Selective Erasure of a Fear Memory
Jin-Hee Han, *et al.*
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treated mice, Foxp3$^+$ T cells still lost Foxp3 expression but did not gain expression of CXCR5, CD40L, and PD-1 (Fig. 4C). Consistent with these observations, blocking CD40-CD40L interactions resulted in a reduction of GC formation in PPs and of B220$^-$ IgA$^+$ plasma cells in the LP (fig. S5, A and B). Thus, the differentiation of Foxp3$^+$ T cells into T_{FH} cells requires B cells and CD40 expression, presumably by either B cells or DCs or both.

Foxp3$^+$ T cells were converted into T_{FH} cells only in PPs; neither T_{FH} cells nor GCs could be detected in spleen or LNs of CD3ε$^{-/-}$ mice adoptively transferred with Foxp3$^+$ T cells (Fig. 4, D and E, and fig. S6). Furthermore, immunization with sheep red blood cells (SRBCs) in the presence of bacterial components failed to induce GCs in spleen of CD3ε$^{-/-}$ mice that received Foxp3$^+$ T cells. In contrast, control mice and CD3ε$^{-/-}$ mice that had received Foxp3$^-$ T cells generated GCs in the spleen after SRBC immunization (Fig. 4, D and E). Thus, the precursors of PP T_{FH} cells are enriched in the Foxp3$^+$ T cell population, whereas other T cells, like Foxp3$^-$ T cells, can differentiate into T_{FH} cells in the spleen on experimental systemic immunization.

Our studies demonstrate that Foxp3$^+$ T cells in PPs can differentiate efficiently into cells with characteristics of T_{FH} cells, which then participate in the induction of GCs and IgA synthesis in the gut. How can we then explain the preferential generation of PP T_{FH} cells from the peripheral Foxp3$^+$ T cell population? Two possible scenarios can be conceived. First, T_{FH} cell differentiation per se may require a Foxp3-dependent molecular program. This is unlikely, however, because adoptively transferred Foxp3$^-$ T cells efficiently generated T_{FH} cells in the spleen on immunization with SRBCs and because *scurfy* T cells, which contain a mutated Foxp3 gene, could generate T_{FH}-phenotype cells in PPs, albeit less efficiently than control T cells (fig. S7). A more likely scenario is that PP T_{FH} cell differentiation may be controlled by the same signals that promote Foxp3 expression in gut T cells (*27*–*30*), such as antigen recognition through the T cell receptor (TCR). Consistent with this idea, adoptive transfer of ovalbumin-specific OT-II TCR transgenic RAG-2$^{-/-}$ CD4$^+$ T cells into CD3ε$^{-/-}$ hosts led to generation of Foxp3$^+$ T cells in the gut LP and T_{FH} cells in the PPs if mice were fed ovalbumin (fig. S8). These data suggest that, depending on the environment, TCR stimulation induces either IL-10–producing "suppressor" or IL-21–producing "helper" T cells (fig. S9). Despite the presence of the same antigens that previously induced Foxp3 expression, the IL-6–, IL-21–, and activated B cell–rich environment of PPs results in many Foxp3$^+$ T cells differentiating into T_{FH} cells. These studies have implications for how the suppression of inflammatory reactions and induction of IgA synthesis occur in the gut.

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Figs. S1 to S9
References

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Selective Erasure of a Fear Memory

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Memories are thought to be encoded by sparsely distributed groups of neurons. However, identifying the precise neurons supporting a given memory (the memory trace) has been a long-standing challenge. We have shown previously that lateral amygdala (LA) neurons with increased cyclic adenosine monophosphate response element–binding protein (CREB) are preferentially activated by fear memory expression, which suggests that they are selectively recruited into the memory trace. We used an inducible diphtheria-toxin strategy to specifically ablate these neurons. Selectively deleting neurons overexpressing CREB (but not a similar portion of random LA neurons) after learning blocked expression of that fear memory. The resulting memory loss was robust and persistent, which suggests that the memory was permanently erased. These results establish a causal link between a specific neuronal subpopulation and memory expression, thereby identifying critical neurons within the memory trace.

Ensembles of neurons are thought to serve as the physical representation of memory (the memory trace) (*1*). However, identifying the precise neurons that constitute a memory trace is challenging because these neuronal ensembles are likely sparsely distributed (*2*). Previous studies detected neurons whose activity is correlated with memory encoding, expression, or both (*3*–*7*). However, correlative studies do not address whether these neurons are essential components of the memory trace. A direct test of this requires specifically disrupting only those activated neurons and determining whether subsequent memory expression is blocked. Establishing this causal role has been difficult, because of the limited ability of current techniques to target a specific subset of neurons within a brain region.

To target neurons whose activity is correlated with memory, we took advantage of our recent findings that LA neurons with relatively increased levels of the transcription factor CREB were preferentially activated by auditory fear memory

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training or testing (*8*). To manipulate CREB levels in a subpopulation (roughly 15%) of LA neurons, a region critical for auditory fear memory (*9*–*12*), we used replication-defective herpes simplex viral (HSV) vectors (*13*). Neurons overexpressing CREB (with CREB vector) were three times as likely to be activated as their noninfected neighbors after fear memory training or testing in wild-type (WT) mice and 10 times as likely to be activated in CREB-deficient mice. Conversely, WT neurons with a dominant-negative CREB vector were 1/12 as likely as their neighbors to be activated by fear training or testing. These findings suggest that neurons with relatively higher CREB function are preferentially recruited into the fear memory trace and that posttraining ablation of just these neurons should disrupt expression of the established fear memory.

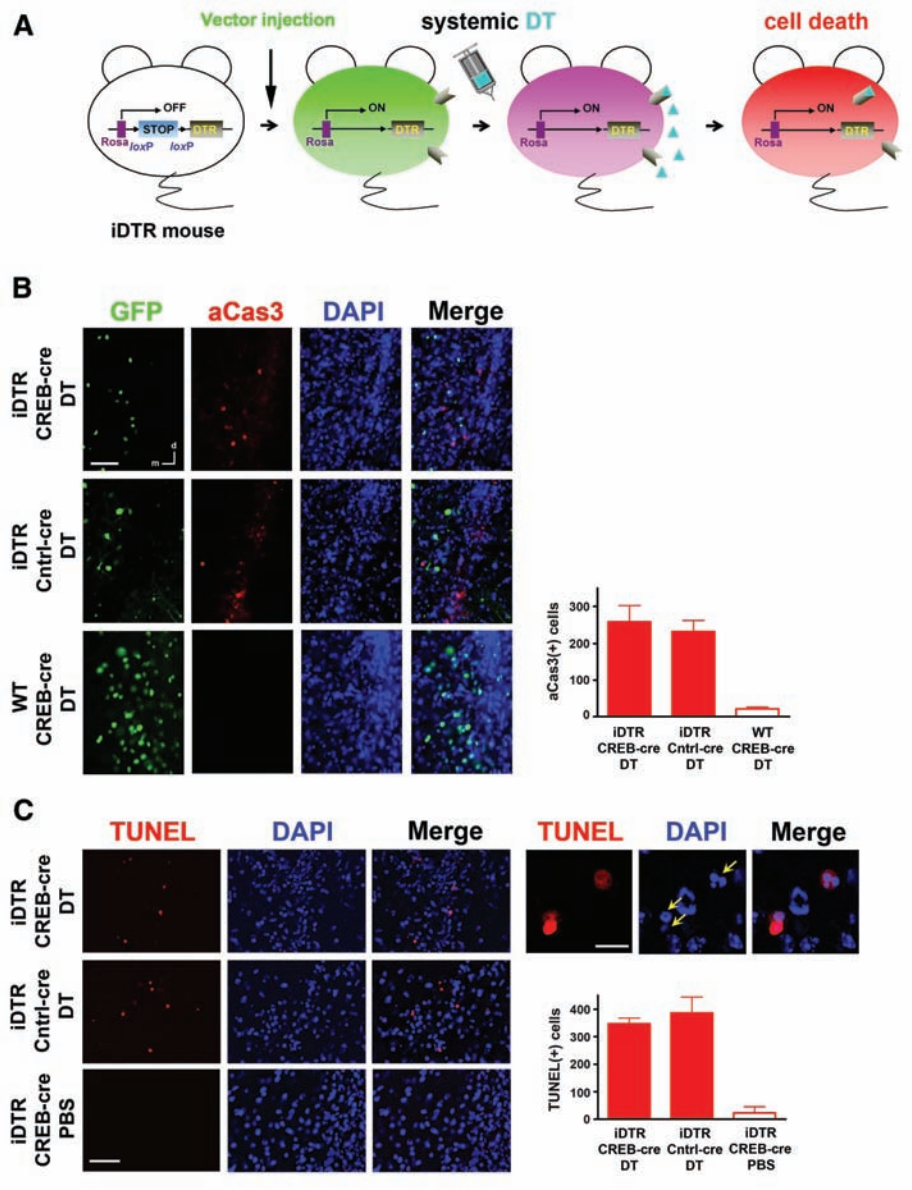

Fig. 1. Selective ablation of LA neurons. (**A**) iDTR mice express DTR under control of a floxed STOP cassette (no DTR expression, indicated by white). CREB-cre or Cntrl-cre microinjected into LA (green). Cre removes STOP cassette, which allows DTR expression (pink). DT (blue triangles) induces apoptosis (red) only in cells that have undergone recombination. (**B**) (Left) Low GFP (with cre vector, green) and high activated caspase-3 (aCas3, red) levels in experimental (iDTR/CREB-cre/DT, iDTR/Cntrl-cre/DT), but not control (WT/CREB-cre/DT), mice. DAPI (4′,6′-diamidino-2-phenylindole)–stained nuclei (blue). Scale bar, 100 μm. (Right) High activated caspase-3 levels in experimental [iDTR/CREB-cre/DT (n = 24), iDTR/Cntrl-cre/DT (n = 17)], but not control [WT/CREB-cre/DT (n = 4)], mice ($F_{2,42}$ = 6.44, P < 0.001). (**C**) (Left) High TUNEL (red) levels in experimental (iDTR/CREB-cre/DT, iDTR/Cntrl-cre/DT), but not control (iDTR/CREB-cre/PBS), mice. Scale bar, 100 μm. (Right, top) Morphological indicators of apoptosis in experimental mice. Scale bar, 50 μm. (Right, bottom) High TUNEL levels in LA of experimental mice [iDTR/CREB-cre/DT (n = 5), iDTR/Cntrl-cre/DT (n = 6) versus control (n = 5); $F_{2,13}$ = 22.31, P < 0.001].

To ablate neurons overexpressing CREB in the present study, we used transgenic mice in which cell death may be induced in a temporally and spatially restricted manner. Apoptosis is induced after diphtheria toxin (DT) binds to the DT receptor (DTR) (*14*). Because mice do not express functional DTRs (*15*, *16*), we used transgenic mice that express simian DTR in a Cre-recombinase (cre)–inducible manner (iDTR mice) (*15*). A loxP-flanked STOP cassette that normally silences DTR expression is excised by cre, which allows constitutive expression of DTR. Injection of DT any time thereafter induces apoptosis only in cells expressing DTR.

We restricted DT-induced ablation to neurons overexpressing CREB by inserting cDNA encoding cre recombinase into our CREB vector (CREB-cre). DTR expression only occurs in neurons expressing cre, which allowed us to persistently tag infected neurons for subsequent ablation (Fig. 1A and fig. S1). As a control, we used Cntrl-cre vector to induce apoptosis in a similar portion of LA neurons that are not preferentially activated by fear testing. To examine cell death, we used two markers of apoptosis, caspase-3 activation and terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling (TUNEL) (*17*). We microinjected CREB-cre, Cntrl-cre, and CREB alone (without cre) vectors into the LA of iDTR transgenic and WT littermate mice, then administered DT or vehicle. We observed substantial cell death only in LA neurons of iDTR mice microinjected with CREB-cre or Cntrl-cre vectors and administered DT (experimental groups, Fig. 1, B and C, and fig. S2) (*15*). Negligible apoptosis was observed in regions outside the LA (figs. S3 and S4) or in control groups that lacked a key component (iDTR, cre or DT) (Fig. 1, B and C). Note that Cntrl-cre and CREB-cre vectors produced equal levels of cell death (Fig. 1, B and C); however, the efficiency of cell death was not complete (see SOM text). Therefore, this system allows temporally specific ablation of tagged neurons.

To verify that LA neurons overexpressing CREB are selectivity activated by fear memory testing with our modified vector, we microinjected WT mice with CREB-cre or Cntrl-cre vector before auditory fear training. To visualize neurons specifically activated by memory expression, we examined Arc (activity-regulated cytoskeleton-associated protein; Arg3.1) RNA (*18*). Neuronal activity induces a rapid, but transient, burst of Arc RNA that is quickly transported to the cytoplasm, which allows nuclear-localized Arc RNA to serve as a molecular signature of a recently (5 to 15 min previously) active neuron (*18*). Five minutes after testing, we removed brains and examined Arc (activated by fear testing) and green fluorescent protein (GFP) (with vector) RNA. Neurons with CREB-cre vector preferentially

expressed Arc after memory testing; neurons with CREB-cre were three times as likely to be activated by fear memory testing as their noninfected neighbors. In contrast, neurons with Cntrl-cre vector and their noninfected neighbors were equally likely to be activated by memory testing (Fig. 2).

We microinjected CREB-cre vector into the LA of iDTR mice before weak auditory fear training. Cntrl-cre vector was used to ablate a similar portion of random LA neurons (i.e., not preferentially activated by fear memory testing). We assessed memory before (test 1) and after (test 2) inducing cell death in tagged neurons by administering DT. The CREB-cre vector enhanced fear memory following weak training (Fig. 3, test 1), consistent with previous results (*8*, *19*–*22*). Selectively deleting neurons with CREB-cre vector completely reversed this enhancement (test 2). Note that CREB-enhanced memory was not blocked if either cre or DT was omitted, consistent with the absence of apoptosis in these control groups. The reversal of CREB-enhanced memory was not due to memory extinction with repeated testing because the control groups froze robustly on test 2. Therefore, increasing CREB in a subpopulation of LA neurons enhances a weak memory and specifically ablating just these neurons reverses this enhancement.

Although increasing CREB in a subpopulation of LA neurons does not further enhance a strong memory, neurons overexpressing CREB are, nevertheless, preferentially activated by fear memory expression (*8*). This suggests that CREB levels dictate which neurons are recruited into a memory trace, even in the absence of behavioral change. To examine the effects of ablating neurons overexpressing CREB on a strong memory, we trained mice that received CREB-cre or Cntrl-cre microinjections using an intense protocol. Strong training produced robust auditory fear memory in both groups before DT administration (Fig. 4A). After DT, only CREB-cre mice showed a loss of auditory fear memory. To investigate whether memory in mice microinjected with CREB-cre is particularly susceptible to the ablation of a small number of neurons, we microinjected both Cntrl-cre and CREB (no cre) vectors, which allowed us to delete only Cntrl-cre neurons after training. Deleting this small portion of neurons (that were not overexpressing CREB) had no effect on memory (fig. S5). Therefore, memory loss was specific; it was not determined by the absolute number of deleted LA neurons but by whether these deleted neurons overexpressed CREB at the time of training.

Administering DT after a fear memory test blocked expression of both CREB-enhanced memory produced by weak training and robust memory produced by strong training in iDTR mice microinjected with CREB-cre (but not Cntrl-cre) vector. Fear memory testing reactivates memory and may trigger a second wave of consolidation (reconsolidation) that, similar to initial consolidation, requires protein synthesis (*23*, *24*). Because DT induces cell death by inhibiting protein synthesis, it is possible that impaired reconsolidation contributes to the memory loss. To assess this, we trained mice but omitted the memory reactivation induced by test 1. Consistent with our previous results, fear memory was blocked in CREB-cre, but not Cntrl-cre, mice (Fig. 4B). Therefore, memory loss was independent of memory reactivation, which ruled out the possibility that blocking reconsolidation accounts for the memory disruption.

If neurons overexpressing CREB during training are critically involved in the subsequent memory trace, then deleting them should permanently block memory expression. To examine the persistence of memory loss, we trained mice, administered DT, and assessed memory 2, 5, and 12 days later. Memory loss in CREB-cre mice was long-lasting, whereas memory remained robust in Cntrl-cre mice (Fig. 4C). Therefore, we found no evidence of memory recovery in mice in which neurons overexpressing CREB were deleted, which suggested that memory was not transiently suppressed. To rule out the possibility that the memory loss was due to a nonspecific impairment in LA function, we showed that CREB-cre mice could relearn (Fig. 4C). Similarly, pretraining deletion of neurons overexpressing CREB did not impair the acquisition, or stability, of a conditioned fear memory (fig. S6). Deleting neurons overexpressing CREB does not affect subsequent learning, presumably because the high portion of remaining (noninfected) neurons are sufficient to encode a new memory. Finally, ablating CREB-overexpressing neurons did not block expression of a memory acquired before surgery (fig. S8). Together, these findings indicate that ablating neurons that were overexpressing CREB at the time of memory encoding blocks memory for that particular learning event, highlighting the specificity of memory loss.

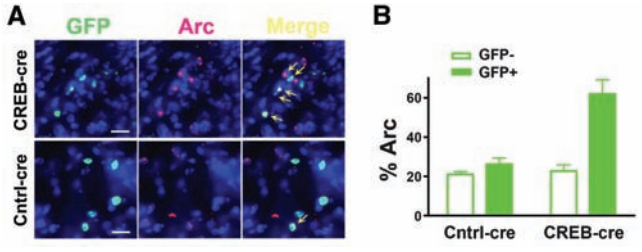

Fig. 2. Neurons overexpressing CREB preferentially activated by fear memory testing. (**A**) Double-labeled nuclei in LA of CREB-cre, but not Cntrl-cre, mice. Nuclei (blue), GFP^+ (with CREB-cre or Cntrl-cre vector, green), Arc^+ (pink), or double-labeled nuclei (GFP^+ and Arc^+; arrows). Scale bar, 20 μm. (**B**) In CREB-cre mice, Arc was preferentially localized in infected (GFP^+), rather than noninfected (GFP^-), neurons. In Cntrl-cre mice, Arc was equally distributed in infected and noninfected neurons (*Vector* × *GFP/Arc colocalization* interaction, $F_{1,5} = 18.74$, $P < 0.05$).

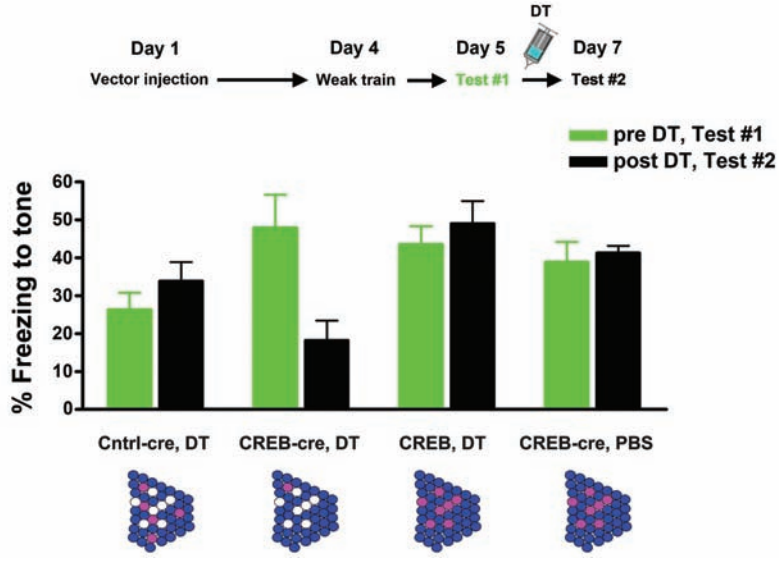

Fig. 3. Overexpressing CREB in LA neurons enhances memory induced by weak training; subsequent ablation of these neurons reverses this enhancement. (**Top**) CREB-cre microinjection enhanced memory after weak training [test 1, CREB-cre/DT ($n = 8$), Cntrl-cre/DT ($n = 9$), $P < 0.001$]. DT administration reversed this memory enhancement (test 2, $P < 0.001$). CREB-enhanced memory was not blocked on test 2 if either cre [CREB/DT ($n = 7$), $P > 0.05$] or DT [CREB-cre/PBS ($n = 6$), $P > 0.05$] was omitted. *Group* × *Test* interaction $F_{3,26} = 13.90$, $P < 0.001$. (**Bottom**) Schematic of LA neurons after DT or PBS. Blue, DAPI-labeled neuronal nuclei; pink, neurons activated by memory; and white, ablated neurons.

Our results show that neurons with increased CREB levels at the time of fear learning are critical to the stability of that memory, because selectively ablating these neurons after training blocks expression of this fear memory. This indicates that these neurons themselves are essential for memory expression in the days after fear conditioning; they are not simply creating a local environment that promotes memory formation (such as releasing trophic factors) (*25*). Deleting neurons whose activity is related to memory expression (overexpressing CREB at the time of training) produced memory loss, whereas ablating a similar number of random neurons (expressing Cntrl vector or overexpressing CREB well before or after training) did not. The observed amnesia was specific, robust, persistent and not due to a disruption in either reconsolidation or overall LA function. Together, these results suggest that ablating neurons overexpressing CREB permanently erases the fear memory (see supporting online material text). Fear learning may generate a broad memory trace that encompasses more LA neurons than affected by our treatment or multiple memory traces throughout the brain (*26*, *27*). However, deleting just neurons overexpressing CREB at the time of training produces amnesia, which suggests that these neurons play an essential role within what is likely a broader fear neuronal network. These results establish a causal link between a defined subpopulation of neurons and expression of a fear memory and, thereby, identify a key component of the memory trace.

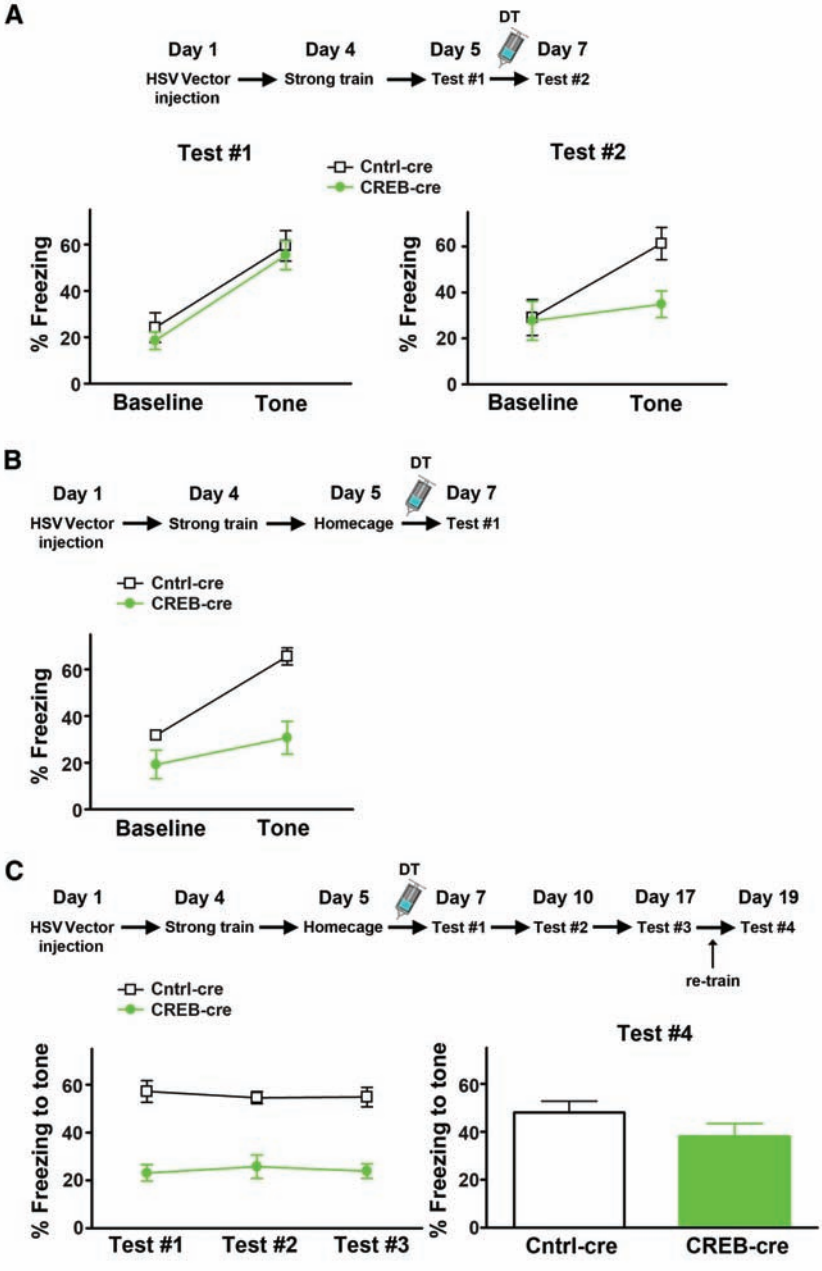

Fig. 4. Specifically ablating LA neurons overexpressing CREB, but not a similar portion of random neurons, blocks expression of strong memory. (**A**) Strong training produced equally robust and specific auditory fear memory (low baseline and high tone freezing) in CREB-cre ($n = 6$) and Cntrl-cre ($n = 6$) mice on test 1. Although Cntrl-cre mice showed robust memory after DT administration (test 2), CREB-cre mice showed impaired memory (low baseline and low tone freezing) (*Vector* × *Test* × *Time* significant three-way interaction, $F_{1,10} = 5.55$, $P < 0.05$). (**B**) Memory loss was not due to disruption of reconsolidation. Cntrl-cre ($n = 5$) mice showed robust auditory fear memory (low baseline but high tone freezing), whereas CREB-cre ($n = 6$) mice showed a loss of auditory fear memory (low baseline and tone freezing) (*Vector* × *Time* interaction, $F_{1,10} = 6.20$, $P < 0.05$). (**C**) Memory loss was persistent in CREB-cre mice, but they could relearn. Over repeated tests (tests 1 to 3), CREB-cre mice ($n = 10$) showed stable, low tone freezing, whereas Cntrl-cre mice ($n = 7$) showed robust tone freezing [*Vector* × *Test* analysis of variance (ANOVA), significant effect of *Vector* $F_{1,15} = 67.81$, $P < 0.05$ only]. (Right) After retraining CREB-cre mice showed an increase in tone freezing ($F_{1,15} = 1.7$, $P > 0.05$).

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Materials and Methods
SOM Text
Figs. S1 to S9
References
Movie S1

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Human Substantia Nigra Neurons Encode Unexpected Financial Rewards

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The brain's sensitivity to unexpected outcomes plays a fundamental role in an organism's ability to adapt and learn new behaviors. Emerging research suggests that midbrain dopaminergic neurons encode these unexpected outcomes. We used microelectrode recordings during deep brain stimulation surgery to study neuronal activity in the human substantia nigra (SN) while patients with Parkinson's disease engaged in a probabilistic learning task motivated by virtual financial rewards. Based on a model of the participants' expected reward, we divided trial outcomes into expected and unexpected gains and losses. SN neurons exhibited significantly higher firing rates after unexpected gains than unexpected losses. No such differences were observed after expected gains and losses. This result provides critical support for the hypothesized role of the SN in human reinforcement learning.

Theories of conditioning and reinforcement learning postulate that unexpected rewards play an important role in allowing an organism to adapt and learn new behaviors (*1*, *2*). Research on nonhuman primates suggests that midbrain dopaminergic neurons projecting from the ventral tegmental area and the pars compacta region of the SN encode unexpected reward signals that drive learning (*3*–*6*). These dopaminergic neurons are phasically activated in response to unexpected rewards and depressed after the unexpected omission of reward (*7*–*9*), and they are major inputs to a larger basal ganglia circuit that has been implicated in reinforcement learning across species (*10*–*15*).

The response of these neurons to rewards has not been directly measured in humans. We recorded neuronal activity in human SN while patients undergoing deep brain stimulation (DBS) surgery for Parkinson's disease performed a probability learning task. Patients with Parkinson's disease show impaired learning from positive and negative feedback in cognitive tasks (*16*–*18*), probably because of the degenerative nature of their disease and the decreased number of dopaminergic neurons capable of mounting phasic changes in activity in response to reward signals (*17*–*19*). We sought to capture remaining viable dopaminergic SN cells in our patients and determine whether they exhibit responses modulated by reward expectation.

We used microelectrode recordings to measure intraoperative activity of SN in 10 Parkinson's patients (6 men, 4 women, mean age of 61 years) undergoing DBS surgery of the subthalamic nucleus (STN) while they engaged in a probability learning task. We rewarded participants in the task with virtual financial gains to motivate learning. We identified SN by anatomic location and its unique firing pattern (Fig. 1A) (*20*). The learning task involved choosing between a red and a blue deck of cards presented on a computer screen (Fig. 1B). We informed partici-

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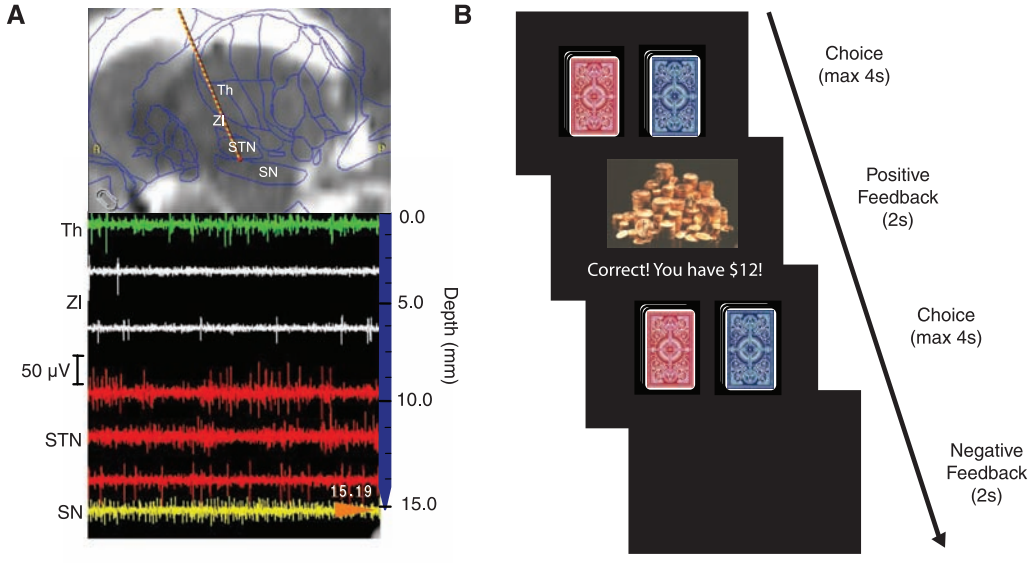

Fig. 1. (**A**) Intraoperative plan for DBS surgery with targeting of the STN. Microelectrodes are advanced along a tract through the anterior thalamic nuclei (Th), zona incerta (ZI), STN, and into the SN to record neural activity. Each anatomical region is identified by surgical navigation maps overlayed with a standard brain atlas (top) and by its unique firing pattern and microelectrode position (bottom). Depth measurements on the right of the screen begin 15 mm above the pre-operatively identified target, the inferior border of STN. In this example, the microelectrode tip lays 0.19 mm below the target. A, anterior; P, posterior. (**B**) Probability learning task. Participants are presented with two decks of cards on a computer screen. They are instructed to repeatedly draw cards from either deck to determine which deck yields the higher reward probability. Participants are given up to four seconds for each draw. After each draw, positive or negative feedback is presented for two seconds. Decks are then immediately presented on the screen for the next choice.