

# CREB Regulates Memory Allocation in the Insular Cortex

Yoshitake Sano,<sup>1</sup> Justin L. Shobe,<sup>1</sup> Miou Zhou,<sup>1</sup> Shan Huang,<sup>1</sup> Tristan Shuman,<sup>2</sup> Denise J. Cai,<sup>1</sup> Peyman Golshani,<sup>2</sup> Masakazu Kamata,<sup>3</sup> and Alcino J. Silva<sup>1,\*</sup>

<sup>1</sup>Departments of Neurobiology, Psychiatry and Biobehavioral Sciences, Psychology, Integrative Center for Learning and Memory and Brain Research Institute

<sup>2</sup>Department of Neurology at David Geffen School of Medicine

<sup>3</sup>Department of Hematology and Oncology

University of California, Los Angeles, Los Angeles, CA 90095, USA

## Summary

The molecular and cellular mechanisms of memory storage have attracted a great deal of attention. By comparison, little is known about memory allocation, the process that determines which specific neurons in a neural network will store a given memory [1, 2]. Previous studies demonstrated that memory allocation is not random in the amygdala; these studies showed that amygdala neurons with higher levels of the cyclic-AMP-response-element-binding protein (CREB) are more likely to be recruited into encoding and storing fear memory [3–6]. To determine whether specific mechanisms also regulate memory allocation in other brain regions and whether CREB also has a role in this process, we studied insular cortical memory representations for conditioned taste aversion (CTA). In this task, an animal learns to associate a taste (conditioned stimulus [CS]) with the experience of malaise (such as that induced by LiCl; unconditioned stimulus [US]). The insular cortex is required for CTA memory formation and retrieval [7–12]. CTA learning activates a subpopulation of neurons in this structure [13–15], and the insular cortex and the basolateral amygdala (BLA) interact during CTA formation [16, 17]. Here, we used a combination of approaches, including viral vector transfections of insular cortex, *arc* fluorescence in situ hybridization (FISH), and designer receptors exclusively activated by designer drugs (DREADD) system, to show that CREB levels determine which insular cortical neurons go on to encode a given conditioned taste memory.

## Results and Discussion

Although CREB has a critical role in memory allocation in the amygdala, it is unclear whether cortical circuits share similar memory allocation mechanisms. To test the hypothesis that taste memory is preferentially allocated to a specific population of neurons expressing higher levels of CREB, we made a lentivirus vector to overexpress CREB tagged with GFP in a subpopulation of insular cortex neurons (vCREB neurons). The CREB vector we derived also coexpresses the inducible hM4Di designer receptors exclusively activated by designer drugs (DREADD) receptor tagged with hemagglutinin (HA) (also referred to as Gi-DREADD). The UbC promoter in the

FG12 plasmid [18] was replaced by a 1.3 kb Ca<sup>2+</sup>/calmodulin-dependent protein kinase (CaMK2a) promoter to direct expression to excitatory neurons. The CREB and hM4Di genes are expressed under the CaMK2a promoter and cloned on either side of a T2A self-processing viral peptide (CREB virus; Figure 1A). This allows them to be coexpressed in the same neurons [19]. The hM4Di receptor is specifically activated by CNO, which does not activate endogenous muscarinic acetylcholine receptor [20]. The activation of hM4Di turns on endogenous G-protein-coupled inwardly rectifying K<sup>+</sup> (GIRK) channels, causing membrane hyperpolarization and decreased neuronal excitability. There is abundant expression of GIRK channels in the cortex [21], which allows the activity of infected neurons to be manipulated by systemic injection of CNO. As a control, we used a lentivirus that expresses dTomato-tagged GFP instead of enhanced GFP (EGFP)/CREB (control virus; Figure 1A).

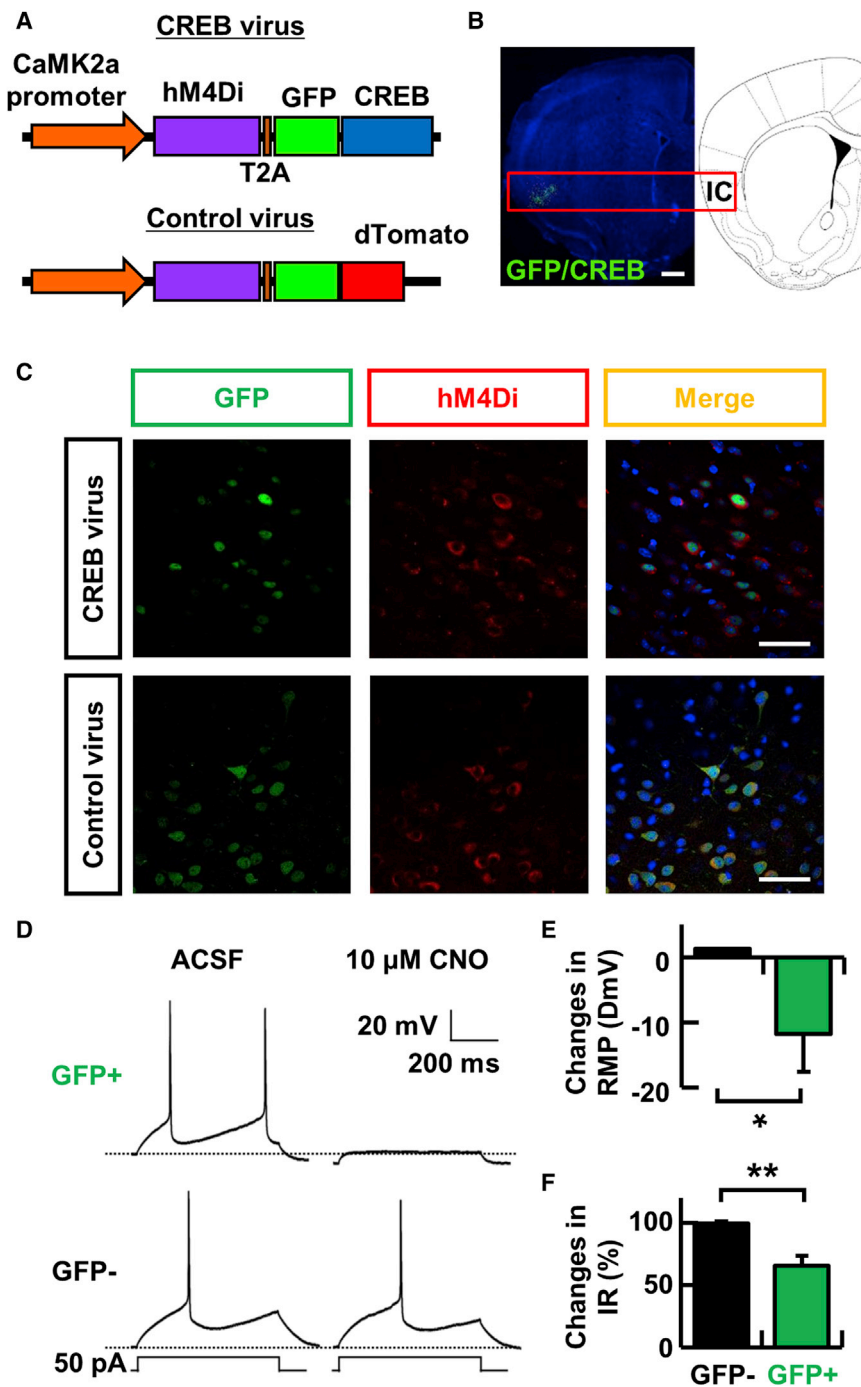
We bilaterally microinjected these viruses into the insular cortex. Immunohistochemical studies with an antibody against GFP detected the expression of this viral gene in a region about 300  $\mu$ m from the injection sites in the insular cortex (Figure 1B). We found that approximately 30% of the cells in the insular cortex expressed EGFP near the injection sites (CREB virus: 25.0%  $\pm$  2.1%; control virus: 31.2%  $\pm$  2.1%; n = 4 mice per group). The expression of the HA-tagged hM4Di receptor was detected using an HA tag antibody. As expected, we found that GFP and hM4Di were coexpressed in the same neurons (Figure 1C). To test whether CNO could selectively silence insular cortex neurons, we performed whole-cell patch-clamp recordings on both visually identified GFP-positive (hM4Di-positive) and GFP-negative neurons in brain slices from mice transfected with the CREB virus. Consistent with previous studies [20, 22, 23], we found that the resting membrane potential (RMP) and input resistance in GFP-positive (GFP+) neurons exposed to CNO were significantly more hyperpolarized and decreased than those in GFP-negative (GFP-) neurons (Figures 1D–1F; change in resting membrane potential [ $\Delta$ mV]: 1.3  $\pm$  1.0, n = 4, GFP-: -11.8  $\pm$  5.8 mV, n = 3, GFP+: p < 0.05; change in input resistance: 99.7%  $\pm$  1.8%, n = 4, GFP-; 65.4%  $\pm$  8.2%, n = 3, GFP+; p < 0.01). We also observed that frequency of firing in insular cortex around GFP+ neurons was decreased after CNO injection in an awake mouse, whereas firing was unchanged in areas of the insular cortex not infected with the virus (Figure S1 available online).

To first determine whether CREB has a role in memory formation in the mouse insular cortex, we used the lentiviruses described above to test the impact of CREB overexpression on memory strength. Previous results showed that overexpression of CREB in either the amygdala or hippocampus leads to enhancements in memory tasks that depend on these structures [3, 6, 24]. Accordingly, our results demonstrated that overexpression of CREB in the insular cortex results in enhancements in conditioned taste aversion (CTA) memory, a finding that is consistent with a role for this transcription factor in learning and memory in this structure (Figures S2A–S2C).

## Selective Silencing of vCREB Neurons Impairs CTA Memory Retrieval

Next, we determined whether silencing the subpopulation of insular cortical neurons that express vCREB disrupts the

\*Correspondence: [silvaa@mednet.ucla.edu](mailto:silvaa@mednet.ucla.edu)



**Figure 1. Selective Modulation of a Set of Genetically Tagged Insular Cortex Neurons**

(A) Schematic map of the lentivirus construct designed to express CREB and hM4Di. The hM4Di and GFP/CREB (or GFP/dTomato) genes located on both side of the T2A sequence are under the control of a Camk2a promoter.

(B) Representative picture showing localized GFP expression in the insular cortex by CREB virus infection. The scale bar indicates 500  $\mu$ m.

(C) Representative pictures showing coexpression of CREB and hM4Di in the same insular cortical neurons. CREB and hM4Di were detected by immunohistochemical staining using specific antibodies to GFP (green) and HA tag (red). Merged images show colocalized expression of either GFP/CREB or GFP/dTomato with hM4Di. GFP/CREB and hM4Di proteins show different subcellular localization, as expected. The scale bar indicates 50  $\mu$ m.

(D) Representative traces showing that CNO (10  $\mu$ M) inactivated transduced (GFP+) neurons in the insular cortex, whereas it had no effect on GFP- neurons. The dashed line represents the resting membrane potential (RMP) before drug administration. The currents injected are shown underneath associated traces. ACSF, artificial cerebrospinal fluid.

(E and F) Summary of the selective effect of CNO on (E) changes in RMP and (F) changes in input resistance (IR) of the insular cortex neurons. \* $p < 0.05$  and \*\* $p < 0.01$ . Data represent mean  $\pm$  SEM (GFP- cells, black columns; GFP+ cells, green columns).

the test. We utilized this CTA protocol to avoid any stress associated with an intraperitoneal injection during training. To evaluate CTA memory, we used a learning index (LI) along with an aversion index that is commonly used in the CTA literature [7]. The advantage of the learning index is that, for each animal tested, it compares avoidance of the salty taste after training with that measured before training (see details in [Experimental Procedures](#)). This comparison is important because not all mice show the same preference for salty tastes before training and differences between mice could bias performance after training.

In support of the hypothesis that CREB modulates memory allocation in

retrieval of a long-term memory for CTA. If vCREB preferentially recruits the neuronal ensemble encoding CTA in the insular cortex, then silencing these neurons should impair recall when compared to mice transfected with the control virus. To test this hypothesis, CREB and control viruses were bilaterally microinjected into the insular cortex 3 weeks before training. During training, mice were given 150 mM LiCl, which tastes salty (conditioned stimulus [CS]) and simultaneously induces malaise (unconditioned stimulus [US]) [25]. Mice were then administered saline or CNO prior to the test. In this test, mice were presented with two choices: water and 150 mM NaCl. Trained mice show a clear avoidance of NaCl during

the insular cortex, memory retrieval was impaired by silencing vCREB-positive neurons (Figure 2A: LI:  $100.0 \pm 11.8$ ,  $n = 8$ , CREB/saline;  $53.3 \pm 15.3$ ,  $n = 9$ , CREB/CNO;  $p < 0.05$ ; Figure S2D: aversion index:  $77.0 \pm 6.7$ , CREB/saline;  $57.0 \pm 6.2$ , CREB/CNO;  $p < 0.05$ ). Importantly, silencing control virus-positive neurons in the insular cortex did not result in impairments of memory retrieval (Figure 2B:  $n = 9$  per group; LI:  $100.0 \pm 8.7$ , control/saline;  $86.4 \pm 7.6$ , control/CNO;  $p = 0.25$ ; Figure S2E: aversion index:  $73.9 \pm 5.3$ , control/saline;  $66.4 \pm 4.7$ , control/CNO;  $p = 0.30$ ). This result suggests that vCREB-positive neurons are preferentially chosen to encode taste memory in the insular cortex because inactivation of a similar population of

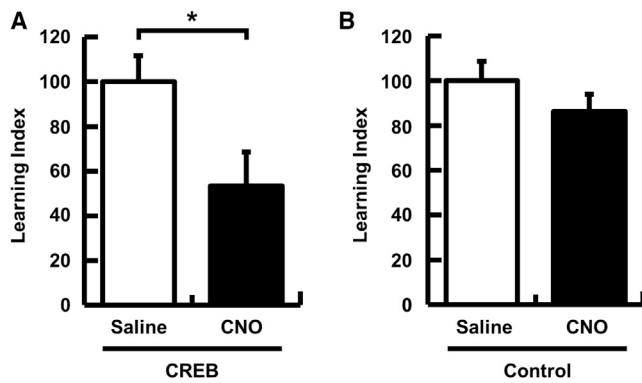


Figure 2. Silencing of CREB+ Neurons in the Insular Cortex Impairs CTA Memory Retrieval

CREB or control lentiviruses were infused into the insular cortex 3 weeks before CTA training. Saline or CNO was systemically injected 45 min before CTA memory retrieval.

(A) CTA memory was significantly impaired by selective silencing of CREB-positive neurons by CNO (saline group,  $n = 8$ ; CNO group,  $n = 9$ ).

(B) CNO injection did not impair CTA memory retrieval in control mice (saline group,  $n = 9$ ; CNO group,  $n = 9$ ).

LI was normalized to 100 for a saline group in each experiment. Data represent mean  $\pm$  SEM. \* $p < 0.05$ . (Saline-injected mice, white columns; CNO-injected mice, black columns).

insular cortical neurons with normal CREB levels did not affect CTA. Taken together, these data suggest that the insular cortex neurons expressing higher levels of CREB are preferentially involved in memory for CTA. Interestingly, Han and colleagues showed that disruption of CREB function in the lateral amygdala before tone fear training did not impair subsequent memory formation, due to the likely exclusion of those neurons from memory allocation, whereas enhancing CREB function in a similar population of neurons before tone fear training biases them toward allocation [3, 4]. These findings suggest that CREB regulates memory allocation during training/memory encoding.

#### vCREB Neurons Are Preferentially Activated during CTA Memory Retrieval

We next imaged the insular cortex following CTA retrieval to further test whether vCREB neurons are preferentially incorporated into neurocircuits encoding CTA. To visualize these neurocircuits, we used the activity-dependent gene *arc* (activity-regulated cytoskeleton-associated protein; also termed *arg3.1*) [26]. Neuronal activity induces a rapid but transient increase in *arc* transcription, such that *arc* RNA expression serves as a molecular signature of a recently (5–15 min) active neuron [26]. CREB and control viruses were bilaterally micro-injected into the insular cortex 3 weeks before CTA training. Five minutes after CTA memory retrieval, we harvested the brain and performed fluorescence in situ hybridization (FISH) to analyze coexpression of *arc* and *gfp* mRNA (denoting viral transfection) in insular cortex neurons. If increased CREB expression biases memory allocation, then vCREB-positive neurons should be more likely to express *arc* mRNA than control virus-positive neurons. Consistent with this hypothesis, the probability of *arc* mRNA expression induced by memory retrieval in vCREB-positive neurons was higher than in control virus-infected neurons (Figures 3A and 3B;  $n = 4$  mice per group; control virus,  $32.9\% \pm 1.9\%$ ; CREB virus,  $50.3\% \pm 4.9\%$ ;  $p < 0.05$ ). There were no differences between the number of *arc*+ neurons between

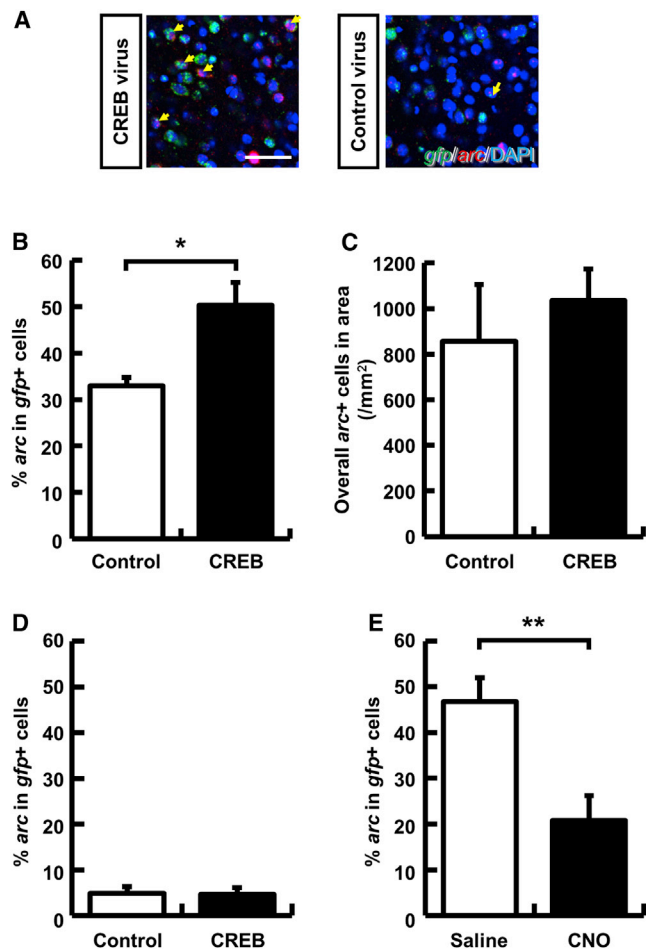


Figure 3. Insular Cortex Neurons with Increased CREB Level Are Preferentially Recruited into Encoding Conditioned Taste Memory

(A) Representative images showing expression of *gfp* (green) and *arc* (red). Yellow arrows indicate double-labeled neurons (*gfp*+ and *arc*+). Nuclei were stained using DAPI. The scale bar indicates 50  $\mu\text{m}$ .

(B) Probability of cells expressing *arc* following taste memory retrieval over cells transduced with the CREB or control viruses in the insular cortex ( $n = 4$  mice per group).

(C) Overall *arc*+ cells in the analyzed area ( $n = 4$  mice per group).

(D) Probability of cells expressing *arc* in home-cage mice in cells transduced with the CREB or control viruses in the insular cortex ( $n = 4$  mice per group).

(E) Probability of cells expressing *arc* following taste memory retrieval with saline or CNO in vCREB-positive cells ( $n = 6$  saline group and  $n = 7$  CNO group).

Data represent mean  $\pm$  SEM; \* $p < 0.05$  and \*\* $p < 0.01$ . (B, C, and D: control virus group, white columns; CREB virus group, black columns; E: saline group, white columns; CNO group, black columns).

the mice transfected with either CREB or control viruses (Figure 3C). To further test whether *arc* expression is induced by vCREB expression independently of memory retrieval, we also measured the probability of *arc* expression in vCREB and control neurons in home cage mice. Our results show that there is no difference between these two groups (Figure 3D;  $n = 4$  mice per group; control virus,  $4.9\% \pm 1.5\%$ ; CREB virus,  $4.7\% \pm 1.5\%$ ;  $p = 0.92$ ). This result is consistent with a similar previous study in which CREB was overexpressed in the amygdala using the herpes simplex virus vector [3]. To further test whether vCREB neurons make a critical contribution to memory retrieval, we analyzed *arc* expression



following CTA memory retrieval in vCREB-positive neurons after CNO or saline treatment. Consistent with the behavioral result (Figure 2A), the probability of *arc* expression in vCREB neurons during retrieval was significantly decreased by silencing these neurons (Figure 3E; saline group, 46.8% ± 5.1%, n = 6; CNO group, 20.9% ± 5.4%, n = 7; p < 0.01). These results further support the hypothesis that CREB levels bias which neurons store memory for CTA in the insular cortex.

The results presented here demonstrate that specific mechanisms regulate memory allocation outside of the amygdala. They showed that CREB plays a critical role in the allocation of CTA memory in the insular cortex. Because CREB plays a role in memory in two very different structures in the mouse brain, it is reasonable to propose that CREB may have a general role in memory allocation in the mammalian brain. By implication, these results also suggest that memory allocation is a general, highly regulated process in the mammalian brain.

We used two independent strategies to determine whether CREB regulates memory allocation in the insular cortex: with the DREADD neuronal-inactivation system, we showed that CTA memory retrieval was impaired by selectively silencing vCREB-positive neurons. Additionally, we used FISH to analyze the expression of the immediate early gene *arc* and showed that insular cortical neurons with virally transduced CREB are preferentially activated after memory retrieval. *Arc* is required for memory formation, and *arc* transcription is turned on in neurons involved in learning and memory [26–28]. All together, these findings indicate that, just as in the amygdala [3–6], taste memory is preferentially allocated into insular cortex neurons with higher levels of CREB. Therefore, these results suggest that CREB is a key component of a general mechanism of memory allocation across brain regions.

Our findings suggest that both the insular cortex and the amygdala use common mechanisms for memory allocation. In the amygdala, the CS and US are thought to converge on the same neurons, where associative memory is stored [29]. The insular cortex receives both taste and visceral information from thalamic areas responding to these different sensory stimuli [30, 31]. However, it is unclear whether this information converges on the same neurons in the insular cortex [29]. Additionally, some studies have shown that the insular cortex is specifically activated by a CS (a novel taste) presentation in the absence of a US (e.g., visceral malaise) [13–15, 32]. These findings suggest that the insular cortex may be more tuned to process CS information. Thus, our results suggest that CREB-dependent mechanisms of memory allocation are not only critical for neurons that encode CS-US associations, they are also critical for memory allocation in circuits that process primarily CS information.

Perhaps CREB-dependent memory allocation links neuronal representations across brain regions; because CREB modulates memory allocation for CTA in the amygdala [6] and insular cortex, it is possible that CREB also coordinates memory allocation between these two regions, presumably strengthening the synaptic connection between these subassemblies. It is possible that coordination of memory allocation across brain regions may facilitate memory retrieval.

In summary, the findings presented here demonstrate that specific mechanisms regulate memory allocation outside of the amygdala and that CREB in the insular cortex plays a critical role in the allocation of CTA memory.

## Experimental Procedures

### Mice

Adult F1 hybrid (C57Bl/6NTac × 129S6/SvEvTac) mice used in behavioral analysis were group housed (two per cage) on a 12 hr light/dark cycle. Four- to five-month-old mice were used for behavioral analyses. All experimental protocols were approved by the Chancellor's Animal Research Committee of the University of California, Los Angeles, in accordance with NIH guidelines.

### Lentivirus Production

Vesicular-stomatitis-virus-G-protein-pseudotyped lentiviral vectors were produced by calcium-phosphate-mediated transient transfection of human embryonic kidney 293 T (HEK293T) cells, as previously described [18]. Lentivirus vectors were titered on HEK293T cells based on EGFP expression. We used about 5 × 10<sup>5</sup> cells/ml titer of CREB and control viruses in this study.

### Surgery

Mice were mounted in a stereotaxic apparatus and anesthetized with isoflurane (1% to 2%). A 2 mm diameter craniotomy was bilaterally performed above the insular cortex. A 1 μl virus solution was bilaterally infused using a Hamilton syringe through a glass micropipette at the following coordinates: relative to bregma (mm): anteroposterior axis (AP): +1.3, mediolateral axis (ML): ±3.7, and dorsoventral axis (DV): –2.5 from dura mater and AP: +0.3, ML: ±3.9, and DV: –2.5 from dura mater, taken from the mouse brain atlas [33] at a rate of 0.1 μl/min. A glass capillary was left in place for an additional 5 min. Behavioral tests were implemented 3 weeks after surgery to allow for recovery and sufficient expression of genes.

### CTA Test

Mice were dehydrated by removal of a water bottle for 24 hr before each test. Pretest: the thirsty mice were presented with two choices, water and 50 mM NaCl, for 3 days (for 12 min at day 1 and 2 and 5 min at day 3). On day 1 and 2, mice were supplied water in their home cage for 48 min after the pretest. Baseline preference to NaCl (NaCl consumed [ml]/total amount of drinking [ml] × 100) in individual mouse was calculated from pretests. Training: on day 3, 150 mM LiCl (presenting US and CS) was presented to a mouse for 10 min after pretest. A water bottle was returned to their home cage 1 hr following training. Test: 3 days after training, a two-choice test was implemented to determine the acquired aversion to a salty taste. The mice were presented with two choices, water and 150 mM NaCl, for 12 min. Saline or 1 mg/kg of CNO (Enzo Life Sciences) was intraperitoneally injected to mice 45 min before this test. Neuronal inactivation by the hM4Di receptor peaks at around 45–60 min after systemic injection of CNO [34]. We used a LI to assess CTA memory. This index normalizes the individual differences for salty taste preference across mice in this task. LI was defined as (1 – [preference to NaCl in test]/[baseline preference to NaCl]) × 100 and then was normalized to 100 for the mice in the saline group in Figure 2 and for control virus mice in Figure S2C. Aversion index was defined as ([water consumed in test]/[water + NaCl consumed in test] × 100) [7]. For the FISH experiment, a separate set of mice was used, and 150 mM NaCl was presented to mice for 5 min in a test designed to trigger the retrieval of the CTA memory. Home-cage mice were taken directly from their home cages.

### Statistical Analyses

For comparisons of two groups, we used unpaired two-tailed t tests. Differences were considered to be statistically significant when the probability value was <0.05.

### Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and two figures and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2014.10.018>.

### Acknowledgments

We would like to thank Sotiris C. Masmanidis, Yong-Seok Lee, and Katie Cai for their assistance. The plasmid containing hM4Di was kindly provided by Dr. Bryan L. Roth (University of North Carolina Chapel Hill Medical School). This research was supported by grant R37 AG013622 and the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation to A.J.S. and the UEHARA Memorial Foundation of Japan to Y.S.

Received: March 23, 2014  
Revised: September 4, 2014  
Accepted: October 8, 2014  
Published: November 13, 2014

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