturing the abdominal wall and skin.

Surgery

Young adult mice (P42 - 49) were anaesthetized with isoflurane (induction, 4%; maintenance, 1-2%) and fixed in a stereotaxic frame (Reward Co.). Body temperature was maintained at 37℃ using a heat pad. Lidocaine was administered subcutaneously. The muscle covering the

auditory cortex was carefully removed with a scalpel. A 2×2 mm^2 region of skull over ACx was removed, exposing the dura. A custom-made double-layered cover glass was used to cover the cortex. UV-cure glue and dental acrylic were used to seal the cover glass. A custom-made stainless steel headplate with a screw hole was embedded into the dental acrylic for repeated imaging. Mice received injection of carprofin (0.3 mg, i.p.) after surgery and given 2 weeks for recovery before imaging.

For mice used for drug infusion, guide cannulae (26 gauge, with screw caps, Plastics One) were implanted bilaterally with coordinates for ACx: 2.5 mm from Bregma, 4.5 mm lateral from the midline, and 1.2 mm vertical from the cortical surface. Implants were fixed to the skull using dental acrylic (Reward Co.). Mice were given 3 d for recovery. For mice used for drug infusion and two photon imaging simultaneously, chronic windows were first implanted in the same way as mentioned above, and then guide cannulae were implanted bilaterally into ACx with an angle of 30°to the cortical surface. Animals were returned to their home cage for recovery. Two-photon imaging began 14 d after the surgery.

For mice used for optogenetic manipulation, chronic windows were implanted over ACx, and then ultra-thin green LEDs (APG-1608ZGC/G, Kingbright, 525 nm, 20 mA) were mounted onto the chronic windows and cemented onto the skull using dental cement. Mice were given 3 d for recovery before behavioral training.

Local drug infusion

Thirty min before fear conditioning or 5 min after fear conditioning, mice were anaesthetized with isoflurane, 30-gauge stainless steel injectors attached to 10 □l syringes (Hamilton) were

inserted into the guide cannulae, and 0.2 μl of muscimol (0.5 mg/ml) or APV (0.5 μg, 1 μg, 2 μg, 10 μg) per hemisphere was delivered bilaterally at 0.5μl/min using a two-channel microinfusion pump (Reward Co.). For experiments in Figure 1f, & 4d, 1 μl of muscimol was injected into ACx. The spread of drugs was determined by injecting 1 μl of fluorescent solution (FITC, Life Sciences, 5 mM).

In experiments testing the involvement of LA-ACx pathway using DRREAD system, the right side of ACx was always blocked using muscimol (1 μl at 0.5 μg/μl) in both control and experimental conditions. At 30 min before fear conditioning or recall test, 1 μl clozapine-*N*-oxide (CNO, Sigma, 1 μg/μl) or saline was infused into the left ACx of mice expressing hM4D or EGFP in left LA neurons, at 0.5 μl/min using a two-channel microinfusion pump (Reward Co.).

Brain Slice Electrophysiology

Mice were anesthetized with isoflurane (Lunan Pharmaceutical) and perfused transcardially with an ice-cold cutting solution containing (in mM) sucrose 213, KCl 2.5, NaH2PO4 1.25, MgSO4 10, CaCl2 0.5, NaHCO3 26, and glucose 11 (300-305 mOsm). The brain was rapidly dissected, and coronal 300μM slices containing the auditory cortex were prepared in the ice-cold cutting solution using a vibratome (Leica VT1200S, Wetzlar, Germany) at slicing speeds of 0.12 mm/s and a blade vibration amplitude of 1 mm. Slices were transferred to the holding chamber and incubated in 34℃ artificial cerebral spinal fluid containing (in mM) NaCl 126, KCl 2.5, NaH_2PO_4 1.25, $MgCl_2$ 2, $CaCl_2$ 2, $NaHCO_3$ 26, and glucose 10 (300-305 mOsm) to recover for 30 min. The slices were then kept at room temperature prior to recordings. Both

cutting solution and ACSF were continuously bubbled with 95%O_2/5%CO_2. Slices were placed on glass coverslips coated with poly-L-lysine (Sigma, St. Louis, MO), and submerged in a recording chamber (Warner Instruments, Hamden, CT). All experiments were performed at near-physiological temperatures (32-34°C) using an in-line heater (Warner Instruments, Hamden, CT) while perfusing the recording chamber with solution at 3-4 ml/min using a pump (BT100-2J, LongerPump). Whole-cell patch-clamp recordings were made from target neurons under IR-DIC visualization and a CCD camera (IR-1000E, DAGE-MTI) using an Olympus BX51WI microscope (Olympus Optical, Tokyo, Japan). Patch pipettes were filled with a Cs-based low Cl–internal solution containing (in mM) $CsMeSO_3$ 130, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 10, QX-314 2, EGTA 11, Mg-ATP 2, Na-GTP 0.3 (pH 7.3, 295 mOsm).

To assess the effects of local CNO infusion in ACx on synaptic transmission of LA axons, we first co-injected AAV-hM4D and AAV-ChR2 into LA, and brain slices were prepared 3 weeks after virus expression. Layer 5 pyramidal neurons in ACx were recorded using whole-cell patch clamp. To record light-evoked EPSCs, 5-ms, 5-mW blue light (Laser: UHP-Mic-LED-475, Prizmatix, Israel) was delivered through the objective to the superficial layers of ACx. Light-evoked EPSCs were recorded before and after perfusion with CNO (6 μM).

Behavior

Fear conditioning and behavioral test for freezing responses took place in different environments. Mice were handled and habituated prior to conditioning. A custom-made cage (22×28×40 cm) with an electrifiable floor connected to a shock generator (MED) was used

for conditioning, and a new context - a round cage (diameter 20 cm, height 40 cm) with a plastic floor - was used for behavioral test. Both cages were sound-proofed. Before conditioning, the conditioning cage was wiped clean with 70% ethanol. Before behavioral test, the test cage was wiped clean with water and sprayed with scented water. The behavior was captured with a camera and recorded with a surveillance system. A custom-made software written using Presentation (Neurobehavioralsystems) was used to control the delivery of sound and foot shocks. The conditioned stimulus is a series of 0.5-s 14 kHz tone beeps interleaved with 0.5-s silence, lasting 10 s, at 80 dB, and the unconditioned stimulus is a 2-s, 0.5-mA foot shock. For mice in behavioral experiments, each mouse was exposed to one presentation of sound and foot shocks. For conditioned mice in imaging experiments, each mouse received five repeats of 10-s sound that co-terminates with the 2-s foot shock. For controls in imaging experiments, five repeats of sound and foot shocks were presented pseudo-randomly without overlapping. The behavioral responses to CS were tested once at 1 d after conditioning for both conditioned and control animals, using CS sound lasting 60 s. Freezing behavior was scored using Noduls (Noldus Information Technology). The mice were considered to be freezing when no obvious difference (non-overlapping region below 10% of the body size) was detected in the images from the video for 2 s. The automatic scoring was verified by an experimenter blind to the experimental conditions. The video recorded 60 s before the test was scored as basal level freezing and subtracted from the freezing time during test. For chemogenetic (hM4D) and optogenetic (eArch3.0) silencing experiments, we silenced the right side of ACx with muscimol, and inhibited LA-ACx pathway on the left side only, due to the low success rate in targeting LA for virus injections.

Two-photon microscopy

Mice were anaesthetized with isoflurane (induction, 4%; maintenance, 1%) and fixated using the implanted headplate. Image stacks were taken every 0.7 μm, from the cortical surface to 100-150 μm deep, with a two-photon microscope (Sutter), controlled by Scanimage (Janelia). The objective used was 25X, 1.05 numerical aperture (Olympus). A digital zoom of 6 is used. A Ti:sapphire laser (Spectra Physics) was used as the light source, and tuned to 920 nm for imaging. YFP and GFP signals were obtained using filters 495/40 and 535/50 (Chrome). 535/50 filter (Channel 1) collected both GFP and YFP signals, 495/40 filter (Channel 2) collected GFP-only signals. By subtracting GFP signals from Channel 1 signals, we got YFP-only images. Mice were first imaged 1 d before conditioning. Second imaging session took place two hours after conditioning. Some mice (data in Fig. 6C) were imaged daily, and others were imaged again at 3 d after conditioning.

Data analysis

All images were analyzed using ImageJ, blind to experimental conditions. For bouton identification, we adopted criteria from ref 22. The fluorescent intensity was profiled along identified axons using ImageJ. Bright swellings were identified as boutons when the peak intensity is over three times brighter than the axon shaft. Spine identification criteria were the same as described before (ref 11). For the dual-color images, a potential bouton-spine pair was visually identified as described in ref 13, when a presynaptic bouton and a postsynaptic spine overlaid in the image plane.

For formation and elimination assays of boutons, spines, or bouton-spine pairs, reconstructed 3D images were used to minimize imaging distortion caused by movements and rotation between imaging intervals. Formation and elimination of bouton (spine, or bouton-spine pairs) were based on comparison of the images collected at two different time points (2 h vs. 1 d before conditioning, or 3 d vs. 1 d before conditioning, in Fig 1&2). Percentages of stable, eliminated and newly formed bouton (spine, or bouton-spine pairs) were all normalized to the initial image at -1 d. The total number of boutons (or spines) we analyzed for each individual mouse was > 200, and that of synaptic pairs was around 100. Data were presented as mean±s.e.m. across animals. Correlation between two parameters was modeled using linear regression and fitted using Matlab curve fitting toolbox.

Statistics

No statistical methods were used to pre-determine sample sizes but our sample sizes are similar to those generally employed in the field. No data point was excluded. Two-sided unpaired student's *t*-test was used for freezing behavior comparisons between different groups. Two-sided paired student's t-test was used for paired comparison in Fig. 1f, Fig.5 & supplementary Fig. 10. Data distribution of mouse freezing responses and spine/bouton turnover rates was assumed to be normal but this was not formally tested. Mann-Whitney U-test was used for comparisons in imaging data. Kolmogorov–Smirnov test was used for Fig. 6c. F test was used for test the significance for linear regression in Supplementary Fig. 7. Statistical test used, test statistics and the *p* values were shown in figure legends. The relevant test statistics, e.g. t-values and degrees of freedom for t-tests, U-values for Mann-Whitney U-

test, were shown in supplementary methods checklist, which is available online.

Histology

After completion of imaging or tracing, mice were deeply anaesthetized and transcardially perfused with 0.1 M phosphate-buffered saline (PBS) solution and then followed with 4% paraformaldehyde (PFA) in PBS. Brains were post-fixed in the same PFA solution overnight, and dehydrated using 30% sucrose. Coronal brain sections of 20-50 μm were cut using a vibratome (Leica Microsystems) and imaged on a stereoscope (Nikon E80i) or confocal microscope (Nikon A1R) to check the virus expression.

Electron microscopy

Mice were deeply anaesthetized and transcardially perfused with PBS and then with 4% PFA and 0.5% glutaraldehyde in 0.1M PBS. The ACx was dissected out with scalpel blade and was immersed in the same fixation solution over 4-6 hours at 4℃, then dehydrated in ascending ethanol series, and finally embedded in araldite over 2 days. Ultrathin slices (70 nm) were cut from the superficial regions of ACx (10-100 μm from the pial surface) to target L1 of ACx, where the *in vivo* images were obtained, and then collected on nickel grids (200 mesh). For immuno-gold EM analysis, YFP-H mice injected with AAV-Cre virus in LA were used. Non-specific binding was blocked by 1% BSA. Slices were incubated at 4℃for 48 h with primary antibodies: 1:40 Mouse anti-GFP (sigma, SAB2702197) for staining apical dendrites that expressed YFP and 1:40 Rabbit anti-Cre (sigma, SAB2700194) for staining LA axon that infected with AAV-Cre. After washed 5×15 min with 0.1 M PBS, slices were incubated with anti-mouse (15 nm, 1:100, Ted Pella, 15752) and anti-rabbit (5 nm, 1:100, Ted Pella, 15725)

Gold conjugates for 2 hours at room temperature. Slices were then washed with 5×15 min 0.1 M PBS and incubated with 1% glutaraldehyde to fix the Nanogold particles. Ultra-thin sections were incubated with methanolic uranyl acetate and lead citrate before observed with a Joel JEM-1230 TEM at the EM core facility in ION.

Data availability

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