Amygdala Reward Neurons Form and Store Fear Extinction Memory

Highlights

- Fear extinction memory requires formation of new engram cells
- Fear extinction engram cells are formed and stored in BLA Ppp1r1b+ neurons
- Fear extinction engram cells and reward cells are functionally interchangeable
- Omission of expected aversive stimuli is rewarding

Authors

Xiangyu Zhang, Joshua Kim, Susumu Tonegawa

correspondence

tonegawa@mit.edu

In Brief

Zhang et al. demonstrate that fear extinction memory requires new engram cells to be formed and stored in BLA neurons that drive reward behaviors. Fear extinction engram cells and reward-responsive cells are functionally equivalent in driving reward behaviors and fear extinction. Fear extinction memory is a newly formed reward memory.
Amygdala Reward Neurons Form and Store Fear Extinction Memory

Xiangyu Zhang,1 Joshua Kim,1 and Susumu Tonegawa1,2,3,*

1RIKEN-MIT Laboratory for Neural Circuit Genetics at the Picower Institute for Learning and Memory, Department of Biology and Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
2Howard Hughes Medical Institute at Massachusetts Institute of Technology, Cambridge, MA 02139, USA
3Lead Contact
*Correspondence: tonegawa@mit.edu
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SUMMARY

The ability to extinguish conditioned fear memory is critical for adaptive control of fear response, and its impairment is a hallmark of emotional disorders like post-traumatic stress disorder (PTSD). Fear extinction is thought to take place when animals form a new memory that suppresses the original fear memory. However, little is known about the nature and the site of formation and storage of this new extinction memory. Here we demonstrate that a fear extinction memory engram is formed and stored in a genetically distinct basolateral amygdala (BLA) neuronal population that drives reward behaviors and antagonizes the BLA’s original fear neurons. Activation of fear extinction engram neurons and natural reward-responsive neurons overlap significantly in the BLA. Furthermore, these two neuronal subsets are mutually interchangeable in driving reward behaviors and fear extinction behaviors. Thus, fear extinction memory is a newly formed reward memory.

INTRODUCTION

The inability to extinguish fear is a hallmark of many psychiatric disorders, such as post-traumatic stress disorder (PTSD) and generalized anxiety disorder (Shalev et al., 2017; Stein and Sareen, 2015). Following Pavlovian fear conditioning (Pavlov and Anrep, 1927), repeated or prolonged presentations of the conditioned stimulus (CS) without an expected aversive unconditioned stimulus (US) diminishes the conditioned fear response, a phenomenon called fear extinction (Herry et al., 2010; Myers and Davis, 2007; Quirk and Mueller, 2008). Fear extinction has been proposed to involve formation of a new memory in competition with the original fear memory (Bouton, 2004; Quirk and Mueller, 2008; Quirk et al., 2010). The amygdala is a key structure for fear memory (Davis, 1992; Duvarci and Pare, 2014; Ehrlich et al., 2009; Maren and Fanselow, 1996) and is also involved in fear extinction memory (Amano et al., 2010; Grewe et al., 2017; Herry et al., 2008). However, it is unknown whether the amygdala is the storage site of fear extinction memory and, if so, which subset of amygdala neurons stores this memory.

Excitatory neurons in the mouse basolateral amygdala (BLA) respond to both positive and negative valence stimuli (Zhang and Li, 2018; Beyeler et al., 2016; Davis and Whalen, 2001; Kim et al., 2016; Namburi et al., 2015; Redondo et al., 2014). All BLA pyramidal neurons are composed of two genetically, functionally, and anatomically distinct neuronal populations (Kim et al., 2016, 2017). R-spondin 2-expressing (Rspo2+) neurons, located in the anterior BLA (aBLA), respond to negative valence stimuli and control negative behaviors and memories, whereas protein phosphatase 1-regulatory inhibitor subunit 1B-expressing (Ppp1r1b+) neurons, located in the posterior BLA (pBLA), respond to positive valence stimuli and control appetitive behaviors and memories (Kim et al., 2016). Furthermore, these two neuronal populations antagonize each other through feedforward inhibition mediated by local inhibitory interneurons (Kim et al., 2016).

In this study, we investigated a potential role of BLA Ppp1r1b+ neurons in fear extinction using a contextual fear extinction paradigm. We found that fear extinction memory engram cells are formed and stored within the BLA Ppp1r1b+ neuronal population and that these engram cells are necessary for suppressing the original fear memory. Furthermore, these fear extinction engram cells and natural reward-responsive cells in pBLA Ppp1r1b+ neurons are mutually interchangeable in driving appetitive behaviors and fear extinction behaviors.

RESULTS

BLA Ppp1r1b+ Neurons Are Activated during Contextual Fear Extinction

Fear extinction phenomena have been observed and studied in both cue-dependent and context-dependent fear conditioning paradigms (Amano et al., 2010; Baldi and Bucherelli, 2014; Herry et al., 2008; Trouche et al., 2013; Zushida et al., 2007). Because the BLA plays a critical role in contextual fear conditioning (Calandreau et al., 2005; Goosens and Maren, 2001; Huff and Rudy, 2004; Redondo et al., 2014), and a substantial amount of information is available regarding the excitatory neuronal subsets in the BLA (Introduction; Kim et al., 2016), we employed a contextual fear extinction paradigm for the purpose of this study. On day 1, the extinction group received contextual fear conditioning (CFC) in a box where the context served as the CS and three rounds of footshocks served as the US. On day 2, mice were returned to the conditioning box for 45 min in the absence of footshocks for contextual fear extinction training.
On day 3, the mice were tested for 5-min extinction memory retrieval in the conditioning box before being sacrificed (Figure 1A). Two control groups were set up as follows. The non-extinction group went through the same protocol as the extinction group but remained in their home cages (HCs) and did not receive extinction training on day 2 (Figure 1A). The non-shock group received the same protocol as the extinction group but received no footshock on day 1 (Figure 1A). The extinction group and non-extinction group, but not the non-shock group, displayed robust levels of freezing behavior on day 1 that increased as more footshocks were presented (Figure 1B). On day 3, the extinction group exhibited a much lower level of freezing than the non-extinction group (Figure 1C).

Double smFISH (single-molecule fluorescence in situ hybridization) was performed to detect activity-dependent expression of the immediate-early gene Fos in Rspo2+ and Ppp1r1b+ cells (STAR Methods). The proportions of Fos+ cells among Rspo2+ cells and Fos+ cells among Ppp1r1b+ cells were quantified across the anterior/posterior (A/P) axis for the aBLA and pBLA, respectively (Kim et al., 2016). The proportion of Fos+/Rspo2+ cells was lower in the extinction group compared with the non-extinction group (Figures 1D, 1E, and 1H). In contrast, the proportion of Fos+/Ppp1r1b+ cells was higher in the extinction group compared with the non-extinction group. No difference was observed between the non-extinction and non-shock groups (Figures 1F, 1G, and 1I). The ratio of Rspo2+/Fos+ cells in the aBLA was lower in the extinction group compared with the non-extinction and non-shock groups, whereas the ratio of Ppp1r1b+/Fos+ cells in the pBLA was slightly lower in the non-extinction group compared with the non-shock group (Figures S1A and S1B). Because BLA Rspo2+ and Ppp1r1b+ cells collectively make up the entire excitatory pyramidal neuronal population in the BLA, these results suggest the involvement of local inhibitory neurons in the switch between high- and low-fear...
Figure 2. Ca²⁺ Activity of BLA Rspo2⁺ and Ppp1r1b⁺ Neurons during Different Behavioral Treatments

(A) Cre-dependent expression of GCamp6f and implantation of the microendoscope above the aBLA of Rspo2-Cre and pBLA of Ppp1r1b-Cre mice, respectively.

(B) The aBLA of Rspo2-Cre mice. Left: representative image showing GCamp6f expression and Gradient-index (GRIN) lens implant. Right: stacked FOV (field of view) image.

(C) The pBLA of Ppp1r1b-Cre mice. Left: representative image showing GCamp6f expression and GRIN lens implant. Right: stacked FOV image.

(D) Behavior protocol for Ca²⁺ recording (see text and STAR Methods).

(E) From left to right: percentages of BLA Rspo2⁺ neurons with increased (up, orange), decreased (down, blue), or no changed (NC, gray) responses to shock, FR, ET, and ER, respectively, compared with habituation. 169 cells total, n = 4 Rspo2-Cre mice.

(legend continued on next page)
states. When BLA Ppp1r1b+ neurons were optogenetically inhibited during extinction retrieval, the neuronal activity of Rsopo2+ cells (Foxs/Rsopo2) as well as freezing levels increased during extinction retrieval (Figures S1C–S1G). This result suggests that Ppp1r1b+ neurons suppress Rsopo2+ neurons during fear extinction retrieval, consistent with the previously reported feedforward inhibition of BLA Rsopo2+ cells by BLA Ppp1r1b+ cells (Kim et al., 2016).

Dynamics of Individual BLA Neurons throughout CFC and Fear Extinction

Next, we performed in vivo calcium imaging with a microendoscope to directly track individual BLA neuronal activity during CFC, followed by contextual FET and retrieval. The genetically encoded calcium indicator GCaMP6f was expressed in BLA Rsopo2+ and Ppp1r1b+ neurons via injecting adeno-associated virus 5 (AAV5)-human synapsin (hsyn):double-floxed inverse open reading frame (DIO)-GCaMP6f into the aBLA of Rspo2-Cre mice and the pBLA of Ppp1r1b-Cre mice, respectively (Figures 2A–2C). The efficiency of Rsopo2- and Ppp1r1b-specific Cre-loxP recombination in Rspo2-Cre and Ppp1r1b-Cre mouse lines is around 90% and 75%, respectively (Kim et al., 2016). We used an automated sorting algorithm to identify individual neurons and tracked their longitudinal activity across days (Figures 2D, S2A, and S2B; STAR Methods; Kitamura et al., 2017; Mukamel et al., 2009; Okuyama et al., 2016). Neuronal activity was analyzed under five conditions: during habitation to the conditioning chamber before CFC (Hab), after footshocks during CFC (shock), fear retrieval (FR), extinction training (ET), and extinction retrieval (ER) (Figure 2D; STAR Methods). To quantify how BLA neurons responded across these conditions, the neuronal activity under each condition was explicitly compared with the activity during habitation that served as a baseline condition (Figures 2E and 2F; STAR Methods). Cells with increased activity were referred to as Up cells, those with decreased activity were referred to as Down cells, and those with unchanged activity were referred to as no change (NC) cells.

Among the responsive neurons, BLA Rsopo2+ cells were predominantly activated by footshocks during CFC and FR and inhibited during ET and ER (Figures 2E, 2G, 2H, and S2C–S2E; Table S1). Conversely, BLA Ppp1r1b+ neurons were predominantly activated during ET and ER and inhibited by footshocks and FR (Figures 2F, 2I, 2J, S2F–S2H; Table S2). In individual Rsopo2-Cre mice, the freezing levels after footshocks were positively correlated with the respective percentages of shock-activated BLA Rsopo2+ cells and inversely correlated with the respective percentages of shock-inhibited BLA Rsopo2+ cells (Figures 2K and 2L). Compared with BLA Rsopo2+ cells, whose activity did not change in response to footshocks (S-NC), shock-activated Rsopo2+ cells (S-Up) were preferentially reactivated during FR (Figure 2M). Compared with BLA Ppp1r1b+ cells with unchanged activity during ET (ET-NC), BLA Ppp1r1b+ cells that were activated during ET (ET-Up) were preferentially reactivated during ER (Figure 2N), demonstrating preferential recruitment of memory-encoding neurons during memory retrieval. Combined with the c-Fos quantification data (Figure 1), these results show that pBLA Ppp1r1b+ neurons are recruited during FET and subsequently reactivated during extinction memory retrieval, whereas shock-activated aBLA Rsopo2+ neurons are suppressed during these processes.

Optogenetic Activation of BLA Ppp1r1b+ Neurons Drives Fear Extinction Learning

We next investigated the role of Ppp1r1b+ and Rsopo2+ neurons in fear extinction at the behavioral level using optogenetic manipulation. We injected a Cre-dependent AAV carrying ChR2 (channelrhodopsin-2) or eArchT (archaerhodopsin) into the aBLA of Rspo2-Cre mice (Figures 3A and S3A) or the pBLA of Ppp1r1b-Cre mice (Figures 3B and S3B). Mice underwent a 3-day contextual fear extinction protocol as mentioned previously (Figure 1A). Starting 5 min after the onset of ET on day 2, a 3-min blue or green laser light pulse was delivered repeatedly during FET at 2-min intervals (Figure 3C). Both inhibition of Rsopo2+ neurons and activation of Ppp1r1b+ neurons facilitated fear extinction learning (Figures 3E and 3F). In contrast, activation of Rsopo2+ neurons and inhibition of Ppp1r1b+ neurons resulted in impairment of fear extinction and extinction memory retrieval (Figures 3D and 3G).

To investigate the role of Ppp1r1b+ neurons in fear extinction memory retrieval, we optogenetically manipulated BLA Ppp1r1b+ neurons during ER on day 3. Activation of Ppp1r1b+ neurons by ChR2 in Ppp1r1b-Cre mice did not decrease the freezing level beyond the level attained in the Cre− control mice (day 3), most likely because of a floor effect of ET (Figure 3H). However, inhibition of Ppp1r1b+ neurons by eArchT in Ppp1r1b-Cre mice impaired fear ER (day 3), suggesting that these neurons are necessary for retention of fear extinction memory (Figure 3I). We did not observe an effect on the baseline freezing levels in a neutral context B that was distinct from the fear conditioning context (Figures S3C–S3E). Optogenetic
Figure 3. Optogenetic Activation of BLA Ppp1r1b+ Neurons and Optogenetic Inhibition of BLA Rspo2+ Neurons Facilitate Contextual Fear Extinction

(A) Left: diagram of bilateral injection of AAV-DIO-ChR2-EYFP or AAV-DIO-eArchT-EYFP virus and optical fiber implant in the aBLA of Rspo2 Cre+ or Cre− mice. Right: representative histology.

(B) Left: diagram of bilateral injection of AAV-DIO-ChR2-EYFP or AAV-DIO-eArchT-EYFP virus and optical fiber implant in the pBLA of Ppp1r1b Cre+ or Cre− mice. Right: representative histology.

(C) Experimental protocol of optogenetic manipulation during FET.

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These results suggest that the pBLA Ppp1r1b+ neuronal population contains fear extinction memory engram cells that are necessary and sufficient for fear extinction learning and memory, whereas the aBLA Rspo2+ neuronal population antagonizes the function of pBLA Ppp1r1b+ neurons in fear extinction.

**Fear Extinction Memory Engram Cells Are Formed and Retained in a BLA Ppp1r1b+ Neuronal Subpopulation**

We then investigated whether the engram cells for fear extinction memory can indeed be found in the pBLA Ppp1r1b+ neuronal population. By using our previously established engram cell identification technology (Liu et al., 2012; Ramirez et al., 2013; Redondo et al., 2014; Ryan et al., 2015; Tonegawa et al., 2015), we injected AAV$_p$-Fos-DIO-tTA (tetracycline transactivator) together with AAV$_p$-TRE (tetracycline response element)-ChR2-EYFP (Ext-ChR2 group) or AAV$_p$-TRE-EYFP (Ext-EYFP group) into the pBLA of Ppp1r1b-Cre mice (Figures 4A and 4B; STAR Methods) and subjected them to a series of engram labeling and behavioral paradigms (Figure 4C; STAR Methods). This genetic manipulation permits labeling of putative engram neurons during memory formation or memory retrieval in a cell type-specific and activity-dependent manner (Figures 4B and S4A–S4D; Khalaf et al., 2018; Liu et al., 2012; Ramirez et al., 2013). During FET, formation of putative fear extinction memory engram cells occurs in parallel with retrieval of the original fear memory. To isolate the putative fear extinction memory engram neurons, we used the retrieval stage of the fear extinction memory for their labeling (day 3) because fear memory expression would have subsided to the background level by then (Figures 4C and 4D). To assess the contribution of food and water supplied in the home cage (HC) to the baseline activity level of Ppp1r1b+ neurons, we set up another negative control group, HC-ChR2 (Figures 4C and 4D). As expected, ChR2-EYFP+ cells in the HC-ChR2 group were sparse compared with the ChR2-EYFP group (Figures 4B, S4C, and S4D).

Three groups of mice, the Ext-ChR2 group, the Ext-EYFP group, and the HC-ChR2 group, exhibited indistinguishable freezing levels during the first round of contextual fear extinction protocol from day 1 to day 3 (Figure 4D). To examine the roles of these labeled neurons in contextual fear extinction, we subjected the mice to another round of CFC in the same box for fear reinstatement (day 5). When these neurons were optogenetically activated during the second round of FET on day 6 (STAR Methods), the Ext-ChR2 group showed accelerated extinction compared with the Ext-EYFP and HC-ChR2 groups (Figure 4E). This reactivation-induced accelerated extinction was not observed in wild-type C57BL/6 mice that underwent the same surgical and behavioral procedures as Ppp1r1b-Cre mice (Figures S4E and S4F). These results demonstrate that extinction memory engram cells are formed in the BLA Ppp1r1b+ neuronal population and that their optogenetic activation is sufficient to drive fear extinction learning.

Because a similar proportion of Rspo2+ cells in the aBLA were also activated during fear ER as Ppp1r1b+ cells (Figure 2E), we tested whether fear extinction engram neurons existed in the aBLA Rspo2+ neuronal population as well (Figures 4F–4H and S4G). Optogenetic activation of BLA Rspo2+ neurons that were activated during fear ER did not affect fear extinction learning behavior (Figure 4I). These results demonstrate that fear extinction engrams are formed and stored specifically in a genetically distinct BLA Ppp1r1b+ neuronal populations.

**Ppp1r1b+ Extinction Engram Cells Are Necessary for Suppressing Rspo2+ Fear Cells in the Fear Extinction State**

We then investigated whether BLA Ppp1r1b+ extinction engram neurons are not only capable of driving fear extinction but also necessary for maintaining extinction memory and suppressing Rspo2+ fear cells. We labeled pBLA Ppp1r1b+ extinction engram cells with ArchT-mCherry during fear extinction memory retrieval (Figures S4A–S4C, S5A, and S5B). Optogenetic inhibition of these ArchT-labeled neurons specifically impaired retrieval of fear extinction memory on day 4 (Figure S5D) and failed to suppress BLA Rspo2+ fear neurons (Figures S5E–S5H). The latter is consistent with feedforward inhibition of Rspo2+ neurons by Ppp1r1b+ extinction engram cells within the BLA (Kim et al., 2016). Optogenetic inhibition of BLA Ppp1r1b+ extinction engram neurons did not affect the baseline freezing level in a neutral context that was different from the conditioning context (Figures S5C and S5D). Together, these results demonstrate a causal role of pBLA Ppp1r1b+ engram cells in extinction memory. These fear extinction engram cells (I) are reactivated during extinction.
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memory retrieval (Figure 2N), (2) have the capacity to drive fear extinction learning (Figure 4E), and (3) are necessary for suppressing the original fear memory (Figure 5D).

**Reward-Responsive Neurons and Fear Extinction Engram Neurons Overlap Significantly in the pBLA**

Our previous study demonstrated that pBLA Ppp1r1b+ neurons respond to a variety of appetitive stimuli (Kim et al., 2016), and our present study shows that fear extinction memory is stored in the pBLA Ppp1r1b+ neuronal population (Figures 1, 2, 3, 4, and 5). Hence, we proceeded to investigate the relationship between these two subsets of Ppp1r1b+ neurons. To label reward-responsive pBLA neurons, the AAVkg-c-Fos-tTA and AAVg-TRE-EYFP viruses were injected into the pBLA of C57BL/6 mice on Dox-ON diet (Figure 6A). One week post-surgery, all mice were partially deprived of water for 24 h on the Dox-ON diet (day 1), and water was given as a reward on the Dox-OFF diet (day 2) (Figure 6B). Mice were then divided into W/W (water/water), W/F (water/food), W/Ext (water/extinction), and W/NonExt (water/non-extinction) groups and kept on the Dox-ON diet throughout the remainder of the experiment (Figure 6B; STAR Methods).

Neurons activated by a water reward on day 2 were labeled with EYFP, and a large fraction (77.7% ± 1.7%) of pBLA neurons activated by a water reward were Ppp1r1b+ (Figure S6A). The proportions of water-responsive neurons among all pBLA neurons (EYFP+/DAPI−) were similar across all four groups (Figures S6C, 6G, S6E, and S6E). No labeling of EYPF− neurons was observed in the anterior BLA (Figures S6B and S6E). Neurons activated by the second condition on day 5—namely, the second delivery of a water reward (W/W group), food reward (W/F group), extinction memory retrieval (W/Ext group), and fear memory retrieval (W/NonExt group)—were detected by immunohistochemistry for endogenous c-Fos. The proportions of c-Fos+ cells among all neurons (c-Fos+/DAPI−) were similar between the W/W group, W/F group, and W/Ext group and were substantially higher compared with the W/NonExt group in the pBLA (Figures S6D, 6G, S6C, and S6E). In contrast, a larger proportion of aBLA neurons was activated in the W/NonExt group compared with the other three groups (Figures S6C and S6E). Two metrics, the ratio of c-Fos and EYFP double-positive cells among EYFP+ cells (c-Fos− EYFP+/EYFP+) and the ratio of c-Fos and EYFP double-positive cells among c-Fos+ only cells (c-Fos+EYFP+/c-Fos+EYFP+), were used to measure the degree of similarity between cells responding to the pair of stimuli used in each mouse group. These ratios were not different among the W/W group, W/F group, and W/Ext group, all of which had higher proportions than the W/NonExt group (Figures 6E–6G, S6D, and S6E). These results show that the overlap of pBLA neurons activated by fear extinction and water reward is as high as the overlap of pBLA neurons activated by two natural rewards (water and food).

We further confirmed these results with calcium imaging, which permits monitoring of the temporal dynamics of the response of individual neurons to various behavioral treatments. Ppp1r1b-Cre mice were injected with AAVV9-hsyn:DIO-GCamp6f in the pBLA and subjected to in vivo calcium imaging using a microendoscope while they longitudinally experienced natural reward presentations (water and food), followed by habituation, footshocks, ET, and extinction memory retrieval sessions (Figures 7A and 7B). The majority of BLA Ppp1r1b+ neurons did not respond to any behavioral treatments. Among the neurons that responded, at the population level, BLA Ppp1r1b+ neurons were predominantly activated by a water reward, food reward, and ER but were predominately inhibited by footshocks (Figures 7C and S7; Table S3). The neurons activated by ER highly overlapped (~40%–60%) with neurons activated by natural rewards, water or food. Notably, the overlap of pBLA Ppp1r1b+ neurons activated by fear extinction and a reward (water or food) was as high as the overlap of pBLA Ppp1r1b+ neurons activated by two types of natural rewards (water and food), both of which were significantly higher than the overlap of pBLA Ppp1r1b+ neurons activated by a reward (water or food) and footshocks (Figures 7D–7H). These results are consistent with those obtained in the c-Fos-based activation study (Figure 6) and indicate that mice respond to fear ER and natural reward presentations.
indiscriminately, as reflected by activation of BLA Ppp1r1b+ neurons. 

**Ppp1r1b**+ Fear Extinction Engram Cells and Reward-Responsive Cells Are Interchangeable at the Behavioral Level

We then examined whether the equivalence of rewards and fear extinction demonstrated at the cellular level is prevalent at the behavioral level. For this purpose, we investigated whether optogenetic activation of fear extinction engram cells can drive appetitive behavior. Four groups of C57BL/6 mice were set up (Figures 8A and 8B). In the Ext-ChR2 and Ext-EYFP groups, Ppp1r1b+ extinction engram cells were labeled with ChR2-EYFP and EYFP during ER on day 3, respectively (Figures 8B, S8A, and S8B). As controls, in the water-ChR2 group and CFC-ChR2 group, neurons activated by a water reward and fearful memory retrieval, respectively, were labeled with ChR2 (Figures 8B, S8C, and S8D). On day 4, all mice were subjected to an optogenetic self-stimulation behavior test or optogenetic real-time place preference test (Figures 8B, S8E, and S8F; STAR Methods). 50% of Ext-ChR2 group mice and 67% of water-ChR2 group mice displayed self-stimulation behavior, but neither the Ext-EYFP group nor the CFC-ChR2 group displayed self-stimulation behavior (Figure 8C). In the optogenetic real-time place preference test, mice in the Ext-ChR2 group and water-ChR2 group spent significantly more time in the light-stimulated side of the chamber, whereas the Ext-EYFP and CFC-ChR2 groups spent an almost equal amount of time in the two sides of the chamber (Figures 8D, 8E, and S8G).

Reciprocally, optogenetic activation of water reward-responsive ChR2-expressing pBLA Ppp1r1b+ neurons accelerated

**Figure 5. BLA Ppp1r1b**+ Fear Extinction Memory Engram Cells Are Necessary for Fear ER and Suppressing Rspo2**+** Fear Cells

(A) Virus-based activity-dependent labeling scheme. Ppp1r1b-Cre mice were injected with AAV$_P$-c-fos-DIO-ITA and AAV$_P$-TRE-eArchT-mCherry or AAV$_P$-TRE-mCherry virus and implanted with optical fibers bilaterally targeting the pBLA.

(B) Experimental protocol. The fear extinction engram was labeled on day 3 during ER and inhibited the following day.

(C) Representative image showing activity-dependent expression of eArchT-mCherry co-stained with PPP1R1B antibody and optical fiber implant in the pBLA.

(D) Inhibition of fear extinction engram cells in pBLA caused significant recovery of fear on day 4. mCherry group, n = 8; ArchT group, n = 8. Unpaired t test, ***p < 0.001. Data are presented as mean ± SEM.

(E) Virus-based activity-dependent labeling scheme of BLA Ppp1r1b+ fear extinction memory engram cells for the experiments in (F) and (H).

(F) Percentage of Fos+ neurons within BLA Rspo2+ neurons across the A/P axis from −0.8 mm to −2.0 mm when pBLA Ppp1r1b+ extinction engram neurons were inhibited during ER on day 4. Data are presented as mean ± SEM.

(G) Average of the percentage of Fos+/Rspo2+ shown in (E). Ext-mCherry group, n = 4; Ext-ArchT group, n = 4. Unpaired t test, ***p < 0.001. Data are presented as mean ± SEM.

(H) Double smFISH of Fos (red) and Rspo2 (green) in the aBLA in Ext-mCherry mice (left) and Ext-ArchT mice (right).
fear extinction learning (Figures 8F and 8G). This facilitatory effect was not observed when water-responsive neurons were labeled with EYFP control virus (EYFP-ON group) or when the optical stimulation was off (ChR2-OFF group) (Figures 8F and 8G). Furthermore, inhibition of water-responsive neurons during ER resulted in impairment of fear ER in the original conditioned context but not in a distinct neutral context (Figures 8H–8K). Thus, fear extinction engram cells can drive appetitive behaviors, and reward-responsive neurons can facilitate fear extinction learning and memory, suggesting functional equivalence between these two neuronal subsets.

DISCUSSION
In this study, we have shown that fear extinction requires new memory engram cells that are formed and stored within the
Figure 7. Calcium Activity of BLA Ppp1r1b+ Neurons during Reward and Fear Extinction

(A) Cre-dependent expression of GCamp6f and implantation of the microendoscope above the pBLA of Ppp1r1b-Cre mice.

(B) Behavior protocol for Ca²⁺ recording during water reward (day 2), food reward (day 5), CFC (day 6), and fear ER (day 8).

(C) From left to right: percentages of BLA Rspo2+ neurons with increased (up, orange), decreased (down, blue), or NC (gray) responses to water reward (295 neurons), food reward (311 neurons), footshocks (259 cells), and ER (252 cells). n = 7 Ppp1r1b-Cre mice.

(D) The percentage of overlapping neurons between water-responsive neurons and neurons activated by one of the other three stimuli. One-way ANOVA. *p < 0.05, ***p < 0.001. Data are presented as mean ± SEM.

(E) The percentage of overlapping neurons between food-responsive neurons and neurons activated by one of the other three stimuli. One-way ANOVA.

(F) A three-set Venn diagram showing the number of neurons that were activated by a water reward, food reward, and ER or combinations of two or three of these conditions.

(G) Heatmap of all 13 cells that were activated by a water reward, food reward, and ER.

(H) Ca²⁺ traces of six representative BLA Ppp1r1b+ neurons that are shown in (G) with corresponding cell numbers.
BLA $Ppp1r1b^+$ reward-responsive subpopulation. Here fear extinction engram cells are defined by their activation during FET, reactivation during ER, and their sufficiency and necessity to drive fear extinction. The overlap of neuronal activation of extinction memory engram neurons and natural reward-responsive neurons is extensive, and these two types of neurons are shown to be mutually interchangeable in driving appetitive and fear extinction behaviors. Thus, the shift from fear to safety in the fear extinction process is mediated by newly formed $Ppp1r1b^+$ appetitive memory neurons and their inhibition of the original $Rspo2^+$ fear memory neurons, both present within the BLA.

Inhibition of $Rspo2^+$ fear neurons by $Ppp1r1b^+$ extinction neurons is likely to be mediated by local inhibitory interneurons in the BLA (Kim et al., 2016), supporting the emerging notion that the switch between fear expression and fear extinction takes place within the BLA (Ehrlich et al., 2009; Grewe et al., 2017; Herry et al., 2008; Pare and Duvarci, 2012). Consistent with previous recording studies (Grewe et al., 2017; Herry et al., 2008), our study shows that fear extinction is due to new learning rather than loss of the original fear memory (Herry et al., 2010; Myers and Davis, 2007; Pavlov, 2010). $Ppp1r1b^+$ extinction neurons may correspond to previously reported BLA neurons identified by in vivo recording, which were activated throughout extinction learning after cue fear conditioning (Herry et al., 2008). However, our findings extend beyond that study because we further demonstrate that the newly formed extinction memory is an appetitive memory that inhibits the original fear memory.

Evaluation of Evidence of Fear Extinction Memory Engram Cells

pBLA $Ppp1r1b^+$ neurons are activated by FET and retrieval (Figures 1 and 2), and they have the capacity to promote fear extinction, as demonstrated by optogenetic activation and inactivation experiments (Figure 3). However, to demonstrate that fear extinction memory engram cells are indeed formed and stored in a subset of the pBLA $Ppp1r1b^+$ population, we showed that these cells were activated by ET and retrieval and that subsequent (e.g., one or more days later) optogenetic reactivation of these neurons with 80%–100% efficiency (Liu et al., 2012; Okuyama et al., 2016) facilitated the fear extinction behavior (Figure 4, sufficiency demonstration), and their optogenetic inhibition after establishing a fear extinction state caused disruption of fear extinction memory (Figure 5, necessity demonstration). These data met the fundamental criteria for the existence of engram cells for fear extinction memory within the pBLA $Ppp1r1b^+$ cell population (Semon, 1921; Tonegawa et al., 2015; Josselyn and Tonegawa, 2020); activation by learning and reactivation by recall, enduring and off-line maintenance of the acquired information, and memory expression via reactivation.

Interestingly, a similar proportion (~12%) of $Rspo2^+$ neurons and $Ppp1r1b^+$ neurons was activated during ER (Figures 2E and 2F). However, in stark contrast to these $Ppp1r1b^+$ neurons, optogenetic activation of these $Rspo2^+$ neurons failed to accelerate the subsequent fear extinction learning (Figures 4H and 4I), supporting the conclusion that fear extinction memory is stored specifically in the genetically marked $Ppp1r1b^+$ neurons in the pBLA. The differential optogenetic outcome of these $Rspo2^+$ and $Ppp1r1b^+$ BLA neurons most likely reflects the differences in their intrinsic properties as well as in their input and output circuits (Kim et al., 2016, 2017). Indeed, despite similar proportions (~12%) being activated during ER, the proportions of cells inhibited during this step were dramatically different between these two BLA populations: 27% in $Rspo2^+$ cells and 2% in $Ppp1r1b^+$ cells. These results suggest that the behavioral outcome of the fear extinction learning is determined not just by the proportion of BLA neurons activated by ER but by the balance of activation and inhibition of the two mutually antagonistic $Rspo2^+$ and $Ppp1r1b^+$ neuronal populations that are genetically predetermined to support negative and positive valence behaviors, respectively (Kim et al., 2016). During extinction memory learning, extinction is not complete, and therefore some $Rspo2^+$ neurons are activated by the conditioned cues, but these neurons are not intrinsically capable of driving extinction learning.

Evaluation of Evidence of the Equivalence of Fear Extinction Engram Cells and Reward-Responsive Cells

We employed two measures: activation of c-Fos (Figure 6) and calcium activity imaging (Figure 7). For the c-Fos measure, we
determined the proportion of Ppp1r1b+ neurons that were activated by water as a natural reward and reactivated 3 days later by the same reward, food as another kind of natural reward, or ER cues (Ext). 10%–12% of Ppp1r1b+ neurons were initially activated, and about 40% of them were reactivated by the second time stimulus. There was no significant difference in the reactivation proportions among the W/W, W/F, and W/Ext combinations, and they were all significantly higher than the overlap proportion of the W/NonExt combination. The activation proportions of 10%–15% by the first presentations of stimuli were well within the range reported in the BLA and lateral amygdala (LA) in previous studies (Beyeler et al., 2016; Han et al., 2009; Kim et al., 2016; Redondo et al., 2014; Ryan et al., 2015).

It has been widely recognized that even when a neuronal population in a particular brain region has the capacity to be activated by a given stimulus at a given moment, only a fraction (5%–20%) of them are eligible (or available) for activation by that or a categorically similar stimuli, presumably because of heterogeneity in the intrinsic cellular excitability (Josselyn and Frankland, 2018; Silva et al., 2009; Tonegawa et al., 2015). It is likely that the limited proportions of Ppp1r1b+ neurons activated or reactivated by natural rewards and fear extinction cues reflect this stimulus- and time-dependent heterogeneity in their eligibility rather than their stimulus-dependent but time-independent heterogeneity of the capacity for activation of the Ppp1r1b+ neuronal population. The crucial points here are that there were no significant differences in the reactivation proportions between the natural reward/natural reward combinations versus natural reward/extinction combinations (Figures 6E, 6F, 7D, and 7E). Additionally, their overlap proportions were all significantly higher than the proportions that would be expected from a chance-level overlap.

The longitudinal monitoring of in vivo calcium activity of individual BLA Ppp1r1b+ cells across multiple behavioral treatments permitted identification of overlapping cells activated by various combinations of the aforementioned stimuli at both the cellular population level and the individual cell level (Figures 7D and 7E). These data confirmed the conclusion drawn by the population analysis made using c-Fos as the readout. The overall results demonstrate the same capacity of BLA Ppp1r1b+ neurons to respond to fear ER as a natural reward stimulus rather than their functional heterogeneity.

**Neural Circuits for Fear Extinction**

The finding that the fear extinction memory is an appetitive memory indicates that the emotional valence associated with a CS is switched from negative to positive during fear extinction. Notably, the finding that activation of fear extinction engram cells can elicit an appetitive effect (Figure 8) supports the notion that the absence of an expected aversive stimulus is, in itself, a reward. Therefore, fear extinction is positively reinforced via reward systems that have been observed in Pavlovian fear conditioning (Nasser and McNaughton, 2012). In addition to direct feedforward inhibition within the BLA, BLA Ppp1r1b+ extinction neurons could also drive fear extinction through their interactions with other brain regions. As downstream targets of BLA, both intercalated cells (ITCs) and the central amygdala (CeA) have been shown to play important roles in fear extinction (Likhtik et al., 2008). Our previous study showed that BLA Ppp1r1b+ neurons provide monosynaptic projections to Prkcd+ neurons located in the lateral subnuclei of the CeA (CeLs), which are also activated during fear ER (Kim et al., 2017). Therefore, fear extinction behavior can also be modulated via BLA Ppp1r1b+-CeA monosynaptic projection or BLA Ppp1r1b+-ITC-CeA di-synaptic projection. The medial prefrontal cortex (mPFC) is another critical brain region for fear learning and extinction behavior. Mounting evidence suggests that two sub-regions of the mPFC bidirectionally regulate conditioned fear responses: the prelimbic (PL) cortex promotes fear expression, whereas the infralimbic (IL) cortex promotes fear suppression (Burgos-Robles et al., 2007; Laurent and Westbrook, 2009; Vidal-Gonzalez et al., 2006). In particular, accumulating findings suggest that the IL is required for consolidation and retrieval of fear extinction memory (Laurent and Westbrook, 2009; Marek et al., 2018; Milad and Quirk, 2002). The antagonistic effects of the PL and IL cortices on fear extinction are believed to be mediated through their reciprocal connections with the amygdala because the PL and IL cortices receive distinct inputs from BLA Rsp02+ and Ppp1r1b+ neurons, respectively, and project to different amygdaloid nuclei (Cho et al., 2013; Kim et al., 2016; Likhtik et al., 2005; Marek et al., 2018; McGarry and Carter, 2016; Quirk et al., 2006; Sotres-Bayon et al., 2012).

Taken together with our findings of BLA Ppp1r1b+ fear extinction memory engram neurons, we propose that fear extinction memory is stored in multiple brain regions that are functionally connected and that each engram cell ensemble contributes to storage of that overall memory quantitatively and qualitatively. Such brain-wide distribution of engram ensembles has also been reported previously for other types of memory paradigms (Kita-mura et al., 2017; Redondo et al., 2014; Ryan et al., 2015).

**Intrinsic Reinforcement Signals during Fear Extinction Learning**

How Ppp1r1b+ cells are activated for acquisition of fear extinction memory is another question that remains to be elucidated. Reinforcement learning theories predict that the acquisition of extinction memory is instructed by negative prediction error, in which the predictive value of the CS with respect to the occurrence of the US is compromised (McNally et al., 2011; Schultz, 2016). Given that there is no obvious external US in the case of fear extinction, this stimulus may be provided internally and preferentially to Ppp1r1b+ neurons in the BLA. Identification of the agent and the circuit that provide this stimulus is of fundamental importance.

Accumulating evidence suggests that dopamine reinforces fear extinction learning by signaling the omission of expected...
We thank X. Zhou, K. Marshall, B. Bane, F.J. Bushard, and W. Yu for technical assistance; C. Sun and C.J. MacDonald for discussions regarding the calcium imaging data analysis; M. Pignatelli, S. Muralidhar, Q. Ferry, R. Lim, K. Flick, and C.J. MacDonald for comments and discussions regarding the manuscript; and all members of the Tonegawa laboratory for their support. This work was supported by the RIKEN Brain Science Institute, the Howard Hughes Medical Institute, and the JPB Foundation (to S.T.).

AUTHOR CONTRIBUTIONS
X.Z., J.K., and S.T. contributed to the study design. X.Z. contributed to data collection and interpretation. X.Z. conducted the surgeries, behavior experiments, and histological analyses. X.Z. and S.T. wrote the paper. All authors discussed and commented on the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES


# STAR★METHODS

## KEY RESOURCES TABLE

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LEAD CONTACT AND MATERIALS AVAILABILITY

All data is available in the main text or the supplementary materials. Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Susumu Tonegawa (tonegawa@mit.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

The Ppp1r1b-Cre (also called Cartpt-Cre) mice were obtained from GENSAT (stock number: 036659-UCD). The Rspo2-Cre transgenic mouse line was developed at RIKEN BSI by the group of Shigeyoshi Itohara and is available through a material transfer agreement. Cre-expressing mice were genetic knock-in mice or have been previously been validated for genetic specificity (16). All Cre transgenic mice were bred using a heterozygous male with females of C57BL/6 background and maintained as heterozygous. Mice had access to food and water ad libitum and were socially housed in numbers of two to five littermates until surgery. After surgery, mice were singly housed. 2- to 6-month old male mice were used for all experiments. For engram labeling, C57/B6 mice were raised on food containing 40 mg kg\(^{-1}\) DOX 1 before stereotaxic surgery and were maintained on Dox diet for the remaining experiment period except during the target Dox-OFF days. All mice were maintained and cared for in accordance with protocols approved by the Massachusetts Institute of Technology (MIT) Committee on Animal Care (CAC) and guidelines by the National Institutes of Health (Andreescu et al., 2015).

METHOD DETAILS

Viral constructs

AAV5-Ef1a-DIO-eArch3.0-EYFP (AV5257), AAV5-Ef1a-DIO-ChR2-EYFP (AV5226B), and AAV5-Ef1a-DIO-eYFP (AV4310D) were obtained from the University of North Carolina at Chapel Hill Vector Core. AAV5-C-fos-DIO-tTA, AAV5-C-fos-tTA, AAV5-TRE-EYFP, AAV5-TRE-ChR2-EYFP, AAV5-TRE-mCherry, and AAV5-TRE-eArchT-mCherry were generated by and acquired from the Gene Therapy Center and Vector Core at the University of Massachusetts Medical School or University of Pennsylvania Vector Core as previously described (Kitamura et al., 2017; Liu et al., 2012; Redondo et al., 2014). Virus plasmids are available through a material transfer agreement. The AAV5-hsyn-flex-GCaMP6f-WPRE-SV40 virus was generated by and acquired from the University of Pennsylvania Vector Core.

Stereotactic surgery

Mice undergoing stereotactic injections were anesthetized under isoflurane. Standard stereotactic procedures were used. Viruses were injected using a mineral oil–filled glass micropipette attached to a 1 µL Hamilton microsyringe. Virus was injected at a speed...
of 80 nl/min, which was controlled by a microsyringe pump. The needle was lowered to the target site and remained for 5 min after the injection. The incision was closed with sutures. Mice were given 0.1 mg/kg slow release Buprenex as analgesic and remained on a heating pad until full recovery from anesthesia. The histology of all injections was verified after behavior experiments.

For optogenetic behavior experiment, 200 μL of AAV5-Cre-dependent virus was bilaterally injected into the anterior BLA of Rspo2-Cre mice (AP −1.4 mm, ML ± 3.4 mm, DV −4.9 mm) and the posterior BLA of Ppp1r1b-Cre mice (AP −2.0 mm, ML ± 3.4 mm, DV −4.9 mm) and incubated for 3–4 weeks before behavioral experiments.

For optical implant surgery, two optic fibers (200 mm core diameter, 5.5 mm length; Doric Lenses) were bilaterally lowered above the injection sites (aBLA: −1.4 mm AP, ± 3.4 mm ML, −4.7 mm DV; pBLA: −2.0 mm AP, ± 3.4 mm ML, −4.7 mm DV). The implants were secured to the skull with two jewelry screws and adhesive cement (C&B Metabond). A protective cap, made using a 1.5 mL black Eppendorf tube, was fixed onto the implant using dental cement (Kim et al., 2016, 2017).

For Ca²⁺ imaging surgery, AAV5-hsyn-flex-GCaMP6f-WPRE-SV40 was unilaterally injected into right aBLA of Rspo2-Cre mice and right pBLA of Ppp1r1b-Cre mice with the following coordinates: aBLA was targeted at −1.4 mm AP, +3.4 mm ML, −4.9 mm DV and pBLA was targeted at −2.0 mm AP, +3.4 mm ML, −4.9 mm DV. Two weeks following AAV5-hsyn-flex-GCaMP6f-WPRE-SV40 virus injection, a GRIN lens (0.5 mm diameter, 8 mm length; Inscopix) was implanted targeting aBLA or pBLA (aBLA: −1.4 mm AP, +3.4 mm ML, −4.7 mm DV; pBLA: −2.0 mm AP, +3.4 mm ML, −4.7 mm DV).

Contextual fear extinction behavior protocol
Behavioral context was 29 × 25 × 22 cm chambers (Med Associates) with grid floors, opaque ceilings, white lighting, and scented with 5% benzaldehyde. Before fear conditioning, mice were habituated to investigator handling for 5 min on three consecutive days in the holding room where the mice were housed. On Day 1, mice were habituated in the behavioral context for 3 min, followed by three footshocks (0.60 mA, 2 s) delivered at 180 s, 250 s and 320 s. Mice remained in the behavior chamber for 80 s after the third footshock and then returned to their home cages in the holding room. 24 hr after fear conditioning, mice returned to the fear conditioning chambers to receive 45 min extinction training without any footshocks. Then mice were put back to their home cages for another 24 hr. On Day 3, mice were placed in the conditioned context for 5 min extinction retrieval test. Behavior videos were recorded with VideoFreeze software and freezing level was scored manually by experimenters who were blinded to conditions or automatically with DeepLabCut behavior analysis toolbox (Mathis et al., 2018).

Used as control in Figures 8K, S3C, and S5C, context B was designed with white plastic floors, curved wall with visual cues, white lighting, and scented with 1% acetic acid to be different from the original conditioning context.

Single molecular fluorescent in situ hybridization
To examine the expression of Fos gene (Figures 1 and 5), single molecule fluorescence in situ hybridization (smFISH) was performed using RNAscope Fluorescent Multiplex Kit (Advanced Cell Diagnostics, ACDBio) as previously described (16, 18). Isoflurane-anesthetized mice were decapitated, their brains harvested, and flash frozen on aluminum foil on dry ice. Brains were stored at −80°C. Prior to sectioning, brains were equilibrated to −16°C in a cryostat for 30 min. Brains were coronally sectioned at 20 μm with cryostat and thaw-mounted onto Superfrost Plus slides (25 × 75 mm, Fisherbrand). Sections from a single brain were serially thaw-mounted onto 10 slides through the entire BLA (anterior-posterior distance from Bregma, −0.8 mm to −2.6 mm). Slides were air-dried for 60 min at room temperature prior to storage at −80°C. smFISH probes for all genes examined were obtained from ACDBio, Fos (Cat #421981), Ppp1r1b (Cat #405901) and Rspo2 (Cat #402001). Slides were counterstained for the nuclear marker DAPI using ProLong Diamond Antifade mounting medium with DAPI (ThermoFisher) (Kim et al., 2016, 2017).

Immunohistochemistry
Mice were perfused transcardially with phosphate buffered saline (PBS), followed by 4% paraformaldehyde (PFA). Brains were post-fixed with 4% PFA solution overnight and then sectioned coronally 50 μm at with a vibratome. Free floating brain sections were washed in PBST (PBS, 3% TritonX) three times for 10 min, blocked for 1 hr in blocking buffer (PBST, 5% normal goat serum), and incubated in primary antibody in blocking buffer overnight at 4°C. On the next day, brains were washed with three 10 min washes of PBST and incubated in secondary antibody in blocking buffer at room temperature for 2 hr. Primary antibodies used were chicken anti-GFP (Invitrogen, A10262, 1:1,000), rabbit anti-c-Fos (Santa Cruz, sc-52, 1:1,000 and Synaptic Systems, Cat. No 226003, 1:1000) and rabbit anti-PPP1R1B (Abcam, ab40801, 1:1,000). Secondary antibodies used were goat anti-chicken Alexa Fluor 488 (Invitrogen, A11039, 1:1,000), goat anti-rabbit Alexa Fluor 555 (Invitrogen, A21428, 1:1,000) and goat anti-rabbit Alexa Fluor 488 (Invitrogen, A27034, 1:1,000). After three more 10-min PBST washes, slides were covered with coverslips and mounted using VectaShield mounting solution containing DAPI (Vector Laboratories).
Calcium imaging surgery and data acquisition

Calcium imaging of BLA Rspo2\(^+\) neurons and Ppp1r1b\(^+\) neurons was performed on Rsopo2-Cre and Ppp1rb-Cre mice, respectively. AAV\(_2\)-hsyn-flex-GCaMP6f-WPRE-SV40 was unilaterally injected into right aBLA of Rsopo2-Cre mice and right pBLA of Ppp1rb-Cre mice with the following coordinates: aBLA was targeted at \(-1.4 \text{ mm AP}, +3.4 \text{ mm ML}, -4.9 \text{ mm DV} \) and pBLA was targeted at \(-2.0 \text{ mm AP}, +3.4 \text{ mm ML}, -4.9 \text{ mm DV} \). Two weeks following AAV\(_2\)-hsyn-flex-GCaMP6f-WPRE-SV40 virus injection, a GRIN lens (0.5 mm diameter, 8 mm length; Inscopix) was implanted targeting aBLA or pBLA (aBLA: \(-1.4 \text{ mm AP}, +3.4 \text{ mm ML}, -4.7 \text{ mm DV} \); pBLA: \(-2.0 \text{ mm AP}, +3.4 \text{ mm ML}, -4.7 \text{ mm DV} \)). Two weeks after GRIN lens implant, a baseplate for the miniaturized microscope camera was attached above the GRIN lens using ultraviolet-light curable glue (Loctite 4305) (Mukamel et al., 2009; Ziv et al., 2013).

For contextual fear extinction behavior in Figure 2, recording was performed during the light cycle. Inscopix endoscope and calcium events were captured at 20 Hz with an Inscopix miniature microscope (nVistaHD 2.0). Inscopix endoscope was synchronized with Med Associate behavior chamber via a TTL signal, which was used for all the other fear extinction behavior experiments in this study. Mice underwent the three-day contextual fear extinction procedure as previously described (Figure 1). During contextual fear conditioning (CFC) on Day 1, freezing behavior and Ca\(^{2+}\) signals of subjects were recorded for 3 min habituation, as well as 3 min when three footshocks were delivered. During 45 min fear extinction training, Ca\(^{2+}\) signals was recorded during the 0-3 min, 5-8 min, 10-13 min, 15-18 min, 20-23 min, 25-28 min, 30-33 min, 35-38 min and 40-43 min timestamps. The 0-3 min epoch was considered as fear retrieval stage. The other eight epochs together were considered as extinction training stage. Recorded calcium-imaging movies were first processed with ImageJ to subtract baseline activity (dividing each image by a low-pass, r = 20 pixels, and high pass, r = 1000 pixels), followed by motion correction using Inscopix Mosaic software (Mukamel et al., 2009). Subsequently, ΔF/F (F-F0)/F0 signal was calculated in Mosaic software (Inscopix), in which F0 was calculated over the entire recording session. A stacked image of ΔF/F recording movie was acquired by maximum intensity projection. Cell identifications were semi-automatically identified and cross-registered for longitudinal recording by visual inspection (Kitamura et al., 2017). The corresponding Ca\(^{2+}\) activity was extracted by applying PCA/ICA analysis in Mosaic software (Inscopix). In total, we recorded 179 BLA Rspo2\(^-\) cells from 5 Ppp1rb-Cre mice and 169 BLA Rspo2\(^+\) cells from 4 Rsopo2-Cre mice. The total number of recorded neurons and the distributions of neurons in each category in each mouse are reported in Tables S1 and S2.

To longitudinally monitor BLA Ppp1rb\(^+\) neurons in response to natural rewards and fear extinction in Figure 7, we injected AAV\(_2\)-hsyn-flex-GCaMP6f-WPRE-SV40 and implanted GRIN lens unilaterally into the right pBLA of Ppp1rb-Cre mice. Animals received water reward after 24 hr water deprivation on Day 2, food reward after 24 hr food deprivation on Day 5, and a three-day contextual fear extinction procedure as previously described (Figure 7B). Calcium events were captured at 20 Hz with an Inscopix miniature microscope (Ppp1rb-Cre #1-5: nVistaHD 2.0; Ppp1rb-Cre #6-7: nVistaHD 3.0). Inscopix endoscope was synchronized with EthoVision XT video tracking software (Noldus Information Technologies) during drinking behavior (Day 2, Figure 7B) and feeding behaviors (Day 5, Figure 7B) via a TTL signal, or Med Associate behavior chamber during CFE (Day 6-8, Figure 7B) via a TTL signal. On Day 2, drinking behavior and Ca\(^{2+}\) signals of subjects were recorded for 3 min before and 5 min after water reward delivery. On Day 5, feeding behavior and Ca\(^{2+}\) signals of subjects were recorded for 3 min before and 5 min after food reward delivery. The data acquisition and analysis of contextual fear extinction behavior was done in the same manner as previously described in Figure 2.

For Ppp1rb-Cre #1-5 (Table S3), the calcium data were analyzed in the same fashion as previously described in Figure 2. For Ppp1rb-Cre #6-7 (Table S3), the calcium data analysis, including the background subtraction, motion correction, ΔF/F (F-F0)/F0 calculation, and PCA/ICA analysis, were all done with corresponding modules in the Inscopix Data Processing Software (IDPS, Inscopix). The detailed neuron numbers recorded from each mouse is reported in Table S3.

Data analysis of Ca\(^{2+}\) events

Ca\(^{2+}\) events were detected with a threshold 15% ΔF/F. We calculated the average time of Ca\(^{2+}\) events, i.e., the sum of all Ca\(^{2+}\) events duration/record time. To identify cells with increased or decreased activity in response to footshocks during CFC (Day 1 in Figures 2D; Day 6 in Figure 7B), we compared the mean ΔF/F of 3 min after delivery of footshocks with that of 3 min habituation stage before footshocks. To identify cells with changed neuronal activities during fear extinction (Figure 2), we compared the mean ΔF/F of the fear retrieval stage (0-3 min) on Day 2 with that of the 3 min habituate period before footshocks on Day 1. To identify cells with increased or decreased activity during fear extinction training, we compared the mean ΔF/F of extinction training with that of habituation stage (0-3 min) (12). To identify cells with increased or decreased activity during extinction retrieval (Day 3 in Figure 2D; Day 8 in Figure 7B), we compared the mean ΔF/F of 5 min extinction retrieval with that of the 3 min habituation stage during CFC. To identify cells with changed neuronal activities in response to water reward (Day 2, Figure 7B), we compared the ΔF/F of 5 min after water delivery (Post Water) with that of the 3 min before water was delivered (Pre Water). To identify cells with changed neuronal activities in response to food reward (Day 5, Figure 7B), we compared the ΔF/F of 5 min after food delivery (Post Food) with that of the 3 min before food was delivered (Pre Food). For each comparison, after shuffling is done on all individual Ca\(^{2+}\) transients 5000 times between the two recording sessions to be compared, a distribution of ΔF/F difference was generated. If the actual difference between mean ΔF/F of two recording sessions was higher than 97.5% of the distribution, the cell was then considered as significantly activated (Up cells). If the actual difference between mean ΔF/F of two recording sessions was lower than 2.5% of the distribution, the cell was then considered as significantly inhibited (Down cells). The rest were considered as no change cells (NC). All the data were analyzed using custom code written in MATLAB (Mathworks).
Longitudinal tracking of individual neurons for in vivo imaging

For BLA Rspo2<sup>+</sup> neurons and Ppp1r1b<sup>+</sup> neurons recorded in Figure 2 and BLA Ppp1r1b<sup>+</sup> neurons recorded in Ppp1r1b-Cre mice #1-5 in Figure 7, ΔF/F recording movie were calculated with Mosaic (Inscopix) software and the stacked images were acquired by maximum intensity projection with ImageJ software. Individual cells were visually tracked based on the cell location and shape in the stacked images (Kitamura et al., 2017; Okuyama et al., 2016), as shown in the supplementary Figures S2A and S2B.

For those BLA Ppp1r1b<sup>+</sup> neurons recorded in Ppp1r1b-Cre #6-7 mice in response to water reward, food reward and fear extinction retrieval in Figure 7, individual cells were tracked with the longitudinal registration function in the Inscopix Data Process Software (IDPS, Inscopix). Multiple recording sessions of the same animal were added to one project and each session was treated as one cell set. Before alignment, a threshold was applied to all cell sets’ images to remove negative values, after which, each image was normalized and aligned to the first cell map that was used as a reference by using a rigid transformation that was composed of translation and rotation. This transformation was estimated using the enhanced correlation coefficient (ECC) image registration algorithm on the two cell maps (Evangelidis and Psarakis, 2008). After the alignment, the cells from each cell set were matched to a common or global list of identified cells. The same cells that appeared to be exactly the same in different cell sets of the same project were longitudinally registered across various behavioral treatments. However, some cells that were only active in particular cell sets and did not overlap with any cell images in another cell set were only analyzed under corresponding conditions and were not included in the overlap analysis.

Cell counting

Images of smFISH counting were acquired using a Zeiss Axiolmager.Z2 under a 10X objective. Consecutive sections with 0.2 mm interval (anterior-posterior distance from Bregma, −0.8 mm to −2.6 mm) were used for quantification. Neurons stained for Fos (red) and against gene marker (green), Rspo2 or Ppp1r1b, were counted using image analysis software (HALO, Indica Labs), which was compatible with RNAscope Fluorescent Multiplex Kit.

Images of immunohistochemistry counting were acquired using confocal microscopy (Zeiss LSM700) under a 10X objective. Maximum intensity projections were generated using ZEN Blue software (Zeiss). Neurons stained against GFP (green), c-Fos (red), mCherry (red), PP1R1B (green or red) and DAPI (blue) were automatically using cell counting software (ImageJ) with manual adjustment of detection thresholds and custom code written in MATLAB (Mathworks).

In Figure 6, For DAPI counts, thresholds were set to only include larger nuclei and to exclude non-neuronal small nuclei as previously described. The acquired images were aligned to the Allen Brain Atlas and the region of interest (ROI) was defined based on anatomical landmarks. All counting was performed blind as to the group and condition that the specimen belonged to.

Optogenetic activation/inhibition

In Figures 1J–1M, AAV<sub>9</sub>-DIO-ArchT-EYFP virus was injected into pBLA of Ppp1r1b-Cre<sup>+</sup> mice or their Ppp1r1b-Cre<sup>−</sup> littermates as control. During fear extinction retrieval on Day 3, the mice received 3 cycles of 1 min optical inhibition (520-550 nm, 8-12 mW, constant) with 1 min interval.

To apply optogenetic activation/inhibition during fear extinction training in Figures 3C–3G, mice were put back to conditioned behavior chamber to receive 45 min fear extinction training. After the first 5 min, the mice received 8 cycles of 3 min optogenetic activation (450-470 nm, 8-12 mW, 20 Hz) or 3 min optical inhibition (520-550 nm, 8-12 mW, constant) with 2 min interval. 450 nm and 520 nm lasers were generated with LED drivers (Doric).

To apply optogenetic inhibition during fear extinction retrieval (Figures 3H and 3I) and control test in context B (Figures S3C-S3E), the mice received 3 cycles of 1 min optical optogenetic activation (450-470 nm, 8-12 mW, 20 Hz) or 1 min optical inhibition (520-550 nm, 8-12 mW, constant) with 1 min interval.

Labeling and optogenetic manipulation of fear extinction engram

To label fear extinction engram neurons in Figure 4, we injected AAV<sub>9</sub>-c-Fos-DIO-tTA together with AAV<sub>9</sub>-TRE-ChR2-EYFP or AAV<sub>9</sub>-TRE-EYFP into the pBLA of Ppp1r1b-Cre mice (Figure 4A) or aBLA of Rspo2-Cre mice (Figure 4F). Then, these mice were subjected to a series of engram labeling and behavioral steps (Figures 4D and 4H). This genetic manipulation permits expression of ChR2-EYFP or EYFP in the cells that were activated during fear extinction memory retrieval through the transcriptional promoter of c-Fos gene when doxycycline (Dox) was removed from the diet. Mice were kept on Dox diet after surgery. In Ext-ChR2 group and Ext-EYFP group (Figure 4C), Rspo2-ChR2 group and Rspo2-EYFP group (Figure 4H), mice were switched to Dox-OFF diet for 24 hr following fear extinction training on Day 2. 60 min after extinction retrieval test on Day 3, mice returned to Dox-ON diet and stayed at home cage on Day 4. In HC-ChR2 group (Figure 4C), mice stayed on Dox diet throughout the first round of contextual fear extinction protocol and were switched to Dox-OFF diet for 24 hr and stayed at home cage on Day 4. On Day 5, mice in all three groups received one immediate footshock (0.75 mA) in the original conditioned context for fear reinstatement. On Day 6, mice received opto-extinction protocol, in which 8 cycles of 3 min-long blue lasers (470 nm, 20 Hz, 8-12 mW) were delivered after the first 5 min. On Day 7, mice were put back to the behavior context for 5 min retrieval test.

In Figure 5, fear extinction engram neurons were labeled in the same fashion as Figure 4, except that the fear extinction engram neurons expressed ArchT-mCherry or mCherry. On Day 4, mice were put back to the conditioned context for 5 min opto-extinction retrieval test, in which mice received three green laser stimulation (520-550 nm, 8-12 mW, constant) at 0-1 min, 2-3 min and 4-5 min
time points. Next day, the same group of mice were placed into context B that was different from the original conditioning context and underwent the same optical inhibition paradigm as described above. For Fos quantification experiment in Figures 5E–5G, mice were sacrificed, and the brain were harvested and subjected to smFISH procedure.

**Labeling and manipulation of reward responsive neurons**

To label reward responsive neurons in Figures 6B, 8B, and 8F, we injected AAV$_9$-c-Fos-tTA together with AAV$_9$-TRE-ChR2-EYFP or AAV$_9$-TRE-EYFP into the pBLA of C57BL/6 mice. One-week post-surgery, mice were deprived of water for 24 hr while on Dox diet. Water-deprived mice were given regular chow food without Dox (Dox-OFF diet) for 24 hr, during which water was given as a reward. 60 min after drinking water, subjects returned to Dox-ON diet to close the labeling window.

After being back to Dox-ON diet, mice were divided into four different groups. Mice in the W/W (Water/Water) group remained on water deprivation for the next three days. On Day 5, W/W group mice received ad libitum water as a reward 90 min before perfusion. Mice in the W/F (Water/Food) group were deprived of food for the next three days and received ad libitum food as a reward 90 min before perfusion. During food deprivation, mice received water that contained 200 ul/L Doxycycline to remain on Dox. Mice in the W/Ext (Water/Extinction) group went through three-day contextual fear extinction protocol and were perfused 90 min after extinction retrieval on Day 5. Mice in the W/NonExt group (Water/NonExtinction) group went through the same procedure as the W/Ext group except that they stayed in home cage (HC) instead of extinction training on Day 4. After perfusion, brains of these four groups of mice were sliced and stained for c-Fos followed by quantification.

In Figure 8F, water reward responsive neurons were labeled with EYFP or ChR2-EYFP depending on the virus injection combination. Mice went through three-day contextual fear extinction protocol. For EYFP-ON group and ChR2-ON group, mice received opto-extinction protocol, in which 8 cycles of 3 min-long blue lasers (470 nm, 20 Hz, 8-12 mW) were delivered after the first 5 min of extinction training. For ChR2-OFF group, mice didn’t receive any optogenetic stimulation during extinction training.

In Figure 8F, water reward responsive neurons were labeled with ArchT-mCherry or mCherry depending on the virus injection combination. Mice went through CFC (Day 3), fear extinction training (Day 4), fear extinction retrieval in the original conditioning context (Day 5), followed by retrieval test in a distinct neutral context B (Day 6). During both retrieval tests on Day 5 and 6, green laser stimulation (520-550 nm, 8-12 mW, constant) was delivered at 0-1 min, 2-3 min and 4-5 min time points.

**Optogenetic open field test**

Mice were placed in a custom-made open field arena (L30 × W30 × H30 cm, white plexiglass). Each trial was 10min in which mice received 3 cycles of 2 min blue laser stimulation (20 Hz, 8-12 mW, 473 nm). Behavioral data were recorded and analyzed with EthoVision XT video tracking software (Noldus Information Technologies).

**Optogenetic self-stimulation test**

The mice were water-restricted overnight before the experiment. The optogenetic self-stimulation test was conducted over a 60-min session on the following day with no prior training required. The water-restricted mice were placed in an operant conditioning chamber (Med Associates) equipped with two nose ports (16, 18). At the beginning of each trial, each nose port contained 0.05 mL sugar water to initiate the nose poke. No additional water reward was given throughout the trial. One of the two nose ports was randomly assigned as active port, which could deliver 5 s duration of optical stimulation (8-12 mW, 20 Hz, 473 nm) upon nose poke, while the other one did not (inactive). The optical laser was generated through a 470 nm LED light source (XLED1, Lumen Dynamics). The number of nose pokes of active port and inactive port were recorded with Med-PC IV software during 60 min test session. If the difference between active pokes and inactive pokes (active-inactive) was greater than 20 pokes, it was accounted as self-stimulation behavior.

**Optogenetic place preference**

Mice were placed in a custom-made behavioral arena (L45 × W15.5 × H30 cm, white plexiglass), where each side of the box contained distinct wall cues. Each trial was 20 min and one side was randomly assigned as the stimulation side for each trial. Mice were place in the non-stimulated side at the onset of the experiment. Each time the mouse crossed over to the stimulation side of the chamber, the mice would receive continuous 20 Hz laser stimulation (8-12 mW, 473 nm) until they cross back to the non-stimulation side. Behavioral data were recorded and analyzed with EthoVision XT video tracking software (Noldus Information Technologies).

**Systemic injection of Kainic acid**

To induce seizure, mice were injected intraperitoneally with 15 mg kg$^{-1}$ kainic acid (KA, Tocris, Cat. No 0222) after being on a Dox off diet for 24 hr. 3 hr after KA injection, mice were returned to Dox diet and perfused next day for immunohistochemistry staining.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

GraphPad Prism (version 8.0 for Mac OS X, GraphPad Software, La Jolla California USA) and MATLAB (Mathworks) were used for statistical analysis. All data are presented as mean ± SEM n indicates number of animals or number of cells. Comparisons
between two-group data were analyzed by two-tailed unpaired t test or two-tailed paired t test. Multiple group comparisons were assessed using a One-way ANOVA with Bonferroni’s correction followed by Turkey’s test or Two-way RM (Repeated-measures) ANOVA followed by Sidak’s multiple comparisons test when significant main effects or interactions were detected. The null hypothesis was rejected at the p < 0.05 level. Data met assumptions of statistical tests. Sample sizes were chosen on the basis of previous studies (Kim et al., 2016, 2017; Liu et al., 2012; Nishi et al., 1997).

DATA AND CODE AVAILABILITY

All data, materials, animals and analysis code are available from corresponding authors upon request.