Fig. S1

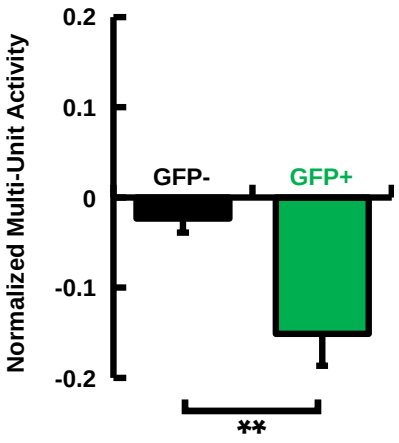
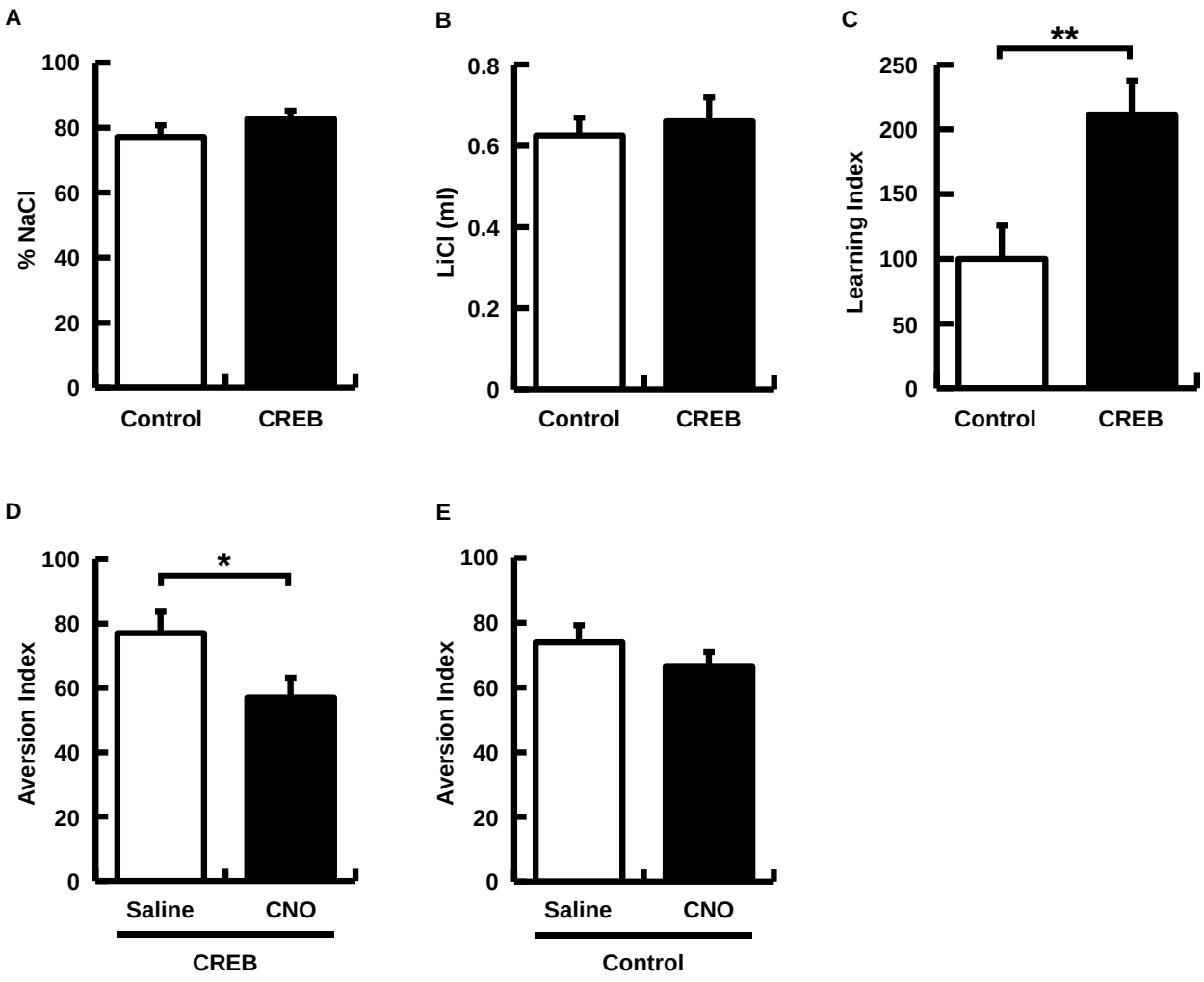


Fig. S2


A
100
80
60
40
20
0
% NaCl
Control
CREB
B
0.8
0.6
0.4
0.2
0
LiCl (ml)
Control
CREB
C
**
250
200
150
100
50
0
Learning Index
Control
CREB
D
*
100
80
60
40
20
0
Aversion Index
Saline
CNO
CREB
E
100
80
60
40
20
0
Aversion Index
Saline
CNO
Control

Supplemental legends

Fig. S1 In vivo activation of hM4Di reduces multi-unit activity in the insular cortex (Related to Fig. 1). Extracellular recordings using silicon probes with 128 electrodes were performed in the insular cortex of two awake, head-fixed mice infected with the CREB virus. In the GFP- mouse (n = 107 channels), the recording site did not overlap with the infected area, while the GFP+ mouse (n = 118 channels) had substantial overlap. Multi-unit activity across each channel was assessed during a 30 minute baseline period as well as 40-70 minutes after CNO injection. Post-injection multi-unit firing rates were normalized by subtracting the baseline and dividing by the sum of the baseline and post-injection period. The GFP+ mouse had significantly lower normalized multi-unit activity than the GFP- mouse. ** $p < 0.01$. Data represent mean ± s.e.m. (GFP- mouse, black columns; GFP+ mouse, green columns).

Fig. S2 Overexpression of CREB in the insular cortex enhances CTA memory formation and silencing of CREB+ neurons in the insular cortex impairs CTA memory retrieval (Related to Fig. 2). CREB or control lentiviruses were infused into the insular cortex 3 weeks before CTA training. (A)

Preference to NaCl in the pretest and (B) drinking amounts of LiCl during training were not different between the CREB and control virus mice (n = 15 per group). (C) Learning index (LI) in CREB mice was significantly higher than that in control mice (n = 15 per group, control mice, 100.0 ± 25.7; CREB mice, 211.5 ± 26.2, $p < 0.01$). LI was normalized to 100 for a saline group in each experiment. (D and E) Saline or CNO was systemically injected 45 min before CTA memory retrieval. (D) CTA memory was significantly impaired by selective silencing of CREB positive neurons by CNO (Saline group, n = 8; CNO group, n = 9). (E) CNO injection did not impair CTA memory retrieval in control mice (Saline group, n = 9; CNO group, n = 9). Data represent mean ± s.e.m. *$p < 0.05$ and **$p < 0.01$. (Control virus mice, white columns (A, B and C); CREB virus mice, black columns in (A, B and C); Saline-injected mice, white columns (D and E); CNO-injected mice, black columns (D and E)).

Supplemental experimental procedures

Immunohistochemistry

Mice were deeply anesthetized with tribromoethanol (avertin) and transcardially perfused with 4 % (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 (PFA). The brains were excised, postfixed with the same fixative at 4 °C overnight, and equilibrated in 30 % (w/v) sucrose in PBS as a cryoprotectant. The brains were embedded in OCT compound (Sakura Finetech), and frozen coronal sections (40 μm) were prepared. Free-floating sections were incubated with 0.4 % (v/v) Triton X-100 in PBS and then blocked with 5 % (v/v) goat serum, 1 % (w/v) BSA and 0.4 % (v/v) Triton X-100 in PBS. Sections were incubated with primary antibodies against to HA-tag (1:500, Cell Signaling technology) and GFP (1:500, aves labs inc.) at 4 °C for overnight. And then, they were incubated with Alexa568-conjugated anti-rabbit IgG and Alexa488-conjugated anti-chicken IgG (life technologies). Nuclei were stained using DAPI (life technologies).

Fluorescent *in situ* hybridization

Brains were quickly perfused with 4 % PFA, 5 min following the CTA memory retrieval test, harvested, post-fixed with 4 % PFA at 4 °C for 3 days, and then

equilibrated in 30 % (w/v) sucrose in PBS. Coronal sections (80 µm) were prepared using a cryostat. All steps were performed at room temperature unless indicated otherwise. Sections were incubated with methanol (MeOH) for 2 h, then washed 3 times for 20 min in PBS containing 0.1 % Tween-20 (PBST), incubated with 10 µg/ml proteinase K (Sigma) in PBST for 10 min, rinsed in PBST, post-fixed in 4 % PFA in 0.1 M PB for 20 min and, finally, washed 3 times for 20 min in PBST. Prior to hybridization, digoxigenin (DIG)- and fluorescein isothiocyanate (FITC)- labeled cRNA probes in hybridization buffer (5 × SSC, 50 % formamide, 0.1 % Tween-20) were denatured at 95 °C for 10 min and then quickly cooled on ice for 10 min. cRNA probes for *arc* and *gfp* were generated using a FITC and DIG RNA labeling kit (Roche). The Arc probe sequence spanned nucleotides 181–2970 of the GenBank sequence, accession no. NM001276684. Hybridization was performed at 58 °C overnight. Sections were washed in 2 × SSC containing 50 % formamide and 0.1 % Tween-20 (SSCT) for 60 min, 2 × SSCT for 15 min, 0.2 × SSCT at 58°C for 60 min, and washed three times in 0.1 M Tris-HCl, pH 7.4 containing 0.15 M NaCl and 0.05 % Tween-20 (TNT) for 20 min. Sections were incubated overnight with peroxidase-conjugated anti-FITC antibody (1:500, Perkin Elmer) in blocking reagent [0.5% blocking

reagent (Perkin Elmer), 0.1 M Tris-HCl, pH 7.4 and 0.15 M NaCl] at 4 °C. The sections were washed three times in TNT for 20 min and then incubated with DNP Amplification reagent (1:100, Perkin Elmer) for 30 min. After three-times wash with TNT, sections were incubated with Alexa488-conjugated anti-DNP antibody (1: 200, life technologies) in blocking reagent [0.5% blocking reagent (Perkin Elmer), 0.1 M Tris-HCl, pH 7.4 and 0.15 M NaCl] for 2 hours. The sections were washed three times in PBST for 20 min and incubated with alkaline phosphatase-conjugated anti-DIG antibody (1:2000, Roche) in blocking reagent [1% blocking reagent (Roche), 10 mM maleic acid, 15 mM NaCl, pH 7.5] at 4°C for overnight. The sections were washed three times in PBST for 20 min. For Fast Red staining, the signal was developed in 1 × Fast Red (Roche) diluted in staining solution (0.1 % Tween-20 and 0.1 M Tris-HCl, pH 8.2). Nucleus was stained using DAPI (life technologies). Sections were imaged by a confocal microscope (Nikon, C2). Confocal images over the insular cortex were acquired using a 60x objective lens and stitched.

Manual cell counts were performed by an experimenter blinded to treatment. Small, bright uniformly DAPI stained nuclei from putative glial cells were not counted. The area of the insular cortex was identified based on a

mouse brain atlas (Paxinos G. and Franklin KB., 2001). Cells were assigned to one of the following 3 categories; 1) GFP+ (virus positive neurons) and Arc+ (activated neurons), 2) GFP+ and Arc- and 3) GFP- and Arc+. Two to six slices were analyzed for each mouse and each treatment group contained 4 mice. The probability of Arc+ on GFP+ neurons was calculated for each mouse studied. The mean number of putative insular cortical neurons counted per mouse in each group was 508 cells in control and 558 cells in CREB virus group. Total number of arc+ cells was normalized by the area (per mm^2) of analyzed field based on the DAPI signal.

***In vitro* electrophysiology**

Brains were rapidly removed from mice 2-3 weeks after virus infusion and placed in an ice-cold cutting buffer containing the following (in mM): 119 Choline chloride, 2.5 KCl, 1 NaH2PO4, 26 NaHCO3, 1 CaCl2, 7 MgSO4, 30 D-glucose, 3 Sodium pyruvate, 1.3 Sodium ascorbate and 1 kynurenic acid. Coronal slices (350 µm thick) containing the insular cortex were prepared with a vibratome (VT1000S, Leica) in an ice-cold cutting buffer and allowed to recover in oxygenated (95% O2/5% CO2) ACSF ([in mM] 120 NaCl, 20 NaHCO3, 3.5 KCl,

1.25 NaH_2PO_4, 2.5 $CaCl_2$, 1.3 $MgSO_4$ and 10 D-glucose [in mM]) supplemented with 1 mM kynurenic acid at 32 °C for 40 min, and then kept in ACSF at room temperature until recording. All solutions were bubbled with 95% O_2/5% CO_2 and perfused over the slice at a rate of ~2 ml/min at 23 – 25 °C. Cells were visualized with an upright microscope using infrared or epifluorescent illumination, and whole-cell current-clamp recordings were done with a Multiclamp 700B amplifier (Axon Instrument). Patch electrodes (3–6 MΩ when filled) were filled with a solution containing the following (in mM): 130 K-methylsulphate, 8 NaCl, 10 HEPES, 2 KH_2PO_4, 2 D-glucose, 4 Mg-ATP, 7 Na-Phosphocreatine, 0.3 Na-GTP and 0.5 Na-ADP (pH 7.4, 285-290 mOsm). Responses were filtered at 2 kHz and digitized at 10 kHz. All data were acquired, stored and analyzed using pClamp 10.0 (Axon Instruments). Access resistance was monitored throughout the experiment. Only cells that maintained the minimal criteria for health and stability (resting membrane potential more negative than −60 mV and with access resistances that changed less than 20 % throughout the experiment) were included in the analyses of this study. Pyramidal neurons in the insular cortex were identified on the basis of their spike frequency adaptation in response to a long depolarizing current injection. Input

resistance was calculated from the I-V curve in a linear range. To investigate the firing properties of neurons, we delivered 20 current injection steps (600-ms duration) from −200–275 pA in 25-pA increments. Whole cell recordings were carried out before and after adding CNO (10 μM) to the perfusing solution.

In vivo electrophysiology

Silicon probe recordings were performed in awake, head-fixed mice. Mice were anesthetized and implanted with an omega-shaped head plate attached to the skull with cyanoacrylate glue and dental cement. A silver ground wire (0.375 mm diameter) was then placed between the dura and bone above the cerebellum and covered with dental cement. Finally, a rectangular craniotomy was performed over the viral injection sites with a length of approximately 1.5mm and a width of approximately 1mm. Mice were placed on the spherical treadmill and the craniotomy was covered with aCSF. Silicon probes were as previously described [S1] and contained 128 channels over 4 shanks 330um apart, with 32 electrodes per shank arranged in a zig zag pattern (25um vertical spacing and 6-10um horizontal spacing between each electrode). Probes were coated with Vybrant® DiI Cell-Labeling Solution (Life Technologies)

and lowered into the insular cortex sagittally with the anterior tip relative to bregma at: AP: + 1.3, ML: ± 3.7, DV: - 2.5. After at least one hour recordings began and data was mutiplexed and amplified by a custom application-specific integrated circuit (ASIC) and digitized at 25kHz per channel using a National Instruments data acquisition board. Following at 30 min baseline recording, CNO was administered (1 mg/kg, i.p.). Approximately 60 minutes after CNO injections, the electrodes were removed. The brains were harvested, post-fixed in paraformaldehyde, sliced (100um), mounted with DAPI, and imaged for probe verification with a confocal microscope.

Multi-unit activity was analyzed by filtering each channel (600-6000hz) and setting a threshold five standard deviations from the mean and counting the number of events above the threshold. Post-injection firing rates were positively skewed and thus were normalized by subtracting the baseline firing rate (bFR) from the post-injection firing rate (pFR) and dividing by the sum of the baseline and post-injection firing rates: Normalized FR = (pFR – bFR) / (pFR + bFR).

Supplemental reference

S1. Du, J., Blanche, T.J., Harrison, R.R., Lester, H.A., and Masmanidis, S.C. (2011). Multiplexed, high density electrophysiology with nanofabricated neural probes. PloS one 6, e26204.