

Supplementary Material

ICER-Controlled Inhibitory Plasticity of Amygdala Engram Neurons Regulates Fear Memory Precision

Hyunyong Lee & Seungho Lee, et al

Corresponding author: Joung-Hun Kim, joungkim@postech.ac.kr

Supplemental contents:

Methods and Materials
Supplementary Figures S1–S17
Supplementary References

Methods and Materials

Animals

Adult mice (3 – 5 months old) were used for all the analyses, while virus injections were made to young adult mice (> 10 weeks old). Mice were group-housed under a 12-hour light/dark cycle with *ad libitum* access to food and water. The following mouse lines were used: C57BL/6J (JAX:000664 from Jackson Laboratory, ME), cFos-tTA (JAX:018306), PV-Cre (JAX:008069), SST-Cre (JAX:013044), VIP-Cre (JAX:010908), CCK-Cre (JAX:012706), VGAT-Cre (JAX:016962), Fos^{CreER} (TRAP1, JAX:021882), Fos^{2A-iCreER} (TRAP2, JAX:030323), Ai14 (JAX:007914), and Grp-Cre (RBRC10694 from RIKEN, Japan). Both sexes were included unless otherwise stated. All procedures were approved by the POSTECH Institutional Animal Care and Use Committee (POSTECH-2025-0105) and conducted in accordance with institutional and national ethical guidelines.

Auditory fear conditioning (AFC) and discrimination testing

Mice underwent AFC in a conditioning chamber (17.75 × 17.75 × 30.5 cm, Coulbourn, MA) with an electrified grid floor connected to a shock generator (Model H13–15, Coulbourn) under infrared illumination. After 2 minutes of habituation, mice received four presentations of an auditory CS (20 kHz tone, 80 dB, 30 s), each co-terminating with an electric foot shock (unconditioned stimulus, US: 0.5 mA, 0.5 s). The CS-US pairings were delivered at pseudorandom intertrial intervals ranging from 60 to 120 s.

Fear recall and discrimination tests were performed in a modified context. A white acrylic chamber was inserted into the original conditioning chamber, supplemented with aspen bedding, peppermint scent, and white overhead lighting to provide contextual distinction from the training environment. After 2 minutes of habituation, mice were presented with a set of auditory tones, including novel stimuli (2, 3, 8, or 14 kHz tones, 80 dB, 30 s) and the original CS (20 kHz). Freezing was quantified using FreezeFrame software (Coulbourn) as the percentage of time immobile during tone presentations, with immobility defined as ≥ 0.25 s.

A discrimination index (DI) was calculated as follows: $DI = (\text{Freezing}^{\text{CS}} - \text{Freezing}^{\text{novel}}) / (\text{Freezing}^{\text{CS}} + \text{Freezing}^{\text{novel}})$, and the resulting values were converted to absolute values. Higher DI values indicate greater discrimination. For the 8 kHz versus 20 kHz comparison, DI values were retained as signed values to preserve directionality.

Adeno-associated viruses (AAVs)

The following commercially available AAVs were used: AAV9-TRE3G-mCherry, AAV9-TRE3G-EYFP, AAV9-TRE3G-DIO-mCherry, AAV9-TRE3G-DIO-ChR2-EYFP, and AAV9-hSyn-DIO-hM4Di-mCherry were purchased from BrainVTA (China); AAV9-Ef1a-DIO-hChR2(H134R)-EYFP, AAV9-Ef1a-DIO-eNpHR3.0-EYFP, AAV9-Ef1a-DIO-EYFP, AAV9-CAG-DIO-Arch-EGFP, AAV5-hSyn-DIO-EGFP, and AAV8-nEF-Cre^{OFF}/Flp^{ON}-eNpHR3.3-EYFP from Addgene (MA). A custom pAAV-TRE3G-DIO-ICER-mCherry construct was packaged by BrainVTA.

For in-house virus production, pAAV-Dlx-DIO-hM4Di-mCherry, pAAV-Dlx-DIO-EYFP, pAAV-hSyn-DIO-ICER-EGFP, pAAV-CMV-ER^{T2}-Cre-ER^{T2}, pAAV-TRE3G-DIO-Gabra1-GFP, pAAV-TRE3G-DIO-EGFP, pAAV-U6-ICER shRNA-Ef1a-EGFP, and pAAV-U6-scramble shRNA-Ef1a-EGFP were packaged as AAV9 using an established protocol [1]. Briefly, AAV plasmids were co-transfected with AAV helper plasmids into HEK293T cells using Lipofector Q (AptaBio, Korea). Viral particles were harvested and purified using iodixanol-gradient ultracentrifugation, followed by concentration and buffer exchange with an Amicon Ultra-15 centrifugal filter unit (Millipore). Final viral titers were $\geq 2.5 \times 10^{12}$ genome copies/mL.

Plasmid construction

For ICER overexpression, mouse CREM cDNA (ORF clone MG53875-CM) was obtained from Sino Biological (China). The coding sequence for *ICER* was subcloned into pAAV-TRE3G-DIO-mCherry and pAAV-hSyn-DIO-EGFP backbones to generate pAAV-TRE3G-DIO-ICER-mCherry and pAAV-hSyn-DIO-ICER-EGFP, respectively. For Gabra1 overexpression, a plasmid encoding the mouse GABA_A receptor $\alpha 1$ subunit tagged with pHluorin (#49168) was obtained from Addgene. The Gabra1-pHluorin coding sequence was subcloned into the pAAV-TRE3G-DIO-EGFP backbone, generating pAAV-TRE3G-DIO-Gabra1-GFP.

For ICER knockdown, a previously validated siRNA target sequence against *ICER* (5'-CAACAUGGCUGUAACUGGGA-3') [2] was used to generate an shRNA cassette, which was cloned into the pAAV-U6-Ef1a-EGFP backbone, generating pAAV-U6-ICER shRNA-Ef1a-EGFP. A scramble shRNA control (SHC016; Sigma-Aldrich, MD) was cloned into the same backbone.

87 *Stereotaxic surgery*

88 Mice were anesthetized with ketamine (100 mg/kg) and xylazine (14 mg/kg) and placed in a
89 stereotaxic frame (Kopf Instruments, CA) with ear bar support. AAVs were bilaterally infused
90 into the LA using the following stereotaxic coordinates relative to bregma: anteroposterior (AP)
91 -1.8 mm, mediolateral (ML) ± 3.3 mm, and dorsoventral (DV) -4.4 mm. A total of 200 nL per
92 hemisphere was delivered at a rate of 1 nL/s using a Nanoject III injector (Drummond Scientific,
93 PA). For optogenetic experiments, bilateral fiber-optic cannulas (200- μ m core diameter, 0.22
94 NA, Newdoon Technologies, China) were implanted just above the injection site.

95 *Labeling AFC-activated ENs*

96 Fear ENs activated during AFC were labeled using the cFos-tTA system with doxycycline
97 (DOX)-regulated, TRE-driven viral expression. cFos-tTA mice were maintained on a DOX-
98 containing diet (60 mg/kg in food pellets) for ≥ 1 week before viral injection. 3 days before
99 AFC, DOX was withdrawn (“Dox-OFF” period), and mice were single-housed to permit
100 activity-dependent labeling during training. Immediately after completion of AFC, mice were
101 returned to a high-dose DOX diet (1 g/kg) to terminate labeling and prevent tagging of neurons
102 activated by unrelated stimuli.

103 To assess whether AFC-labeled neurons were reactivated during fear-memory retrieval,
104 a retrieval-reactivation experiment was performed 1 day after AFC. cFos-tTA mice received
105 intra-LA injections of AAV-TRE-mCherry and underwent AFC with a 20-kHz tone during the
106 Dox-OFF period, after which the DOX-containing diet was restored. 1 day later, mice were
107 exposed to four 30-s presentations of either the conditioned 20-kHz tone or a novel 2-kHz tone.
108 Mice were sacrificed 90 minutes after tone exposure, and brain sections were processed for c-
109 Fos immunohistochemistry.

110 *Stimulation of AFC-tagged Grp⁺ LA neurons*

111 Grp-Cre;cFos-tTA mice injected with AAV-TRE-DIO-ChR2-EYFP into the LA were used to
112 evaluate whether artificial reactivation of AFC-tagged Grp⁺ neurons was sufficient to induce
113 freezing behavior. AFC-activated Grp⁺ LA neurons were labeled as described above.
114 Optogenetic recall (opto-recall) was performed twice per animal: a pre-AFC session 3 days
115 before AFC, while mice were maintained on DOX, to establish baseline responses to optical
116 stimulation, and a post-AFC session 1 day after AFC to test whether optogenetic activation of
117 AFC-tagged Grp⁺ neurons could evoke a conditioned freezing response (**Fig. S3B, C**). Each

opto-recall session consisted of three consecutive phases: ① OFF (baseline), a 2-min period without optical stimulation, ② ON (stimulation), a 2-min period of 473-nm laser stimulation at 30 Hz with a 15% duty cycle, and ③ OFF (post-stimulation), a 2-min period without stimulation to assess residual freezing responses.

Ex vivo electrophysiology

Acute coronal brain slices (300 μ m) containing the amygdala were prepared using a vibratome (VT1000S; Leica) in ice-cold sucrose cutting solution (in mM: 185 sucrose, 2.5 KCl, 1.2 NaH₂PO₄, 10 MgSO₄, 26 NaHCO₃, 25 glucose, 1 CaCl₂, 1 sodium L-ascorbate), continuously bubbled with 95% O₂/5% CO₂. Slices recovered for 30 minutes in artificial cerebrospinal fluid (aCSF; in mM: 124 NaCl, 2.5 KCl, 2 CaCl₂, 2 MgSO₄, 1.2 NaH₂PO₄, 26 NaHCO₃, 12.5 glucose), followed by recording at room temperature under continuous perfusion.

Whole-cell recordings were performed using a MultiClamp 700B amplifier and Digidata 1550B (Molecular Devices, CA). Miniature EPSCs were recorded at -70 mV with a CsMeSO₃-based internal solution (in mM: 135 CsMeSO₃, 8 NaCl, 0.5 EGTA, 10 HEPES, 2 MgATP, 0.1 NaGTP; pH 7.2) in the presence of picrotoxin (100 μ M) and TTX (1 μ M). Miniature IPSCs were recorded at -70 mV using a CsCl-based internal solution (in mM: 145 CsCl, 1 EGTA, 10 HEPES, 2 MgATP, 0.1 NaGTP; pH 7.2) in the presence of DNQX (20 μ M) and TTX (1 μ M). Events were detected with Mini Analysis (Synaptosoft, NJ) and visually inspected. Mean event amplitude and event frequency were calculated per cell over a 15-minute recording period.

Evoked synaptic responses were obtained by stimulating thalamic afferents (internal capsule) with a bipolar electrode (0.5-ms pulses at 0.05 Hz; 25–100 μ A). EPSCs were recorded at -70 mV and IPSCs at $+10$ mV. Pharmacological validation of disynaptic IPSCs was performed using DNQX (20 μ M) and APV (50 μ M). For interneuron subtype contributions to FFI, halorhodopsin-expressing PV-, SST-, VIP-, or VGAT-INs were inhibited using 530-nm illumination (40 ms, starting 5 ms before stimulation), and the change in IPSC amplitude was used to quantify subtype-specific contributions. In subsequent experiments measuring PV-FFI, halorhodopsin-expressing PV-INs were inhibited using 595-nm illumination with the same timing relative to afferent stimulation.

Optogenetically evoked PV-IN-mediated IPSCs (PV-IPSCs) were elicited using 470-nm LED pulses (0.5 ms at 0.05 Hz) and recorded at -70 mV with CsCl-based internal solution.

For quantal PV-IPSCs (qIPSCs), CaCl₂ was replaced with SrCl₂ (4 mM), and events were analyzed in a 450-ms window beginning 50 ms after light onset.

For pathway-specific input analysis, ChR2-expressing medial geniculate nucleus or auditory cortex axons were stimulated with 470-nm pulses (0.5 ms, 0.05 Hz) while IPSCs were recorded at +10 mV. Interneuron subtype contributions were measured by inhibiting halorhodopsin-expressing interneurons using 617-nm illumination (40 ms, starting 5 ms before stimulation) and quantifying percent reductions in IPSC amplitude.

Single-cell RNA sequencing analysis

Single-cell RNA-seq data were obtained from the publicly available dataset of Hochgerner et al. (2023) [3]. Analyses were performed in R using Seurat (v4). Data were normalized and variance-stabilized using SCTransform, followed by PCA, clustering, and uniform manifold approximation and projection (UMAP) visualization. LA pyramidal neurons were identified as Grp/Cpne8 co-expressing cells. Module scores for immediate early gene (IEG) expression (*Arc*, *Bdnf*, *Btg2*, *Fos*, *Fosl2*, *Homer1*, *Npas4*, *Nr4a1*) were calculated, and 2-hour (h) post-AFC cells were stratified into high- versus low-IEG populations based on module score thresholds (≥ 0.5).

Immunohistochemistry (IHC) and imaging

Mice were transcardially perfused with phosphate-buffered saline followed by 4% paraformaldehyde. Brains were post-fixed overnight at 4°C, cryoprotected in 30% sucrose, embedded in OCT, and sectioned coronally (20 μ m). Sections underwent antigen retrieval (citrate buffer, 95–100°C, 13 minutes), permeabilization (0.3% Triton X-100), and blocking (CAS Block; 008120, Invitrogen, MA). Primary antibodies were incubated overnight at 4°C and detected with fluorophore-conjugated secondary antibodies. Sections were coverslipped in mounting medium with or without DAPI.

Primary antibodies included (1:500 unless otherwise noted): rabbit anti-ICER (ab230543, Abcam, UK); guinea pig anti-NeuN (ABN90P, Sigma-Aldrich); rabbit anti-GABA(A) α 1 Receptor (AGA-001, Alomone lab, Israel); rabbit anti-c-Fos (2250S, Cell signaling, MA); rabbit anti-SYT2 (ab113545, Abcam); mouse anti-VGAT (131 011, Synaptic Systems, Germany); guinea pig anti-PV (195 004, Synaptic Systems); chicken anti-c-Fos (226 009, Synaptic Systems); guinea pig anti-SST (366 004, Synaptic Systems); mouse anti-NeuN (MAB377, Sigma-Aldrich); mouse anti-GAD67 (MAB5406, Sigma-Aldrich); mouse anti-PV (MAB1572, Sigma-Aldrich); rabbit anti-VIP (20077, Immunostar, WI).

Confocal imaging was performed using an FV-3000 microscope (Olympus, Japan). Quantification of puncta (e.g., GABA_AR α 1, VGAT, Syt2) was performed using MetaMorph 7.7 (Molecular Devices) with threshold-based segmentation, and the analyses were restricted to perisomatic regions. 3D reconstructions were generated using IMARIS (Bitplane, United Kingdom). ICER fluorescence intensity was quantified in ImageJ (NIH, MD) within NeuN⁺ somata.

Immunogold electron microscopy (EM)

Post-embedding immunogold EM was performed using established protocols [1]. cFos-tTA mice injected with AAV-TRE3G-mCherry were perfused 3 days after AFC with 4% PFA/0.5% glutaraldehyde. LA tissue was resin-embedded and sectioned into 100-nm ultrathin slices, which were mounted on 200-mesh copper grids (EMS200-Cu, Electron Microscopy Sciences, PA). Sections were incubated with antibodies against mCherry (1:50, NBP1-96752, Novus biologicals, CO) and GABA_AR α 1 (1:50, AGA-001, Alomone lab) followed by gold-conjugated secondary antibodies (Jackson ImmunoResearch, PA). Sections were counterstained with uranyl acetate and Reynolds lead citrate and imaged using a JEM-1011 transmission electron microscope (JEOL, Japan). Fear ENs were identified by mCherry labeling (18-nm gold particles), and GABA_AR α 1 immunogold particles (12 nm) were quantified when located within 30 nm of the perisomatic plasma membrane.

Interneuron manipulation, PV-iLTD induction, and behavioral assays

For chemogenetic experiments, clozapine-N-oxide [CNO; 5 mg/kg, intraperitoneally (i.p.); Tocris, MN] or JHU37160 (J60; 0.5 mg/kg, i.p.; Hello Bio, UK) was administered 30 minutes before behavioral testing. CNO was used for the initial chemogenetic screening of PV-, CCK-, and SST-INs in loss-of-function experiments during the fear discrimination test (**Fig. S6**). J60 was used for the PV-IN inhibition experiment coupled with EN reactivation analysis (**Fig. 4A–K**), because its high *in vivo* potency permitted hM4Di activation at a lower systemic dose, thereby reducing potential nonspecific ligand-related influences on neural activity [4,5]. Ligand-matched control virus-injected groups were included in each chemogenetic experiment.

For *in vivo* optogenetic inhibition of PV-INs, the inhibitory opsin Arch was activated using a 532 nm laser (GL532T3; Shanghai Laser & Optics Century, China) during tone presentations. PV-Cre mice injected with either AAV-DIO-Arch-EGFP or control AAV-DIO-EGFP were exposed to a serial sequence of 2, 8, 14, and 20 kHz tones (30 s each), with each

tone presented 4 times. For each tone frequency, laser illumination was delivered during either the first 2 or the last 2 tone presentations, such that laser-ON and laser-OFF trials were counterbalanced across animals. Laser light was delivered through implanted optic cannulas throughout the 30-s tone presentation to inhibit PV-IN activity.

To identify an effective protocol for inducing PV-IN inhibitory long-term depression (PV-iLTD) selectively onto fear ENs, optogenetic stimulation of PV-INs was first performed in acute brain slices. Stimulation consisted of 100 light pulses at 10 Hz, delivered either once or repeated two, three, or four times with 10-second intertrial intervals [6]. These protocols were tested in the absence of pharmacological antagonists (e.g., DNQX, APV, or picrotoxin) to facilitate subsequent *in vivo* translation. Among the tested conditions, four repetitions of 100-pulse stimulation at 10 Hz reliably induced significant depression of PV-IPSCs in fear ENs, but not in non-ENs, and this protocol was therefore applied *in vivo* (**Figs. S7 and S8**). One day after AFC, mice underwent a pre-iLTD recall test (exposure to 2 and 20 kHz tone). The following day, *in vivo* PV-iLTD was induced using 473nm laser stimulation: 100 pulses at 10 Hz, repeated four times (10 s intervals). A post-iLTD recall test was conducted 1 hour after iLTD induction. Freezing levels and DI between 2 and 20 kHz tones were compared before and after the induction of iLTD.

Anxiety-like behavior and locomotor activity were assessed using the elevated plus maze (EPM) and open field test (OFT), with automated video tracking using SMART v3.0 software (Panlab, Spain). The EPM consisted of four arms (50 × 10 cm), elevated 50 cm above the floor, with two open arms and two enclosed arms. Mice were placed in the central zone facing an open arm, allowed a 30-second acclimation period, and monitored for 10 minutes. The OFT was conducted in a white square arena (40 × 40 cm), in which mice were placed in the center, allowed 30 seconds for acclimation, and monitored for 10 minutes. The arena was virtually divided into a central zone (20 × 20 cm) and a peripheral zone.

Innate fear responses were assessed using predator odor exposure with 2,5-dihydro-2,4,5-trimethylthiazoline (TMT; 97%; BioSRQ, FL). TMT-spotted filter paper was placed in one corner of a white acrylic chamber supplemented with aspen bedding, peppermint scent, and overhead white lighting. Mice were placed in the chamber for 10 minutes, and freezing behavior was quantified using FreezeFrame software (Coulbourn).

Labeling of tone frequency-specific auditory inputs (Tone tagging)

The tone-tagging procedure used to label tone-specific auditory neurons in the MGN/ACx was performed as follows [7,8]. cFos-CreER^{T2} mice received tamoxifen (Sigma-Aldrich, 150 mg/kg, i.p., 20 mg/mL in corn oil) and were immediately placed in an isolated, quiet context shielded from external auditory and other sensory stimuli. This context consisted of a black acrylic chamber with aspen bedding, no odor cues, and alternating light/dark cycles. 24 hours after tamoxifen administration, mice were exposed to the designated auditory tone for tagging. Auditory tones were presented as 5-s tone pips at 0.05 Hz for 30 min, with the 30-min tone-exposure session repeated 4 times.

To validate the specificity of the tone-tagging system, both histological and functional analyses were performed in cFos-CreER^{T2}/Ai14 and cFos-CreER^{T2} mice. For histological validation, cFos-CreER^{T2}/Ai14 double-transgenic mice underwent either 2 kHz or 20 kHz tone tagging using the procedure described above. Tone exposure induced Cre-mediated tdTomato (tdT) expression in neurons activated by the designated tone. 2 to 3 weeks later, mice were exposed to the 20 kHz tone in a different novel chamber consisting of a white acrylic chamber with aspen bedding, a mild peppermint scent, and constant light. A 30-s 20 kHz tone was presented 4 times with 5-s intertrial intervals. Mice were sacrificed 90 min after tone exposure, and brains were collected and processed for c-Fos immunostaining. Co-localization of tdT⁺ and c-Fos⁺ neurons was quantified to assess reactivation of tone-tagged neurons upon exposure to either the same tone used for tagging or a distinct tone.

For functional validation, cFos-CreER^{T2} mice received MGN injections of AAV-DIO-hChR2-EYFP and optic-fiber implantation. After a 2-week recovery period, mice underwent either 2 kHz or 20 kHz tone tagging using the same procedure described above. 4–5 weeks later, both groups underwent AFC using the 20 kHz tone as the CS. Recall testing was conducted in a novel context on the following day. After a baseline period, the previously tagged MGN neurons were optically stimulated twice (Optical recall; 473 nm, 30 Hz, 15% duty cycle, 30 s per trial). Optical stimulation was delivered in the absence of tone presentation. These optical recall trials were subsequently followed by two 30-s presentations of the 20 kHz CS (CS recall). This design allowed comparison of conditioned freezing evoked by optogenetic activation of tone-tagged MGN neurons with that evoked by CS presentation.

Classification of subject animals based on behavioral indices

Fear discrimination tests were performed at 2, 6, 12, and 24 hours after AFC in a novel context

using the 20 kHz CS and a novel 3 kHz tone. Both male and female mice were included. Animals were classified as discriminators or generalizers using weighted K-means clustering based on 3 key behavioral parameters [9,10]: ① the freezing consistency ratio (FCR), defined as the ratio of freezing during the second novel-tone presentation to that during the first novel-tone presentation, ② freezing during the second novel-tone presentation, and ③ the DI between the novel tone and the CS, which reflects the degree of discrimination between the two tones (Fig. S13A, E, I, and M).

For each post-AFC timepoint, the three behavioral features were standardized across animals to z-scores before clustering. The standardized freezing value during the second novel-tone presentation was multiplied by 3, whereas the other two standardized features were left unchanged. This weighting was based on the biological rationale that freezing during the first novel-tone presentation may include an initial orienting or novelty response, whereas freezing during the second presentation provides a more direct measure of the animal's fear response to the novel tone [11]. Weighted k-means clustering was performed using squared Euclidean distance with 1,000 random centroid initializations.

The optimal number of clusters was evaluated by comparing the mean silhouette coefficient [12,13] across clustering solutions containing two to five clusters. At each post-AFC timepoint, the two-cluster solution yielded the highest mean silhouette coefficient and was therefore selected for subsequent analyses. The cluster with the higher mean DI was designated the discriminator group, whereas the other cluster was designated the generalizer group. Individual silhouette values were subsequently calculated for animals in the selected two-cluster solution. To assess whether classification was associated with sex, the proportions of male and female mice classified as discriminators or generalizers were compared using two-sided Fisher's exact tests.

The stability of individual cluster assignments was assessed using bootstrap resampling. For each post-AFC timepoint, 2,000 bootstrap datasets were generated by sampling animals with replacement, with each bootstrap sample containing the same number of observations as the original dataset. Within each bootstrap iteration, the three behavioral features were re-standardized using the mean and standard deviation of the bootstrap sample, after which the same predefined weighting scheme was applied. A two-cluster k-means model was then fitted using 100 random centroid initializations. The original animals were transformed using the scaling parameters derived from each bootstrap sample and assigned to

the nearest bootstrap-derived cluster centroid. Because cluster labels are arbitrary across k-means runs, bootstrap-derived clusters were matched to the original discriminator and generalizer clusters based on centroid correspondence before calculating classification stability. For each animal, bootstrap classification stability was defined as the proportion of bootstrap iterations in which its assigned cluster agreed with its classification in the primary clustering solution. Dashed reference lines at a bootstrap classification stability of 0.90 and an individual silhouette value of 0.25 were included in **Fig. S13B, F, J, and N** to facilitate visual assessment of classification robustness. These values were used as descriptive reference thresholds and were not treated as formal statistical cutoffs.

Cluster-level stability was additionally quantified using the mean Jaccard similarity [14] between each original cluster and its corresponding bootstrap-derived cluster across bootstrap iterations. The mean Jaccard indices for the discriminator and generalizer clusters were 0.987 and 0.968 at 2 h, 0.970 and 0.932 at 6 h, 0.992 and 0.978 at 12 h, and 0.964 and 0.923 at 24 h, respectively.

Classification decoder based on GABA_AR $\alpha 1$ puncta in ENs

To examine whether fear generalization would be accompanied by any changes of inhibitory postsynaptic structures, cFos-tTA mice injected with AAV-TRE3G-EYFP underwent AFC after a 3-day Dox-off period and were subjected to the discrimination task on the next day. Immediately after completion of the discrimination tests, brains were collected and processed to analyze perisomatic GABA_AR $\alpha 1$ puncta (size and density) in ENs between discriminators and generalizers through IHC. Mean punctum density and size were quantified for each animal and used as predictor variables in a random forest classifier [15] trained to distinguish discriminators from generalizers. Animals were randomly divided into training and test sets at an 80:20 ratio using stratified random sampling. Hyperparameters were optimized within the training set using randomized search with fivefold stratified cross-validation. The search evaluated the number of trees, maximum tree depth, minimum numbers of observations required to split a node or form a terminal leaf, and the number of predictor variables considered at each split. The optimized model was evaluated on the held-out test set using the area under the receiver-operating-characteristic (ROC) curve.

Quantification of Creb1 and ICER in the LA

C57BL/6J wild-type mice underwent auditory fear conditioning followed by fear

discrimination testing at 2, 6, 12, or 24 hours after conditioning. Based on behavioral performance, mice were classified as discriminators or generalizers using K-means clustering. Unconditioned, naïve mice (0 hours) served as controls.

Immediately after behavioral testing, mice were euthanized by isoflurane overdose followed by transcardial perfusion with phosphate-buffered saline. Brains were rapidly extracted and sectioned into 400- μ m-thick coronal slices using a vibratome (VT1000S; Leica) in ice-cold phosphate-buffered saline. The lateral amygdala was microdissected using a fine angled dissecting knife (10056-12, Fine Science Tools, CA). Total RNA was extracted using the miRNeasy Micro Kit (#217084, Qiagen, CA), and complementary DNA was synthesized using SuperScript IV Reverse Transcriptase and oligo(dT)₍₂₀₎ primers (Invitrogen).

Gene expression was quantified by reverse transcription quantitative polymerase chain reaction (RT-qPCR), and relative expression levels were calculated using the $\Delta\Delta$ Ct method with *GAPDH* as the internal reference. Primer sequences were as follows: *Creb1*, forward 5'-CACAGACCACTGATGGACAGCA-3' and reverse 5'-AGGACGCCATAACAACCTCCAGG-3' [16]; *ICER*, forward 5'-ATGGCTGTAAGTGGAGATGAACT-3' and reverse 5'-GTAGGAGCTCGGATCTGGTAAGT-3' [17]; *GAPDH*, forward 5'-CACTGAAGGGCATCTTGG-3' and reverse 5'-TTACTCCTTGGAGGCCATG-3' [18].

The temporal profile of ICER protein expression in the LA was examined using two technically distinct experimental settings. For analysis at 6, 12, and 24 hours after AFC, cFos-tTA mice injected with AAV-TRE-mCherry into the LA underwent AFC during the DOX-off period. Mice underwent fear discrimination testing at 6, 12, or 24 hours after AFC and were classified as discriminators or generalizers. Immediately after testing, mice were sacrificed, and brains were collected and processed for ICER IHC. ICER pixel intensity was quantified separately in mCherry⁺ ENs and neighboring mCherry⁻ non-ENs in the LA. Mean ICER fluorescence intensity was measured within individual NeuN⁺ somata and compared between discriminators and generalizers at each time point.

For analysis at 2 hours after AFC, C57BL/6J wild-type mice were used because cFos-tTA/TRE-driven mCherry expression was insufficient for reliable identification of ENs at this early time point [19,20]. Mice underwent AFC and fear discrimination testing and were classified as discriminators or generalizers. Brains were collected 2 hours after AFC and processed for combined c-Fos and ICER IHC. Unconditioned, naïve mice (0 hours) served as

controls. ICER pixel intensity was quantified separately in c-Fos⁺ and c-Fos⁻ LA neurons. Mean ICER fluorescence intensity was measured within individual NeuN⁺ somata and compared among the 0 h control, discriminator, and generalizer groups. The same ICER IHC, image-acquisition, and fluorescence-intensity quantification procedures were applied across both experimental settings. Data obtained using the cFos-tTA/TRE-mCherry approach at 6, 12, and 24 hours and those obtained using c-Fos IHC at 0 and 2 hours were analyzed and presented separately (**Fig. S15**).

ICER overexpression in the LA

For ICER overexpression in ENs, cFos-tTA/Grp-Cre double transgenic mice stereotactically received either AAV-TRE-DIO-ICER-mCherry or control AAV-TRE-DIO-mCherry into the LA and remained for 2 – 3 weeks. After a 3-day Dox-off period, mice underwent AFC, which allowed for transgene expression in ENs. Brains were collected one day after AFC. ENs overexpressing ICER (ICER-OE) and control mCherry (mCherry-OE) were compared for thalamic-evoked synaptic responses or GABA_AR $\alpha 1$ expression by IHC. To compare PV-FFI, cFos-tTA/Grp-Cre/PV-Flp triple transgenic mice were generated and were co-injected with either AAV-TRE-DIO-ICER-mCherry or AAV-TRE-DIO-mCherry, along with AAV-nEF-Cre_{OFF}/Flp_{ON}-eNpHR3.3-EYFP to confine halorhodopsin expression in PV-INs. AFC and Dox-off protocols were identical to those described above (**Fig. 5M–O**).

For concomitant overexpression of ICER and Gabra1 in ENs, Grp-Cre/cFos-tTA double transgenic mice received stereotaxic injections of AAV-TRE-DIO-ICER-mCherry together with AAV-TRE-DIO-Gabra1-GFP, or the corresponding control AAV pairs into the LA. Mice were allowed 2–3 weeks for viral expression. Following a 3-day Dox-off period, mice underwent AFC and were tested for fear discrimination one day later. Brains were collected one day after the discrimination test, and GABA_AR $\alpha 1$ expression in ENs was assessed by IHC.

To investigate the functional consequences of general overexpression of ICER in LA pyramidal neurons, wild-type mice were bilaterally co-injected with AAV-CMV-ER^{T2}-Cre-ER^{T2} and either AAV-hSyn-DIO-ICER-EGFP or AAV-hSyn-DIO-EGFP. After a 2-week recovery, mice underwent AFC with 20 kHz tone. Immediately after AFC, mice received a single i.p. injection of 4-hydroxytamoxifen (4-OHT, Sigma-Aldrich, 25 mg/kg) to activate Cre recombination and trigger random overexpression of ICER or EGFP in the LA. One day after

AFC, mice were subjected to fear discrimination tests (**Fig. S16C–H**).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 10 (GraphPad Software, MA). Parametric tests, including Student's *t* test and one-way or two-way analysis of variance (ANOVA) with appropriate *post hoc* comparisons, were used for normally distributed data. For non-normally distributed data, the Mann–Whitney *U* test was applied. Data are presented as mean ± SEM. Significance thresholds were defined as follows: ns, non-significant; **p* < 0.05; ***p* < 0.01; ****p* < 0.001. If no statistical significance is depicted in the figure or described in the figure legend, such results are considered statistically not significant. Exact sample sizes, statistical tests, and *post hoc* analyses are reported in each figure legend.

Supplementary Figures

Fig. S1

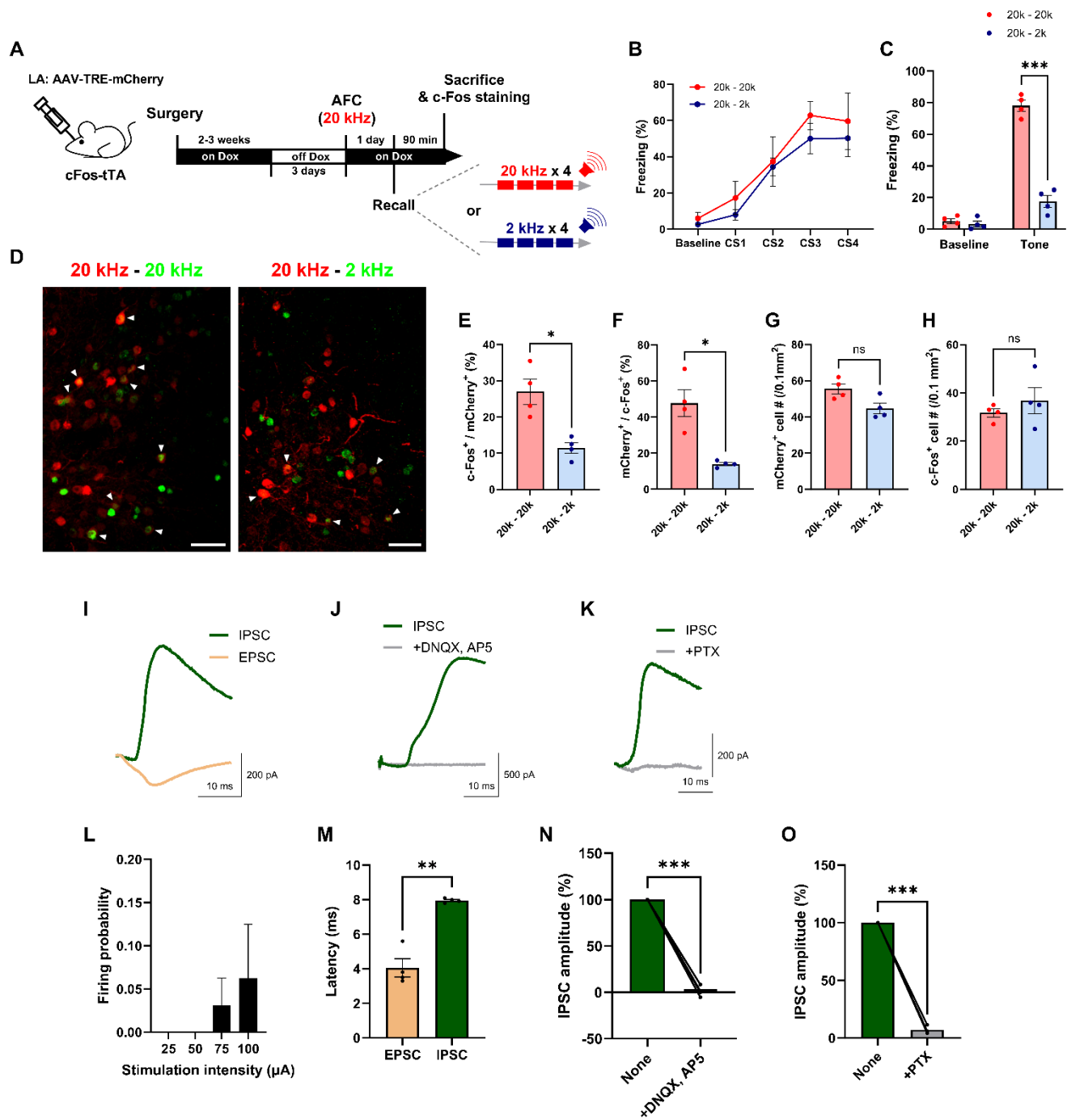


Fig. S1. CS-specific reactivation of AFC-labeled neurons and electrophysiological properties of EPSCs and IPSCs in LA pyramidal neurons.

(A) Schematic of the AAV injection strategy and experimental timeline. After AFC with a 20 kHz tone, mice were exposed one day later to four presentations of either the conditioned 20 kHz tone (20k-20k group) or a novel 2 kHz tone (20k-2k group). (B) Freezing during AFC in the 20k-20k and 20k-2k groups ($N = 4$ mice per group). (C) Freezing during the baseline period and tone presentation during the recall session. Data are analyzed using two-way ANOVA followed by Šidák's *post hoc* test, *** $p < 0.001$. Each dot represents an individual mouse. (D) Representative images showing mCherry-labeled neurons (red) and c-Fos expression (green). White arrowheads indicate mCherry⁺/c-Fos⁺ double-positive cells. Scale bars, 50 μ m. (E) Percentage of mCherry⁺/c-Fos⁺ cells among all mCherry⁺ cells. (F) Percentage of mCherry⁺/c-Fos⁺ double-positive cells among all c-Fos⁺ cells. (G) Density of c-Fos⁺ cells following exposure to the conditioned 20 kHz tone or the

novel 2 kHz tone. **(H)** Density of mCherry⁺ cells in the 20k–20k and 20k–2k groups. Cell densities are expressed as the number of cells per 0.1 mm². Data are analyzed using Mann-Whitney *U* test, **p* < 0.05. Each dot represents the mean value obtained from an individual mouse. **(I–O)** Electrophysiological characterization of thalamic pathway-evoked synaptic responses in LA pyramidal neurons from naïve C57BL/6J mice. **(I)** Representative traces of monosynaptic EPSCs and disynaptic IPSCs evoked by thalamic pathway stimulation. **(J)** Representative IPSC traces recorded in the absence and presence of 20 μM DNQX and 50 μM APV. **(K)** Representative IPSC traces recorded before and after application of 100 μM picrotoxin (PTX), a GABA_A receptor antagonist. **(L)** Firing probability of LA pyramidal neurons (*n* = 7 cells from *N* = 2 mice) in response to increasing thalamic stimulation intensities (25–100 μA). **(M)** Onset latencies of EPSCs and IPSCs following thalamic pathway stimulation (*n* = 4 cells from *N* = 2 mice). Paired *t* test. **(N)** IPSC amplitudes before and after application of DNQX and APV, normalized to the no-drug condition (*n* = 4 cells from *N* = 2 mice). Paired *t* test. **(O)** IPSC amplitudes before and after application of PTX, normalized to the no-drug condition (*n* = 3 cells from *N* = 2 mice). Paired *t* test. Data are presented as mean ± SEM. ***p* < 0.01, ****p* < 0.001.

Fig. S2

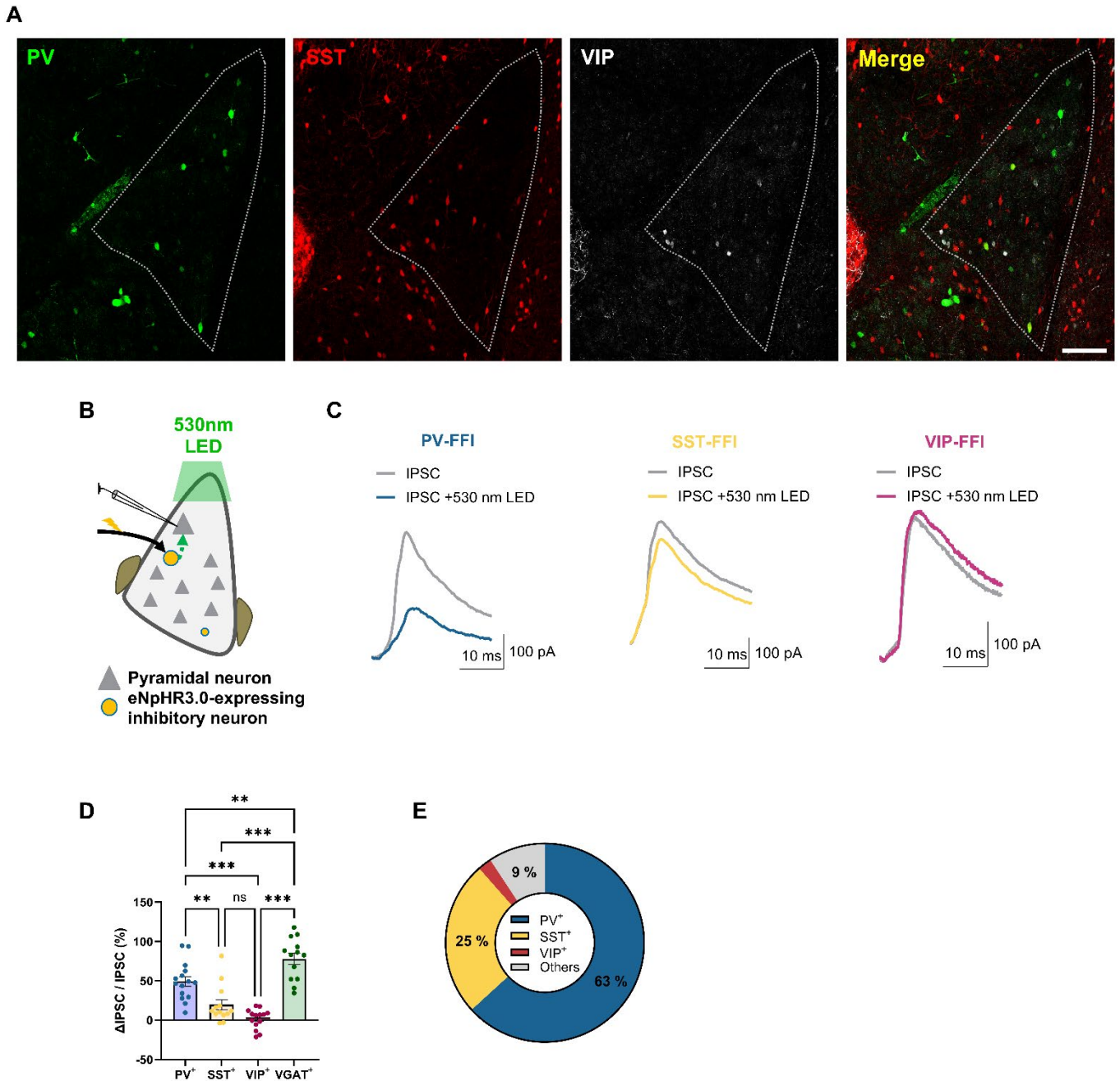


Fig. S2. Differential contributions of PV-, SST-, and VIP-INs to FFI in the LA.

(A) Representative fluorescence images showing PV (green) and VIP (white) immunoreactivity and tdTomato-labeled SST-INs (red) in the LA of SST-Cre/Ai14 mice. Scale bar, 100 μ m. (B) Schematic of thalamic stimulation-evoked IPSC recordings during optogenetic inhibition of individual IN subtypes in naïve PV-Cre, SST-Cre, or VIP-Cre mice injected with AAV-DIO-NpHR into the LA. (C) Representative IPSC traces before and during photoinhibition of PV-INs (left), SST-INs (middle), and VIP-INs (right). (D) Relative contributions of PV-INs ($n = 15$ cells from $N = 3$ mice), SST-INs ($n = 13$ cells from $N = 3$ mice), VIP-INs ($n = 14$ cells from $N = 3$ mice), and VGAT⁺ GABAergic neurons ($n = 13$ cells from $N = 3$ mice) to FFI. One-way ANOVA followed by Tukey's *post hoc* test. Data are presented as mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$. (E) Relative contributions of each IN subtype to VGAT⁺ neuron-mediated FFI: PV-FFI ($63 \pm 8\%$), SST-FFI ($25 \pm 9\%$), and VIP-FFI ($2 \pm 4\%$). The residual fraction of FFI not accounted for by PV-, SST-, and VIP-INs is denoted as 'Others'.

Fig. S3

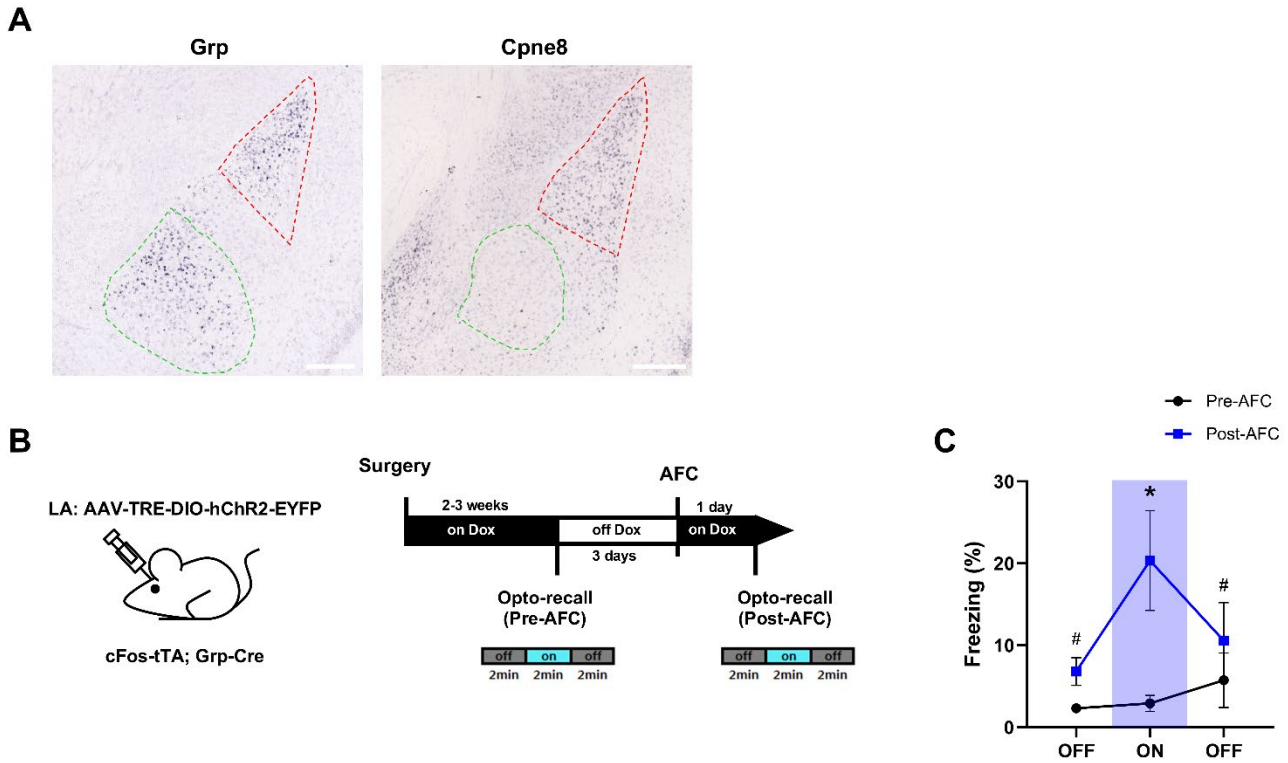


Fig. S3. Optogenetic reactivation of AFC-tagged Grp⁺ LA neurons induces freezing behavior.

(A) Representative *in situ* hybridization images from the Allen Brain Atlas showing *Grp* expression (left) in the LA (red outline) and basomedial amygdala (BMA; green outline), and *Cpne8* expression (right) predominantly in the LA. Scale bar, 300 μ m. (B) Schematic of the AAV injection strategy and experimental timeline. (C) Freezing levels during laser-OFF and laser-ON phases 3 days before AFC (Pre-AFC; black line) and 1 day after AFC (Post-AFC; blue line) ($N = 5$ mice). Two-way ANOVA followed by Fisher's *post hoc* test. $*p < 0.05$, Pre-AFC vs. Post-AFC during the laser-ON phase; $\#p < 0.05$, laser-OFF vs. laser-ON during Post-AFC recall. Data are presented as mean $\pm$ SEM.

Fig. S4

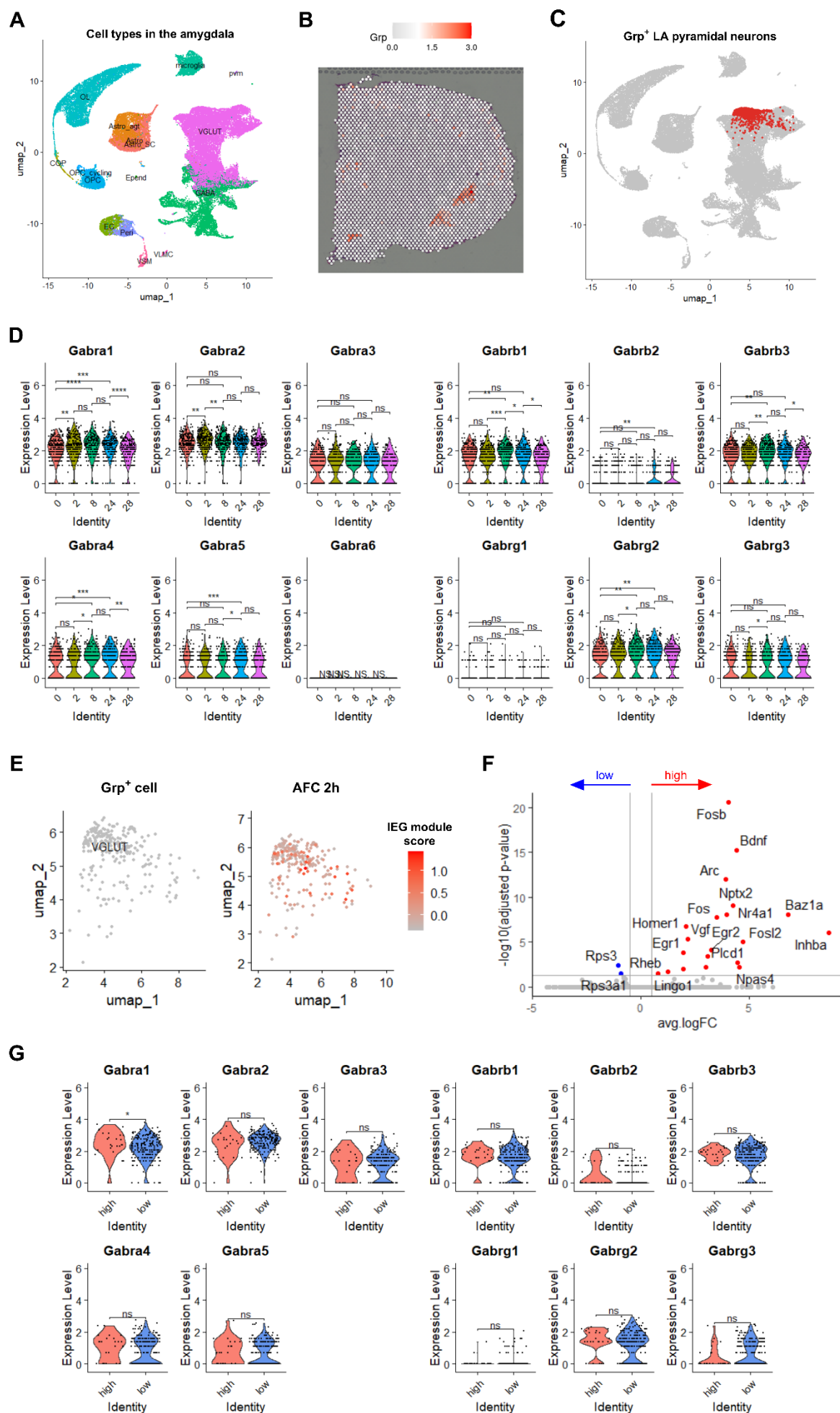


Fig. S4. Analysis of GABA_AR subunit gene expression in LA pyramidal neurons and putative fear ENs using transcriptomic data from Hochgerner et al. (2023).

(A) Uniform Manifold Approximation and Projection (UMAP) clustering of amygdala cell types based on single-cell transcriptomic profiles. GABA, GABAergic neurons; VGLUT, glutamatergic neurons; Astro, telencephalic astrocytes; Astro_agt, angiotensinogen-positive diencephalic astrocytes; Astro_SC, astrocyte-like neural stem/progenitor cells; COP, committed oligodendrocyte precursors; EC, endothelial cells; Epend, ependymal cells; OL, oligodendrocytes; OPC, oligodendrocyte precursor cells; OPC_cycling, cycling OPCs; Peri, pericytes; VLMC, vascular and leptomeningeal cells; VSM, vascular smooth muscle cells; microglia, microglia; pvm, perivascular macrophages. (B) Spatial distribution of *Grp* mRNA in an adult mouse brain section from the 10x Genomics Visium dataset. (C) UMAP embedding showing the Grp⁺/Cpne8⁺ cell cluster within the VGLUT⁺ glutamatergic neuron cluster. (D) Expression levels of GABA_AR subunit genes in the Grp⁺ cell cluster at 0 h (naïve, $n = 262$ cells), 2 h ($n = 238$ cells), 8 h ($n = 215$ cells), 24 h ($n = 206$ cells), and 28 h ($n = 169$ cells) after AFC. Data are analyzed using the Kruskal-Wallis test followed by pairwise Wilcoxon rank-sum tests. (E) UMAP of Grp⁺ cell cluster at 2 h after AFC, shown without (left) and with (right) IEG module scores overlaid. (F) Volcano plot showing differentially expressed genes between high-IEG (red, $n = 25$ cells) and low-IEG (blue, $n = 213$ cells) Grp⁺ cells, using thresholds of adjusted $p < 0.05$ and $|\log_2\text{fold change}| > 0.5$. Genes enriched in IEG-high cells are shown in red and genes enriched in IEG-low cells in blue. (G) Comparative expression levels of GABA_AR subunit genes between high-IEG and low-IEG Grp⁺ cells. Wilcoxon rank-sum test. Data are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Fig. S5

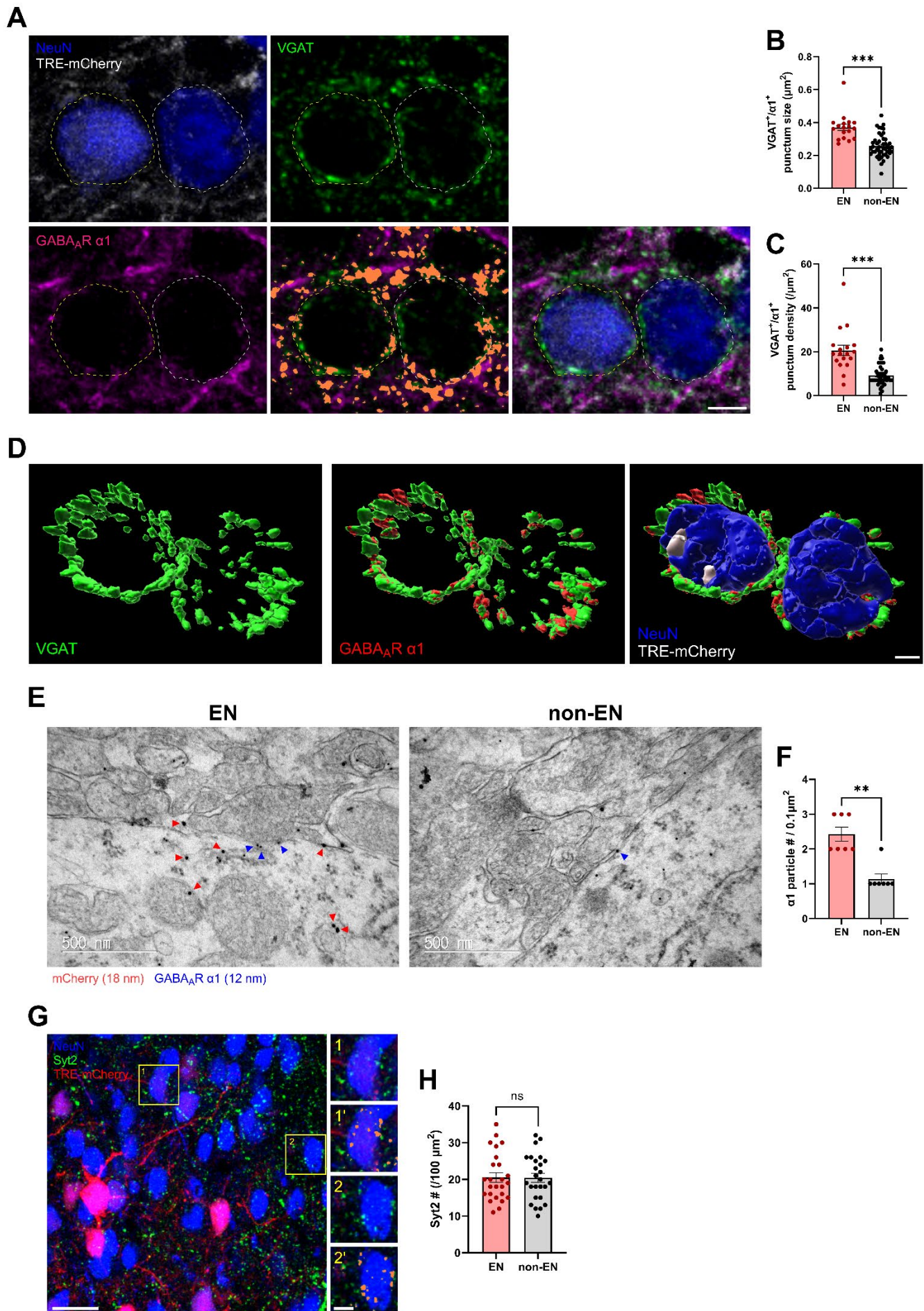


Fig. S5. Localization of $\alpha 1$ -containing GABA_ARs at GABAergic perisomatic synapses.

(A) Representative immunofluorescence images showing NeuN (blue), a TRE-mCherry-labeled EN (white), VGAT (green), GABA_AR $\alpha 1$ (magenta), and merged channels in the LA of cFos-tTA mice injected with AAV-TRE-mCherry. Mice underwent AFC during a DOX-off period and were sacrificed one day after AFC. Dashed outlines indicate the perisomatic regions of an EN and a non-EN, respectively. Perisomatic VGAT⁺/ $\alpha 1$ ⁺ puncta are highlighted in pseudocolor (orange). Scale bar, 5 μ m. (B, C) Quantification of the mean size (B) and density (C) of perisomatic VGAT⁺/ $\alpha 1$ ⁺ puncta in ENs ($n = 18$ cells from $N = 3$ mice) and non-ENs ($n = 45$ cells from $N = 3$ mice). Mann-Whitney U test, *** $p < 0.001$. Data are shown as mean $\pm$ SEM. (D) 3D reconstruction of perisomatic inhibitory synapses showing VGAT (green), VGAT-colocalized GABA_AR $\alpha 1$ (red), and merged channels with NeuN (blue) and TRE-mCherry (white). (E) Representative post-embedding immunogold electron micrographs of an EN (left) and a non-EN (right) in the LA of cFos-tTA mice injected with AAV-TRE-mCherry. Mice underwent AFC during a DOX-off period and were sacrificed one day after AFC. mCherry was labeled with 18 nm gold particles (red arrowheads), and GABA_AR $\alpha 1$ was labeled with 12 nm gold particles (blue arrowheads denote postsynaptic GABA_AR $\alpha 1$). Scale bars, 500 nm. (F) Quantification of postsynaptic GABA_AR $\alpha 1$ ⁺ particle density per 0.1 μ m² of perisomatic membrane in ENs ($n = 7$ cells) and non-ENs ($n = 7$ cells). Mann-Whitney U test, ** $p < 0.01$. Data are presented as mean $\pm$ SEM. (G) Representative immunofluorescence image showing NeuN (blue), Syt2 (green), and TRE-mCherry-labeled ENs (red) in the LA of cFos-tTA mice injected with AAV-TRE-mCherry. Mice underwent AFC during a DOX-off period and were sacrificed one day after AFC. Insets show perisomatic regions of an EN (1, 1') and a non-EN (2, 2'); NeuN⁺/Syt2⁺ puncta are highlighted in pseudocolor orange (1', 2'). Scale bars: 20 μ m (left), 5 μ m (insets). (H) Quantification of perisomatic Syt2⁺ puncta density in ENs ($n = 25$ cells from $N = 3$ mice) and non-ENs ($n = 26$ cells from $N = 3$ mice). Mann-Whitney U test. Data are presented as mean $\pm$ SEM.

Fig. S6

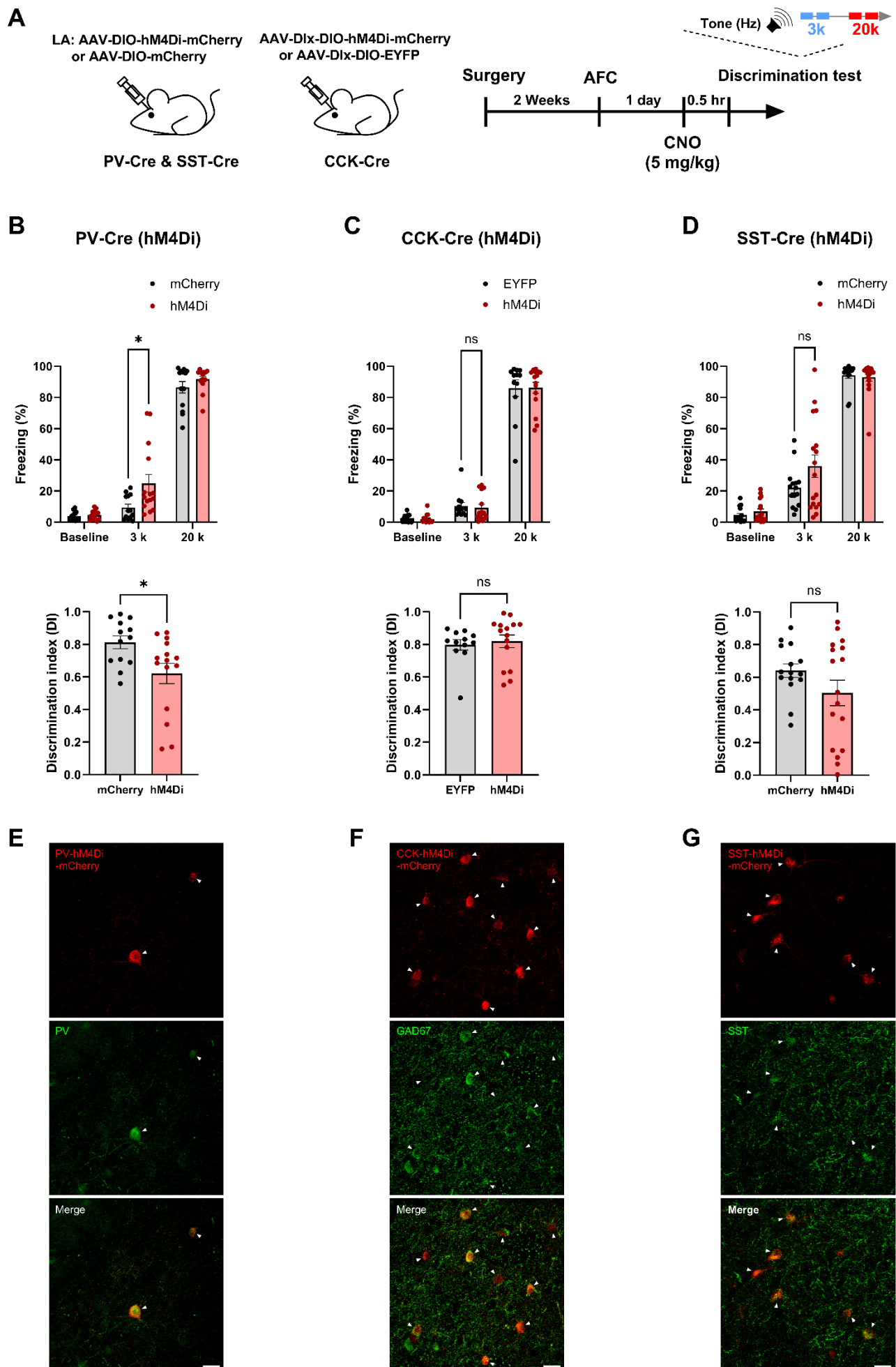


Fig. S6. Effects of IN subtype-specific suppression on fear discrimination.

(A) Schematic of the AAV injection strategy and experimental timeline. (B–D) Freezing levels during baseline and presentations of the 3 kHz and 20 kHz tones (top), and corresponding DIs (bottom) in mice expressing the inhibitory DREADD hM4Di or control fluorophore in PV-INs (B; hM4Di, $N = 15$ mice; mCherry, $N = 13$ mice), CCK-INs (C; hM4Di, $N = 15$ mice; EYFP, $N = 12$ mice), or SST-INs (D; hM4Di, $N = 17$ mice; mCherry, $N = 15$ mice). Data in the top panels are analyzed using two-way ANOVA followed by Fisher's *post hoc* test, and data in the bottom panels are analyzed using Mann-Whitney *U* test. Data are presented as mean $\pm$ SEM. $*p < 0.05$. (E) Representative images showing hM4Di-mCherry expression (red) in PV-INs (top), PV immunoreactivity (green; middle), and merged channels (bottom). White arrowheads indicate double-labeled cells. Scale bar, 20 μ m. (F) Representative images showing hM4Di-mCherry expression (red) in CCK-INs (top), GAD67 immunoreactivity (green; middle), and merged channels (bottom). White arrowheads indicate double-labeled cells. Scale bar, 20 μ m. (G) Representative images showing hM4Di-mCherry expression (red) in SST-INs (top), SST immunoreactivity (green; middle), and merged channels (bottom). White arrowheads indicate double-labeled cells. Scale bar, 20 μ m.

Fig. S7

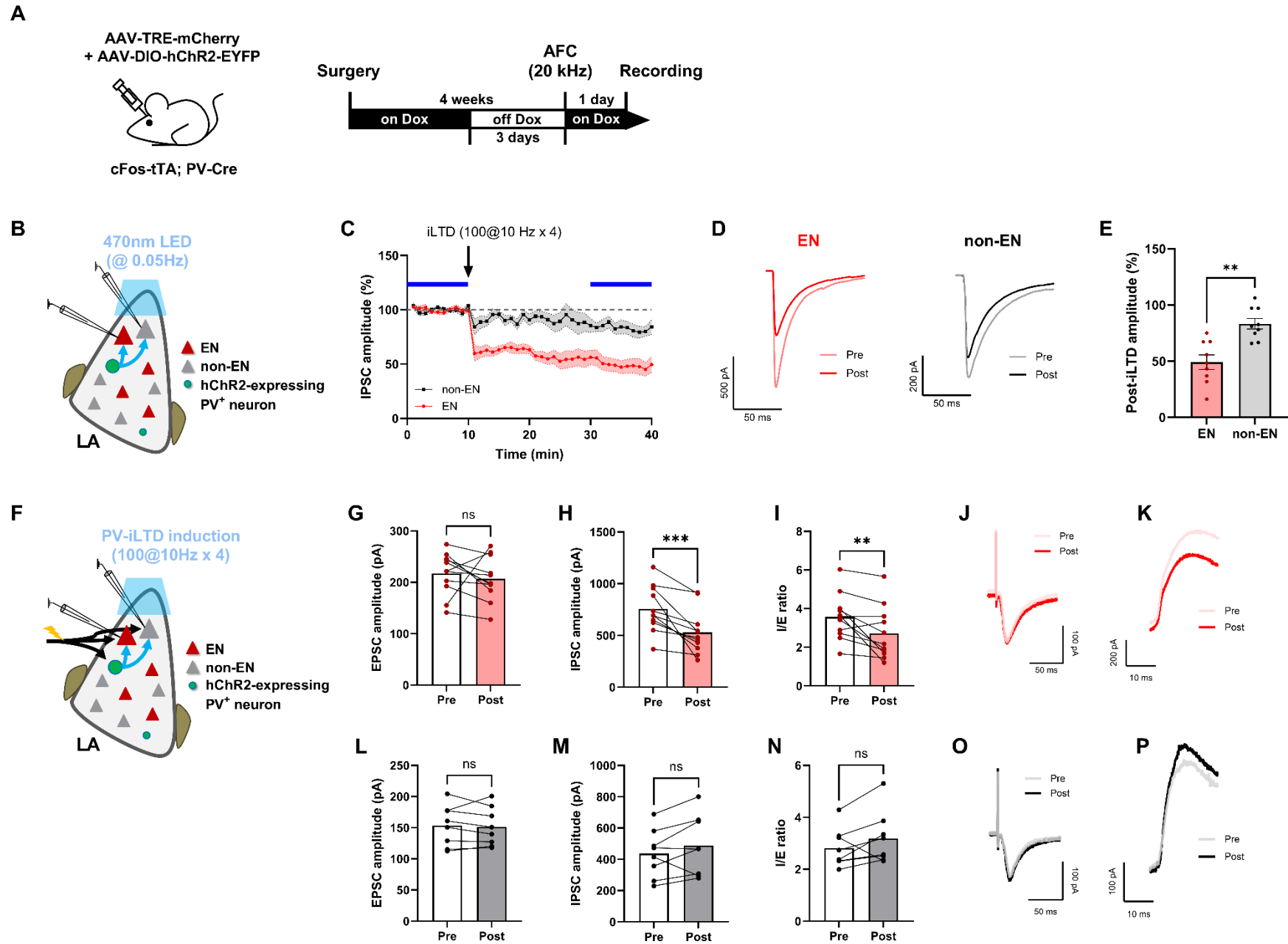


Fig. S7. *Ex vivo* validation of an optical PV-iLTD protocol for selective depression of PV-FFI to ENs.

(A) Schematic of the AAV injection strategy and experimental timeline. (B) Schematic of PV-IPSC recordings from ENs and non-ENs before and after PV-iLTD induction. (C) Time course of PV-IPSC amplitudes in ENs (red, $n = 9$ cells from $N = 3$ mice) and non-ENs (black, $n = 9$ cells from $N = 3$ mice) before and after PV-iLTD induction. Blue bars indicate the analysis windows. (D) Representative PV-IPSC traces from ENs and non-ENs before and after PV-iLTD induction. (E) PV-IPSC amplitudes after PV-iLTD, normalized to baseline, in ENs ($n = 9$ cells from $N = 3$ mice) and non-ENs ($n = 9$ cells from $N = 3$ mice). Mann-Whitney U test. (F) Schematic of thalamic stimulation-evoked EPSC and IPSC recordings from ENs and non-ENs before and after PV-iLTD induction. (G–I) EPSC amplitudes (G), IPSC amplitudes (H), and I/E ratios (I) in ENs ($n = 11$ cells from $N = 3$ mice) before and after PV-iLTD induction. Wilcoxon signed-rank test. (J, K) Representative EPSC (J) and IPSC (K) traces from ENs before and after PV-iLTD induction. (L–N) EPSC amplitudes (L), IPSC amplitudes (M), and I/E ratios (N) in non-ENs ($n = 8$ cells from $N = 3$ mice) before and after PV-iLTD induction. Wilcoxon signed-rank test. (O, P) Representative EPSC (O) and IPSC (P) traces from non-ENs before and after PV-iLTD induction. Data are presented as mean $\pm$ SEM. $**p < 0.01$, $***p < 0.001$.

Fig. S8

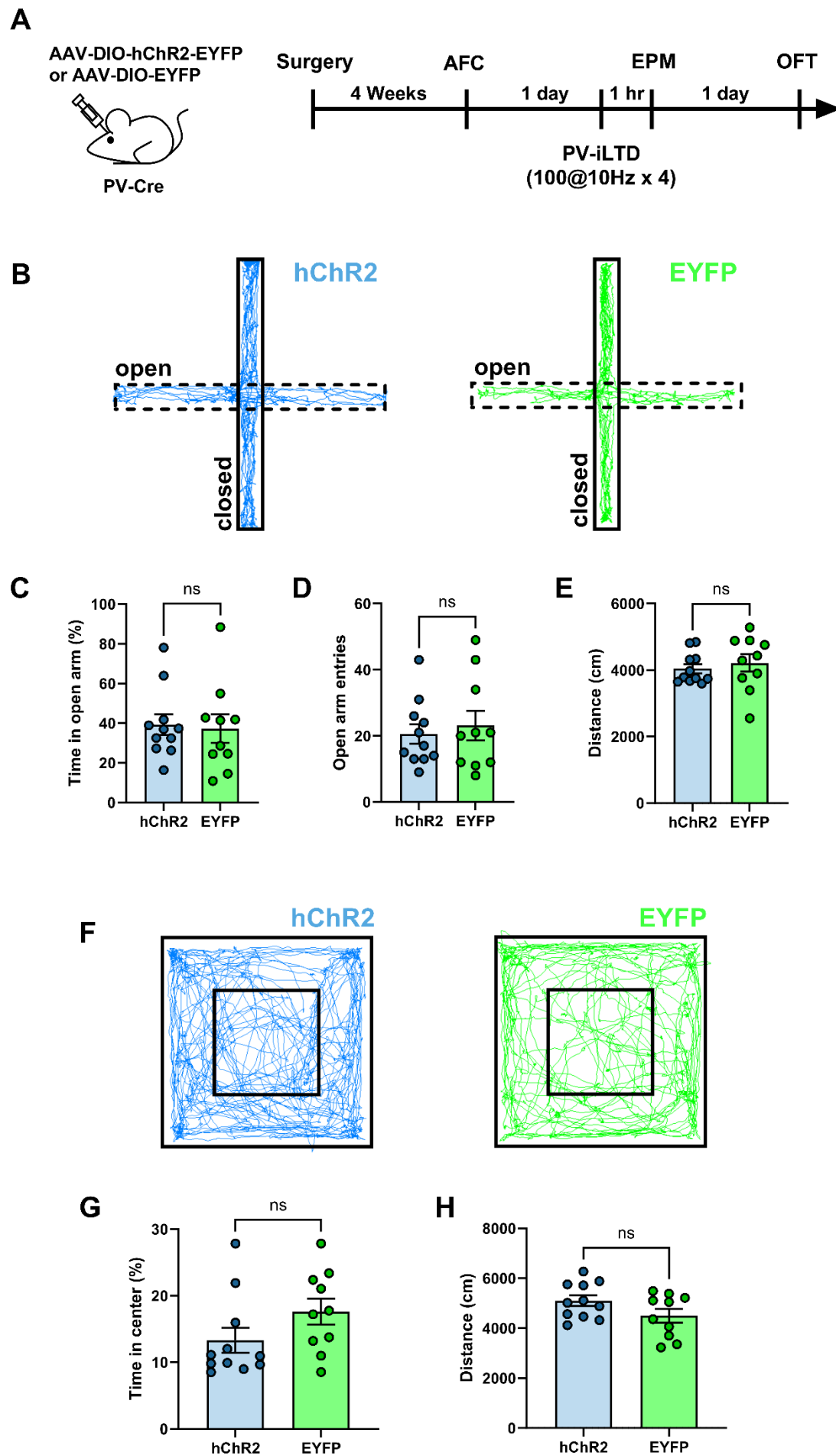


Fig. S8. PV-iLTD does not affect anxiety-like behavior or locomotion.

(A) Schematic of the AAV injection strategy and experimental timeline. (B) Representative locomotor trajectories of PV-hChR2-expressing mice (hChR2 group; left) and PV-EYFP-expressing control mice (EYFP group; right) in the elevated plus maze (EPM). Closed arms are outlined with solid lines and open arms with dashed lines. (C–E) Quantification of EPM behavior following PV-iLTD: percentage of time spent in open arms (C), number of open arm entries (D), and total distance traveled (E) in hChR2 ($N = 11$ mice) and EYFP ($N = 10$ mice) groups. (F) Representative locomotor trajectories of hChR2 (left) and EYFP (right) mice in the open field test (OFT). The center zone is virtually outlined by the inner solid lines. (G, H) Quantification of OFT behavior following PV-iLTD: percentage of time spent in the center zone (G), and total distance traveled (H) in hChR2 ($N = 11$ mice) and EYFP ($N = 10$ mice) groups. Mann-Whitney U test. Data are presented as mean $\pm$ SEM.

Fig. S9

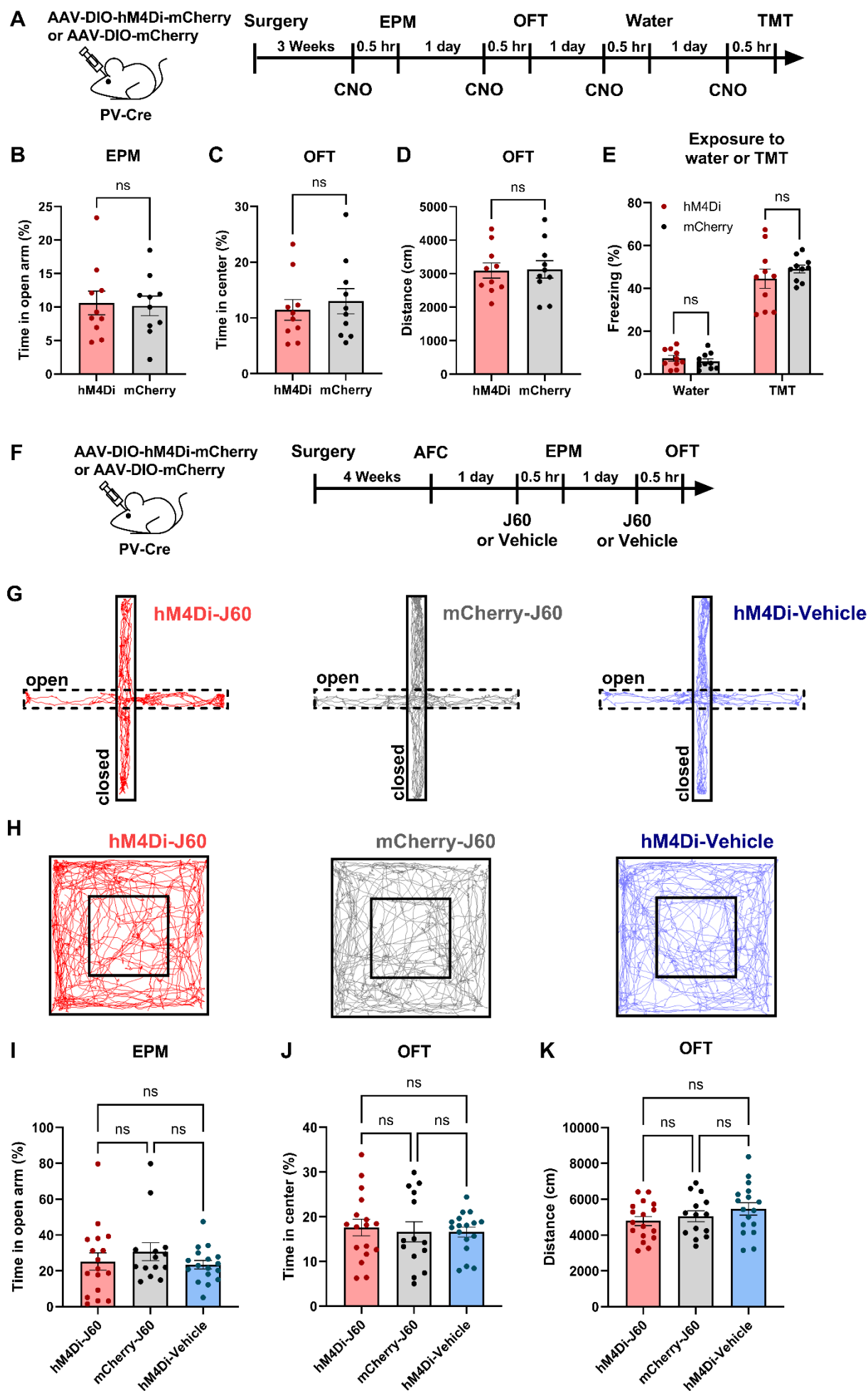


Fig. S9. Chemogenetic suppression of PV-INs does not alter anxiety-like behavior, locomotion, or innate fear.

(A) Schematic of the AAV injection strategy and experimental timeline for testing naïve PV-Cre mice injected with AAV-DIO-hM4Di-mCherry ($N = 10$ mice) or AAV-DIO-mCherry ($N = 10$ mice). Mice received CNO (5 mg/kg, i.p.) 30 min before behavioral testing in the EPM, OFT, and innate fear test using predator odor 2,5-dihydro-2,4,5-trimethylthiazoline (TMT) or water as a control. (B–E) Behavioral outcomes in naïve PV-Cre mice: percentage of time spent in the open arms of the EPM (B), percentage of time spent in the center zone of the OFT (C), total distance traveled in the OFT (D), and percentage of freezing during exposure to water or TMT (E). Mann-Whitney U test (B–D); mixed-effects model followed by Šídák's *post hoc* test (E), ns, not significant, $p > 0.05$. (F) Schematic of the AAV injection strategy and experimental timeline. Mice were tested in the EPM one day after AFC and in the OFT two days after AFC. Mice received J60 (0.5 mg/kg, i.p.) or vehicle 30 min before each behavioral test. (G, H) Representative locomotor trajectories in the EPM (G) and OFT (H) for each group (hM4Di-J60, red, $N = 17$ mice; mCherry-J60, gray, $N = 14$ mice; hM4Di-Vehicle, blue, $N = 17$ mice). Closed arms are outlined with solid lines and open arms with dashed lines (G). The center zone is virtually outlined by the inner solid lines (H). (I–K) Behavioral outcomes in fear-conditioned PV-Cre mice: percentage of time spent in the open arms of the EPM (I), percentage of time spent in the center zone of the OFT (J), and total distance traveled in the OFT (K). One-way ANOVA followed by Šídák's *post hoc* test, ns, $p > 0.05$. Data are presented as mean $\pm$ SEM.

Fig. S10

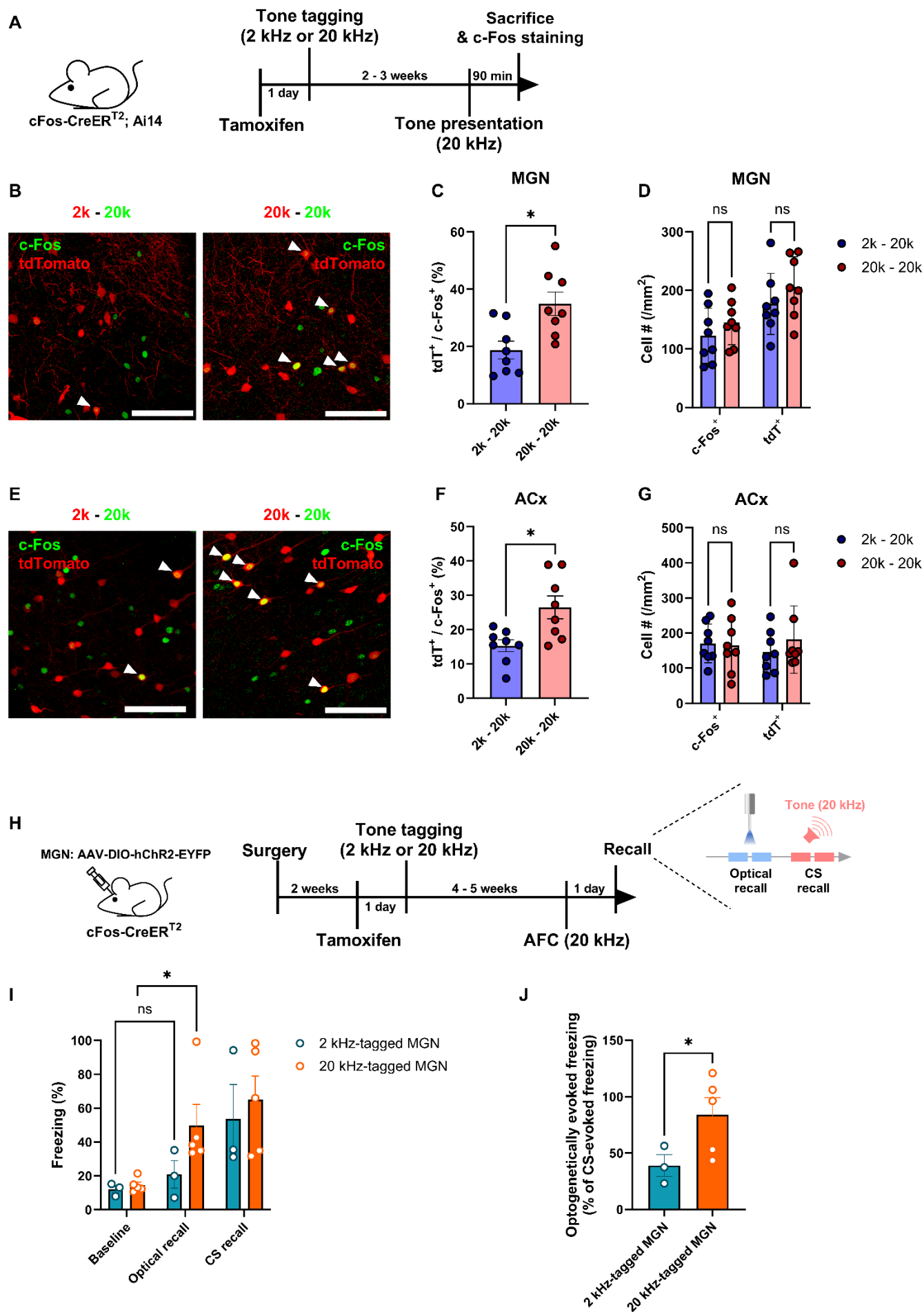


Fig. S10. Validation of the tone-tagging system used for labeling frequency-specific neurons in the MGN and ACx.

(A) Schematic of the experimental timeline for activity-dependent tone-tagging in the MGN and ACx. (B) Representative images showing c-Fos expression (green) in the MGN following 20 kHz tone exposure in mice previously tagged with the 2 kHz tone (left; 2k–20k group) or 20 kHz tone (right; 20k–20k group), with tdTomato (tdT) labeling previously tone-tagged neurons (red). White arrowheads indicate tdT⁺/c-Fos⁺ double-labeled neurons. Scale bars, 100 μ m. (C) Proportion of tdT⁺/c-Fos⁺ double-labeled neurons among c-Fos⁺ neurons in the MGN of mice in the 2k–20k (blue, $N = 8$ mice) and 20k–20k (red, $N = 8$ mice) groups. Mann-Whitney U test. (D) Numbers of c-Fos⁺ and tdT⁺ neurons in the MGN of the 2k–20k and 20k–20k groups. Two-way ANOVA followed by Šídák's *post hoc* test. Each dot represents the mean value obtained from an individual mouse. (E) Representative images showing c-Fos expression (green) in the ACx following 20 kHz tone exposure in mice previously tagged with the 2 kHz tone (left; 2k–20k group) or 20 kHz tone (right; 20k–20k group), with tdT labeling previously tone-tagged neurons (red). White arrowheads indicate tdT⁺/c-Fos⁺ double-labeled neurons. Scale bars, 100 μ m. (F) Proportion of tdT⁺/c-Fos⁺ double-labeled neurons among c-Fos⁺ neurons in the ACx of mice in the 2k–20k (blue, $N = 8$ mice) and 20k–20k (red, $N = 8$ mice) groups. Mann-Whitney U test. (G) Numbers of c-Fos⁺ and tdT⁺ neurons in the ACx of the 2k–20k and 20k–20k groups. Two-way ANOVA followed by Šídák's *post hoc* test. Each dot represents the mean value obtained from an individual mouse. (H) Schematic of the AAV injection strategy and experimental timeline for the 2 kHz-tagged ($N = 3$ mice) and 20 kHz-tagged ($N = 5$ mice) groups. (I) Freezing during the prestimulation baseline, optical stimulation of tagged MGN neurons (optical recall), and presentation of the 20 kHz CS (CS recall). Optical recall and CS recall values represent the mean freezing across the two respective 30-s trials. Two-way ANOVA followed by Šídák's *post hoc* test. (J) Optogenetically evoked freezing normalized to CS-evoked freezing in the 2 kHz-tagged and 20 kHz-tagged groups. Welch's t test. Data are presented as mean $\pm$ SEM. * $p < 0.05$.

Fig. S11

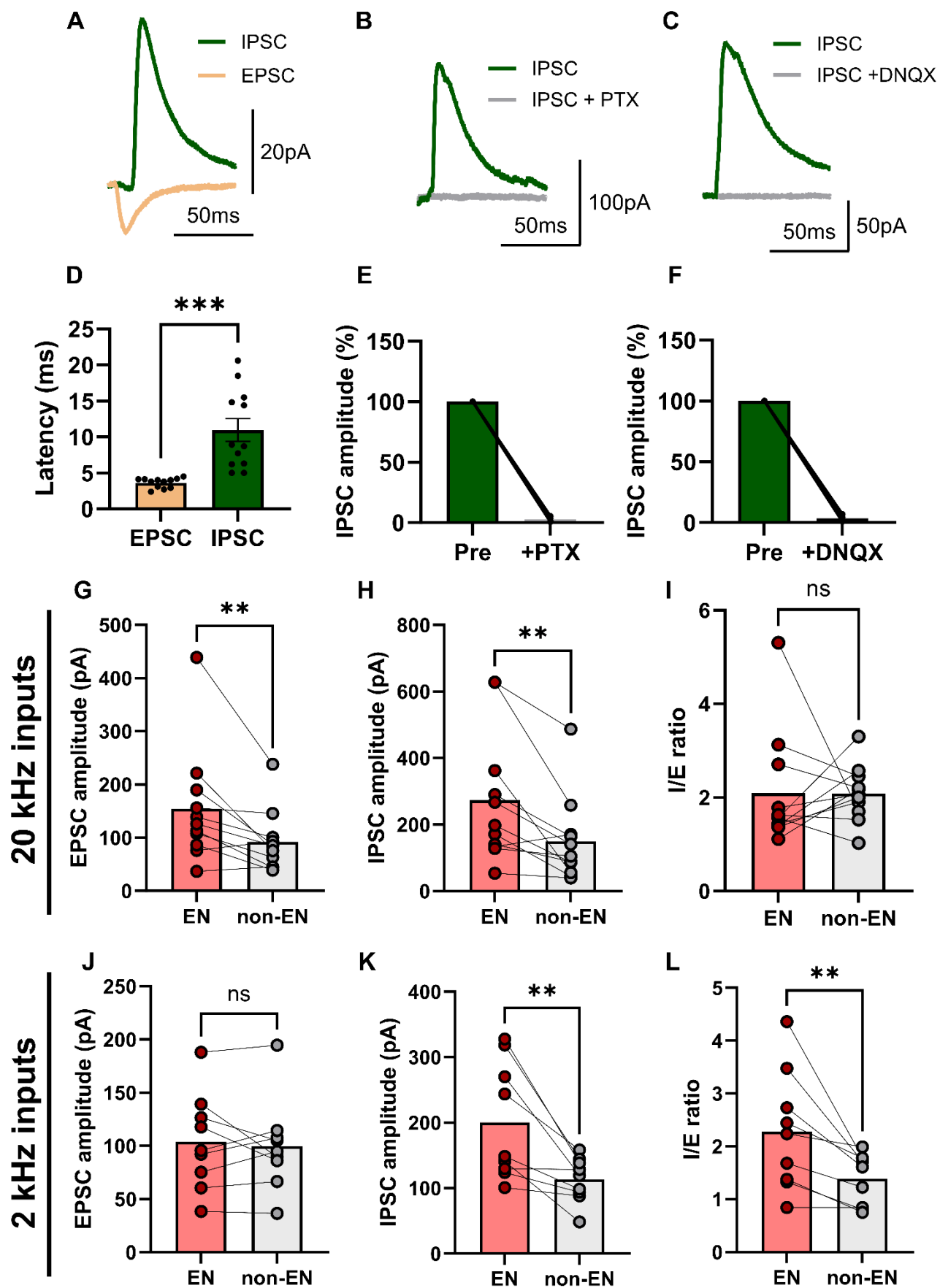


Fig. S11. Characterization of postsynaptic currents evoked by optogenetic stimulation of auditory inputs.

(A–F) Characterization of optogenetically evoked synaptic responses in LA pyramidal neurons of naïve C57BL/6J mice expressing ChR2 under the CaMKII α promoter in the MGN and ACx. (A) Representative traces of optogenetically evoked EPSCs and IPSCs recorded from LA pyramidal neurons. (B) Representative IPSC traces recorded in the absence and presence of 100 μ M PTX. (C) Representative IPSC traces recorded in the absence and presence of 20 μ M DNQX. (D) Onset latencies of EPSCs and IPSCs ($n = 12$ cells from $N = 3$ mice). Mann-Whitney U test. (E, F) IPSC amplitudes before and after application of PTX (E, $n = 5$ cells from $N = 3$ mice) or DNQX (F, $n = 7$ cells from $N = 3$ mice), normalized to the pre-drug condition. (G–L) Mouse-level averaged synaptic responses in LA ENs and non-ENs of cFos-CreER^{T2}/cFos-tTA mice injected with AAV-DIO-ChR2 into both the MGN and ACx and AAV-TRE-mCherry into the LA. Tone-tagging was performed 2 weeks after AAV injection, followed by AFC during a DOX-off period. Mice were sacrificed one day after AFC. Cell-level data are presented in Fig. 4O–R. (G–I) 20 kHz input-evoked EPSC amplitudes (G), IPSC amplitudes (H), and I/E ratios (I) in ENs and non-ENs ($N = 11$ mice). Each pair of dots represents the mean EN and non-EN values obtained from an individual mouse. Wilcoxon signed-rank test. (J–L) 2 kHz input-evoked EPSC amplitudes (J), IPSC amplitudes (K), and I/E ratios (L) in ENs and non-ENs ($N = 9$ mice). Each pair of dots represents the mean EN and non-EN values obtained from an individual mouse. Wilcoxon signed-rank test. Data are presented as mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$.

Fig. S12

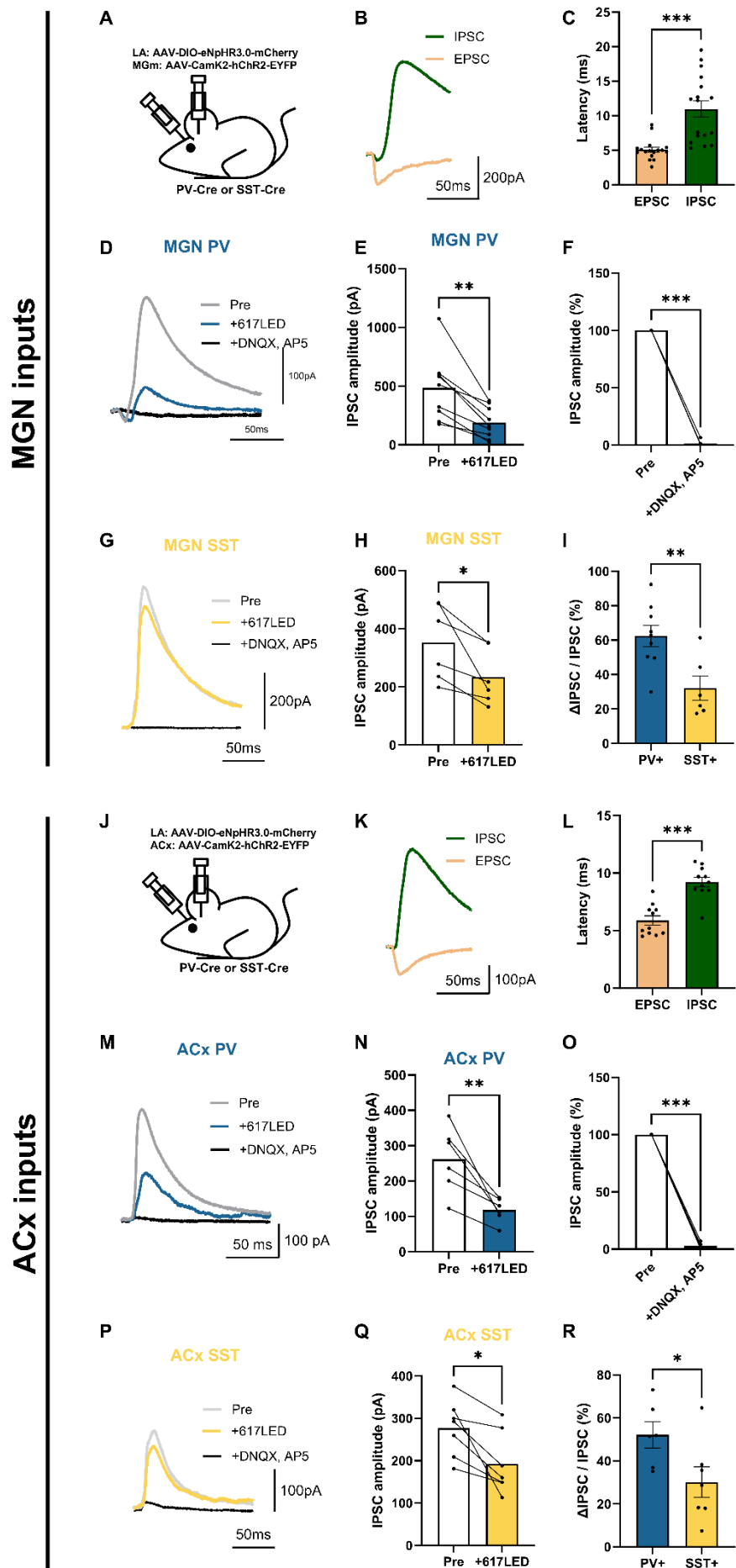


Fig. S12. Contributions of PV-INs and SST-INs to FFI in the MGN–LA and ACx–LA pathways.

(A) Schematic of the AAV injection strategy targeting the MGN and LA. (B) Representative traces of EPSCs and IPSCs recorded from LA pyramidal neurons following optogenetic stimulation of MGN inputs. (C) Onset latencies of EPSCs and IPSCs ($n = 17$ cells from $N = 4$ mice). Mann-Whitney U test. (D) Representative traces of MGN input-evoked IPSCs recorded before (Pre) and during photoinhibition of PV-INs in the LA (+617LED), and following application of 20 μ M DNQX and 50 μ M APV (+DNQX, AP5). (E) MGN input-evoked IPSC amplitudes before and during PV-IN photoinhibition ($n = 9$ cells from $N = 3$ mice). Paired t test. (F) IPSC amplitudes before and after application of DNQX + APV, normalized to the pre-drug condition ($n = 7$ cells from $N = 2$ mice). Paired t test. (G) Representative traces of MGN input-evoked IPSCs recorded before (Pre) and during photoinhibition of SST-INs in the LA (+617LED), and following application of 20 μ M DNQX and 50 μ M APV (+DNQX, AP5). (H) MGN input-evoked IPSC amplitudes before and during SST-IN photoinhibition ($n = 6$ cells from $N = 3$ mice). Paired t test. (I) Relative contributions of PV-INs ($n = 9$ cells from $N = 3$ mice) and SST-INs ($n = 6$ cells from $N = 3$ mice) to MGN–LA FFI, quantified as Δ IPSC/IPSC (%). Unpaired t test. (J) Schematic of the AAV injection strategy targeting the ACx and LA. (K) Representative traces of EPSCs and IPSCs recorded from LA pyramidal neurons following optogenetic stimulation of ACx inputs. (L) Onset latencies of EPSCs and IPSCs ($n = 11$ cells from $N = 3$ mice). Mann-Whitney U test. (M) Representative traces of ACx input-evoked IPSCs recorded before (Pre) and during photoinhibition of PV-INs in the LA (+617LED), and following application of 20 μ M DNQX and 50 μ M APV (+DNQX, AP5). (N) ACx input-evoked IPSC amplitudes before and during PV-IN photoinhibition ($n = 6$ cells from $N = 3$ mice). Paired t test. (O) IPSC amplitudes before and after application of DNQX + APV, normalized to the pre-drug condition ($n = 5$ cells from $N = 2$ mice). Paired t test. (P) Representative traces of ACx input-evoked IPSCs recorded before (Pre) and during photoinhibition of SST-INs in the LA (+617LED), and following application of 20 μ M DNQX and 50 μ M APV (+DNQX, AP5). (Q) ACx input-evoked IPSC amplitudes before and during SST-IN photoinhibition ($n = 7$ cells from $N = 3$ mice). Paired t test. (R) Relative contributions of PV-INs ($n = 6$ cells from $N = 3$ mice) and SST-INs ($n = 7$ cells from $N = 3$ mice) to ACx–LA FFI, quantified as Δ IPSC/IPSC (%). Unpaired t test. Data are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. S13

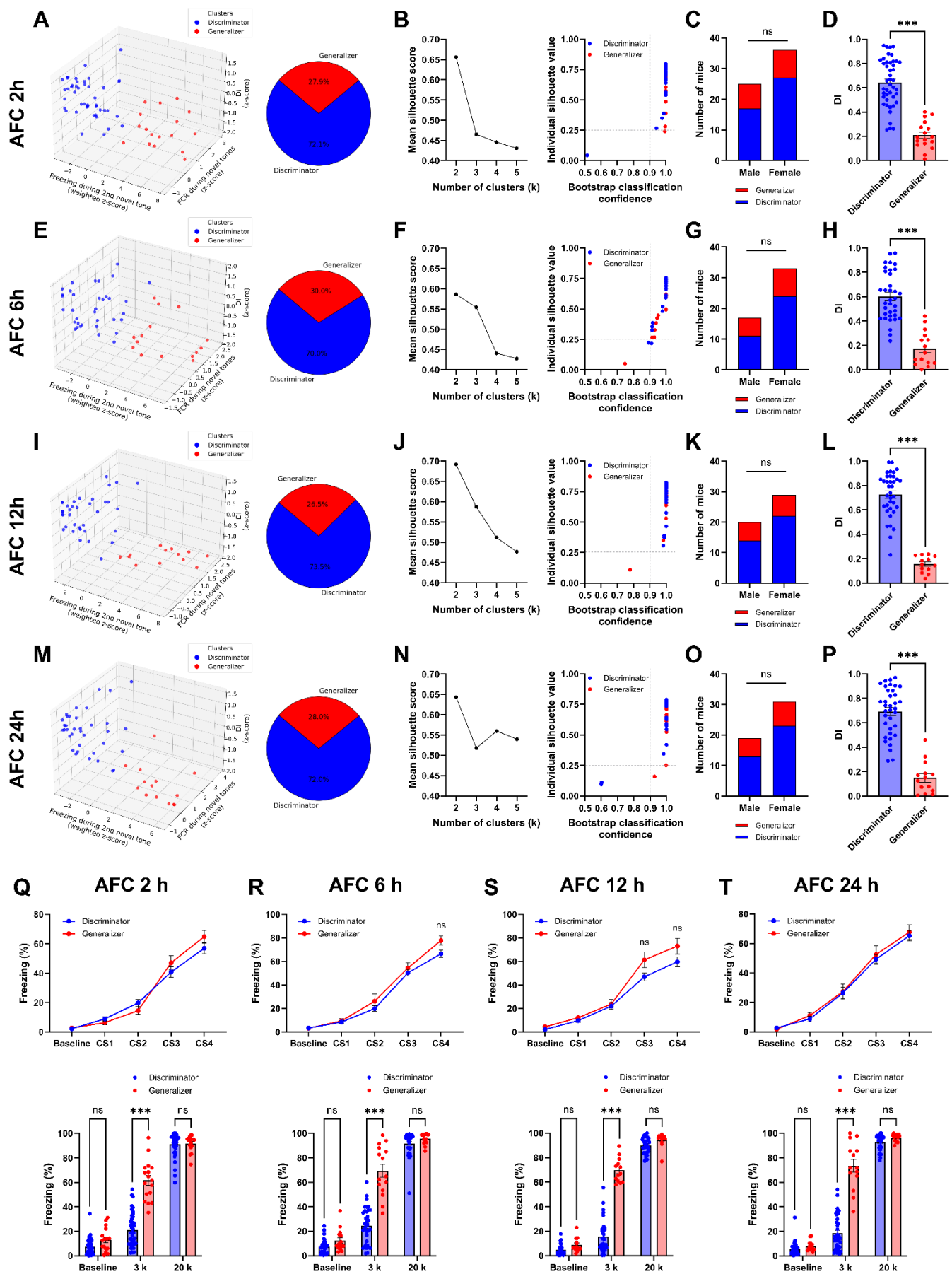


Fig. S13. Stable proportions of discriminators and generalizers across post-AFC timepoints.

(A, E, I, M) Three-dimensional visualization of behavioral features following weighted K-means clustering and corresponding proportions of mice classified as discriminators and generalizers at 2 h (A; discriminators, $N = 44$; generalizers, $N = 17$), 6 h (E; discriminators, $N = 35$; generalizers, $N = 15$), 12 h (I; discriminators, $N = 36$; generalizers, $N = 13$), and 24 h (M; discriminators, $N = 36$; generalizers, $N = 14$) after AFC. Blue and red symbols indicate discriminators and generalizers, respectively. (B, F, J, N) Left, mean silhouette scores for clustering solutions containing two to five clusters. Right, individual silhouette values plotted against bootstrap classification stability for each mouse. Dashed lines indicate descriptive reference values of 0.90 for bootstrap classification stability and 0.25 for the individual silhouette value. (C, G, K, O) Numbers of male and female mice classified as discriminators or generalizers at each time point. The proportions of discriminators and generalizers between male and female mice were compared using two-sided Fisher's exact tests. (D, H, L, P) DI values of discriminators and generalizers at each time point. Statistical comparisons were performed using Mann-Whitney U test. $***p < 0.001$. (Q–T) Freezing during AFC training (top) and during the discrimination test with the novel 3 kHz tone and 20 kHz CS (bottom), shown separately for discriminators (blue) and generalizers (red) at 2 h (Q), 6 h (R), 12 h (S), and 24 h (T) after AFC. Data are analyzed by two-way ANOVA followed by Šidák's *post hoc* test, $***p < 0.001$. Data are presented as mean $\pm$ SEM. Dots represent individual mice.

Fig. S14

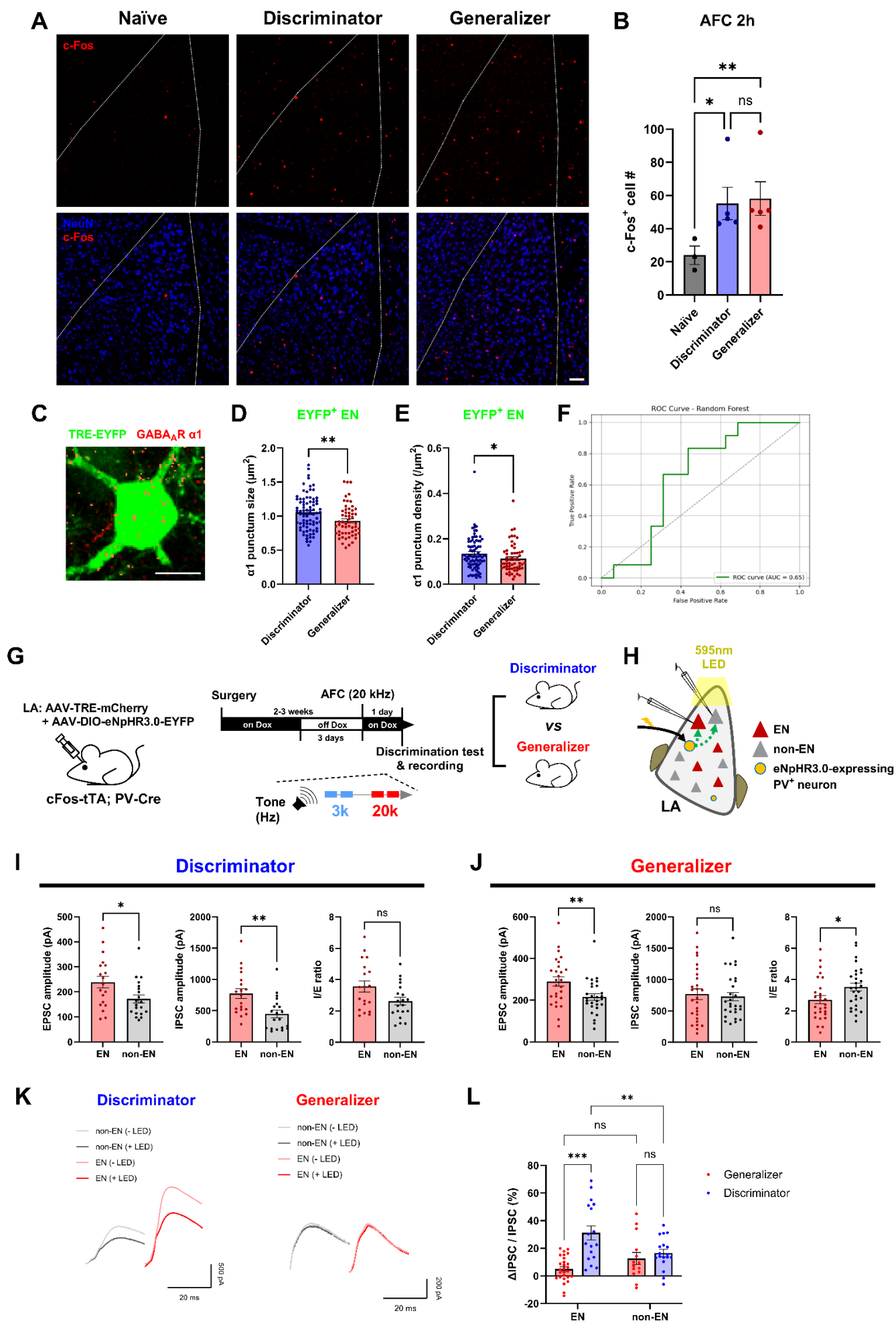


Fig. S14. AFC-induced potentiation of PV-FFI to ENs is absent in generalizers.

(A) Representative fluorescence images showing c-Fos expression (red) and NeuN-labeled neurons (blue) in naïve C57BL/6J mice and in discriminators and generalizers 2 h after AFC. AFC-trained C57BL/6J mice underwent a fear discrimination test 2 h after AFC and were classified as discriminators or generalizers. Mice were sacrificed immediately after the discrimination test for c-Fos analysis. Scale bar, 50 μm . (B) Numbers of c-Fos⁺ neurons in the LA per 0.2 mm² in naïve mice ($N = 3$), discriminators ($N = 5$), and generalizers ($N = 5$). Each dot represents the mean value obtained from an individual mouse. Kruskal-Wallis test followed by Dunn's *post hoc* test. (C) Representative image showing GABA_AR $\alpha 1$ (red) and EYFP-labeled ENs (green) in cFos-tTA mice injected with AAV-TRE-EYFP. Mice underwent AFC during a DOX-off period and a fear discrimination test one day after AFC, after which they were classified as discriminators or generalizers and then sacrificed. EYFP⁺/GABA_AR $\alpha 1$ ⁺ double-positive puncta in the perisomatic regions of EYFP-labeled ENs are highlighted in pseudocolor orange. Scale bar, 10 μm . (D, E) Quantification of GABA_AR $\alpha 1$ ⁺ punctum size (D) and density (E) in ENs of discriminators ($n = 80$ cells from $N = 5$ mice) and generalizers ($n = 57$ cells from $N = 4$ mice). Mann-Whitney U test. (F) Receiver operating characteristic (ROC) curve from a random forest classifier trained to distinguish discriminators from generalizers based on GABA_AR $\alpha 1$ ⁺ punctum size and density in ENs. (G) Schematic of the AAV injection strategy and experimental timeline. (H) Schematic of thalamic stimulation-evoked IPSC recordings from ENs and non-ENs during optogenetic suppression of PV-INs. (I) EPSC amplitudes (left), IPSC amplitudes (middle), and I/E ratios (right) in ENs ($n = 19$ cells from $N = 3$ mice) and non-ENs ($n = 20$ cells from $N = 3$ mice) of discriminators. Mann-Whitney U test. (J) Same measurements as in I, but in generalizers: ENs ($n = 27$ cells from $N = 4$ mice), and non-ENs ($n = 28$ cells from $N = 4$ mice). Mann-Whitney U test. (K) Representative IPSC traces from ENs and non-ENs of discriminators and generalizers before and during PV-IN suppression. (L) Relative contribution of PV-FFI to total FFI, quantified as $\Delta\text{IPSC}/\text{IPSC}$ (%), in ENs and non-ENs of discriminators ($n = 17$ and 18 cells, respectively, from $N = 3$ mice) and generalizers ($n = 27$ and 14 cells, respectively, from $N = 4$ mice). Mixed-effects model followed by Fisher's *post hoc* test. Data are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. S15

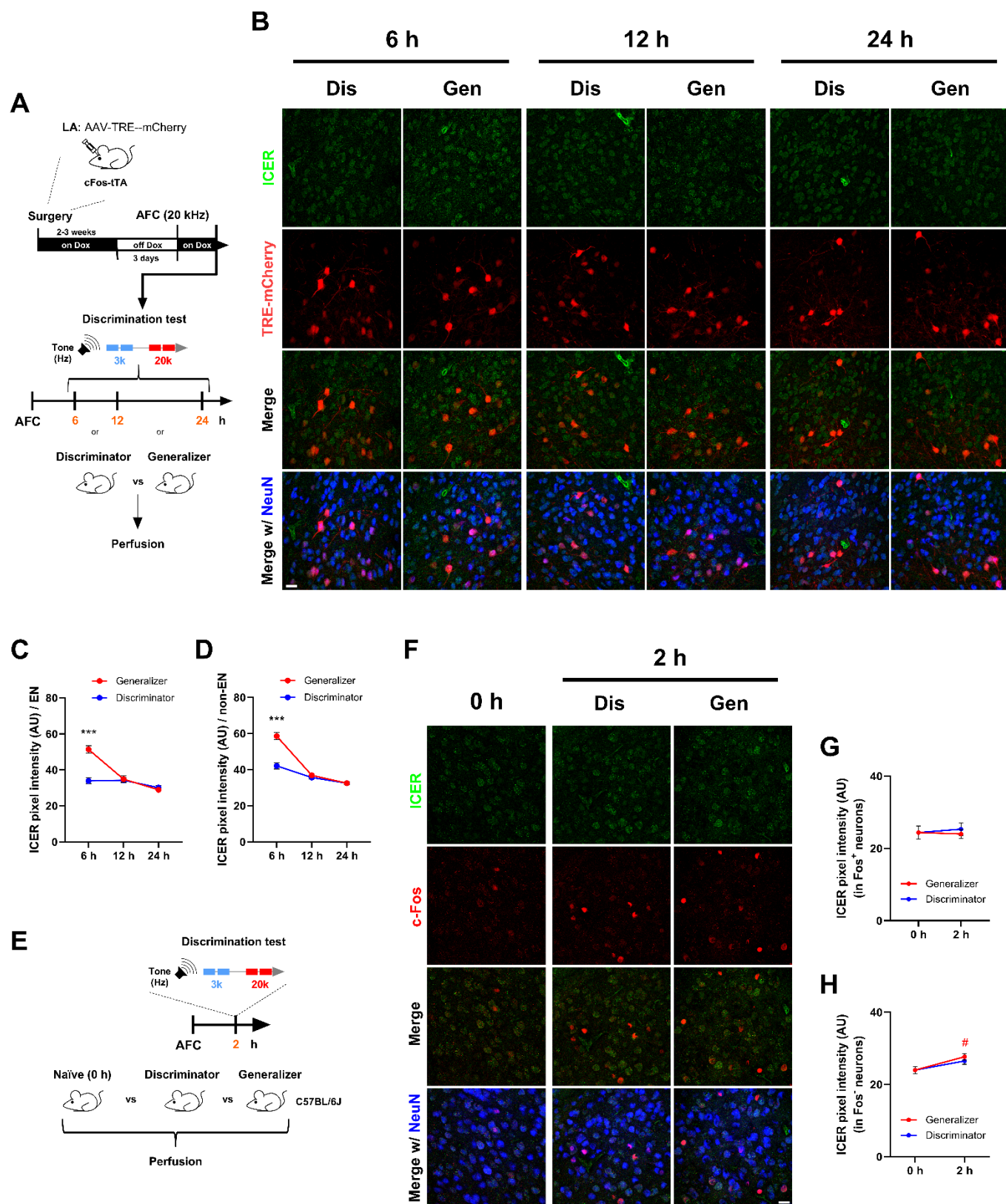


Fig. S15. Temporal profile of ICER protein expression in the LA before and after AFC.

(A) Experimental timeline and analysis design for quantifying LA ICER expression in AFC-tagged ENs and non-ENs at 6, 12, or 24 h after AFC. (B) Representative images showing NeuN (blue), ICER (green), and mCherry⁺ ENs (red) in discriminators (Dis) and generalizers (Gen) at 6, 12, and 24 h after AFC. Scale bar, 20 μ m. (C) Quantification of ICER pixel intensity in mCherry⁺ ENs at 6, 12, and 24 h after AFC (6 h: Dis, $n = 39$ cells; Gen, $n = 43$ cells; 12 h: Dis, $n = 38$ cells; Gen, $n = 41$ cells; 24 h: Dis, $n = 37$ cells; Gen, $n = 40$ cells; $N = 5$ mice per group at each timepoint). Mixed-effects model followed by Šidák's *post hoc* test, *** $p < 0.001$

comparing Dis vs. Gen. **(D)** Quantification of ICER pixel intensity in mCherry⁻ non-ENs at 6, 12, and 24 h after AFC (6 h: Dis, $n = 79$ cells; Gen, $n = 59$ cells; 12 h: Dis, $n = 93$ cells; Gen, $n = 95$ cells; 24 h: Dis, $n = 107$ cells; Gen, $n = 113$ cells; $N = 5$ mice per group at each timepoint). Mixed-effects model followed by Šídák's *post hoc* test, *** $p < 0.001$ comparing Dis vs. Gen. **(E)** Experimental timeline and analysis design for quantifying LA ICER expression in naïve C57BL/6J mice at 0 h and in Dis and Gen C57BL/6J mice 2 h after AFC using c-Fos IHC. **(F)** Representative images showing NeuN (blue), ICER (green), and c-Fos⁺ neurons (red) in 0 h naïve controls and in Dis and Gen mice 2 h after AFC. Scale bar, 20 μ m. **(G)** Quantification of ICER pixel intensity in c-Fos⁺ neurons from 0 h naïve controls and from Dis and Gen mice 2 h after AFC (0 h: $n = 21$ cells, $N = 3$ mice; 2 h: Dis, $n = 39$ cells, $N = 5$ mice; Gen, $n = 52$ cells, $N = 5$ mice). Mixed-effects model followed by Šídák's *post hoc* test. **(H)** Quantification of ICER pixel intensity in c-Fos⁻ neurons from 0 h naïve controls and from Dis and Gen mice 2 h after AFC (0 h: $n = 120$ cells, $N = 3$ mice; 2 h: Dis, $n = 107$ cells, $N = 5$ mice; Gen, $n = 103$ cells, $N = 5$ mice). Mixed-effects model followed by Šídák's *post hoc* test, # $p < 0.05$ comparing 0 h vs. 2 h Gen. Data are presented as mean $\pm$ SEM.

Fig. S16

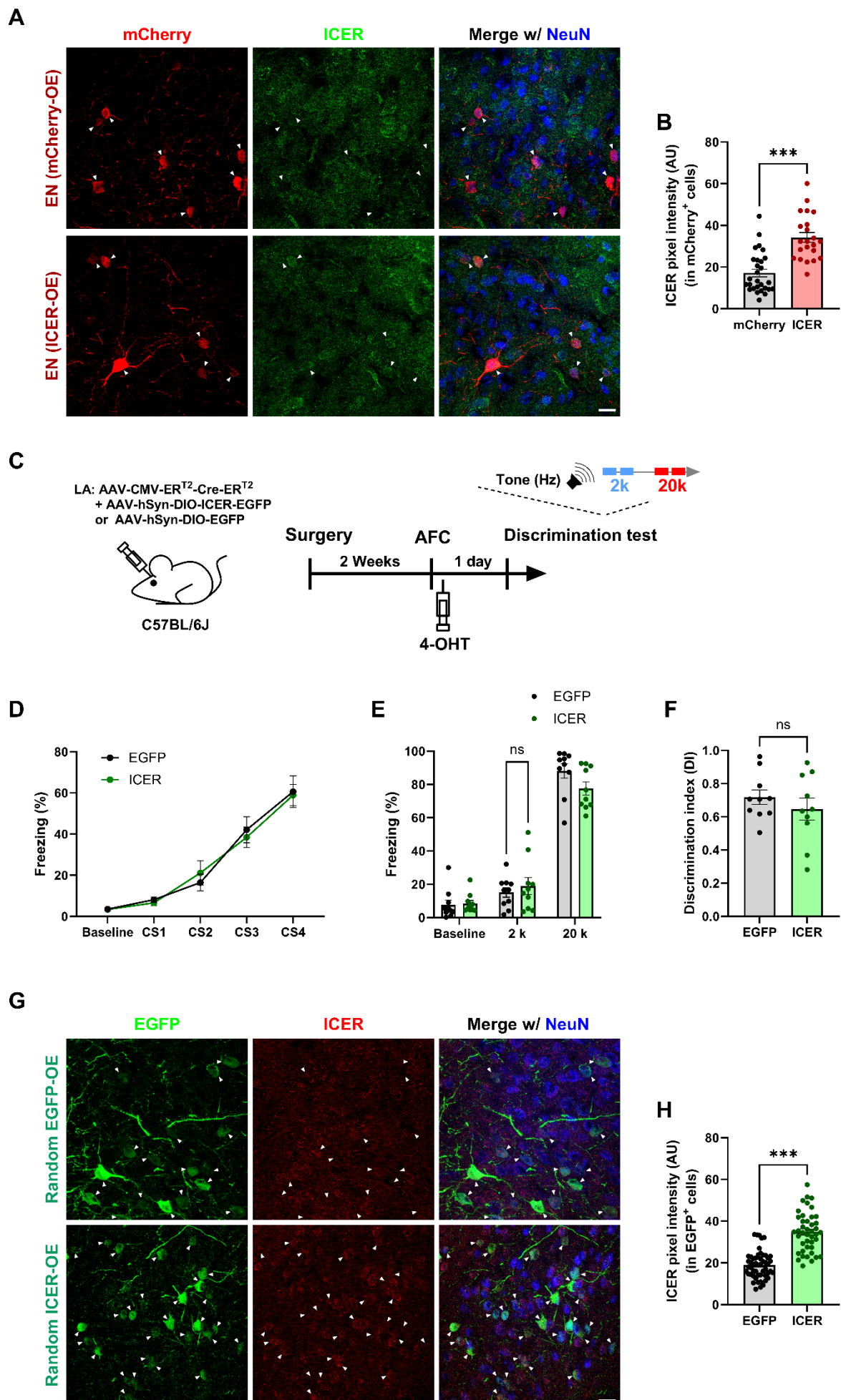
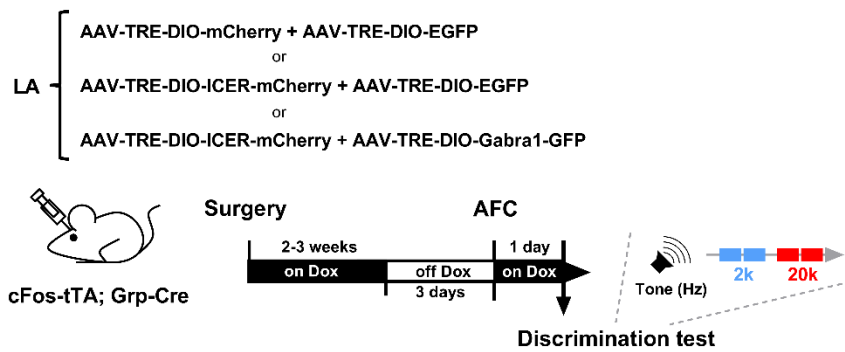


Fig. S16. Validation of EN-specific and random ICER overexpression in the LA.

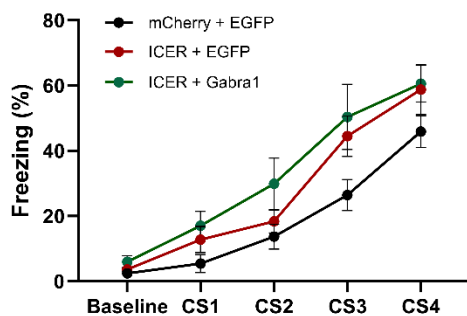
(A) Representative images showing ICER expression (green) in mCherry-labeled ENs (red) from control mCherry-OE mice (top) and ICER-OE mice (bottom). NeuN (blue) was counterstained. Scale bar, 20 μ m. (B) Quantification of ICER pixel intensity in mCherry-labeled ENs from mCherry-OE ($n = 28$ cells from $N = 3$ mice) and ICER-OE ($n = 22$ cells from $N = 3$ mice) groups. Mann-Whitney U test. (C) Schematic of the AAV injection strategy and experimental timeline for random ICER overexpression in the LA. (D) Freezing levels during AFC in mice with random ICER or EGFP overexpression in the LA ($N = 10$ mice per group). (E) Freezing levels during baseline and presentations of the novel 2 kHz tone and 20 kHz CS in ICER- or EGFP-overexpressing mice. Two-way ANOVA followed by Šídák's *post hoc* test. (F) DI between the 2 and 20 kHz tones in ICER- and EGFP-overexpressing mice. Mann-Whitney U test. (G) Representative images showing ICER expression (red) in EGFP-labeled AAV-transduced LA neurons (green) from control EGFP-OE mice (top) and ICER-OE mice (bottom). NeuN (blue) was counterstained. Scale bar, 20 μ m. (H) Quantification of ICER pixel intensity in EGFP-labeled AAV-transduced LA neurons from EGFP-OE ($n = 51$ cells from $N = 3$ mice) and ICER-OE ($n = 41$ cells from $N = 3$ mice) groups. Mann-Whitney U test. Data are presented as mean $\pm$ SEM. *** $p < 0.001$.

Fig. S17

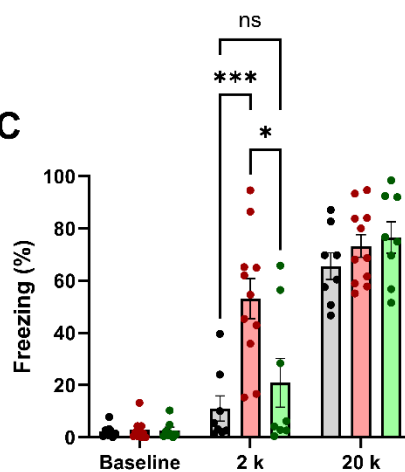
A



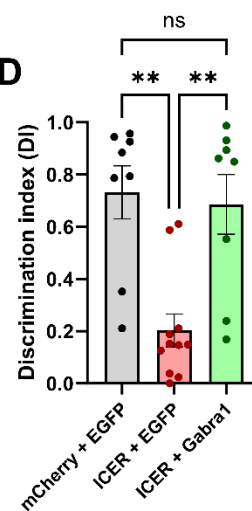
B



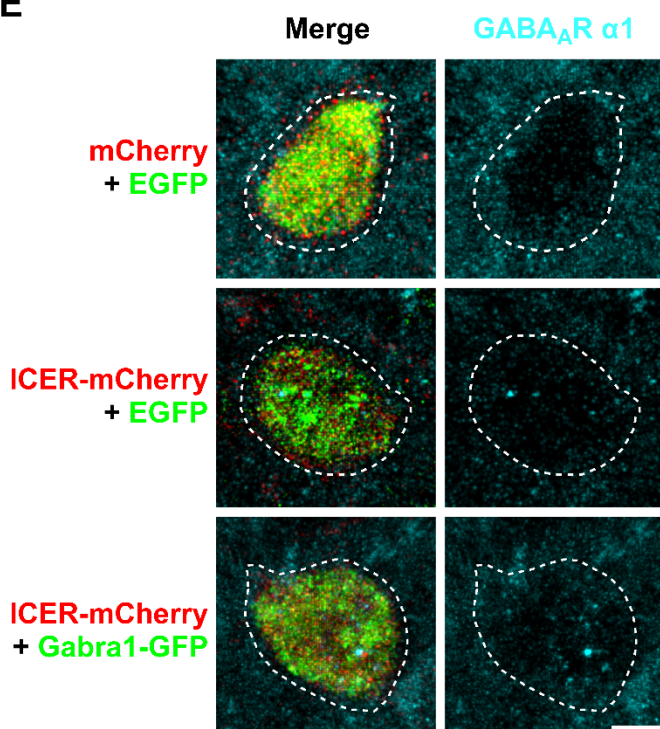
C



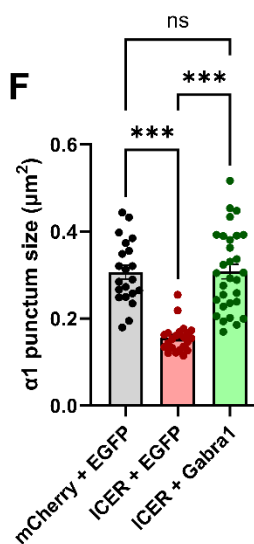
D



E



F



G

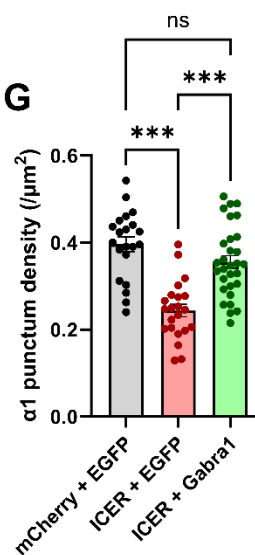


Fig. S17. Gabra1-mediated rescue of ICER-induced fear overgeneralization.

(A) Schematic of the AAV injection strategy and experimental timeline. (B) Freezing levels during AFC in mice expressing control mCherry and EGFP (mCherry+EGFP; black, $N = 8$ mice), ICER-mCherry and EGFP (ICER+EGFP; red, $N = 11$ mice), or ICER-mCherry and Gabra1-GFP (ICER+Gabra1; green, $N = 8$ mice) in ENs. Data are analyzed using two-way ANOVA followed by Šídák's *post hoc* test. (C) Freezing levels during baseline and presentations of the 2 kHz novel tone and 20 kHz CS in the mCherry+EGFP, ICER+EGFP, and ICER+Gabra1 groups. Each dot represents an individual mouse. Data are analyzed using two-way ANOVA followed by Šídák's *post hoc* test, *** $p < 0.001$, * $p < 0.05$. (D) DI between the 2 and 20 kHz tones in the mCherry+EGFP, ICER+EGFP, and ICER+Gabra1 groups. Data are analyzed using Kruskal-Wallis test followed by Dunn's *post hoc* test, ** $p < 0.01$. (E) Representative images showing mCherry or ICER-mCherry (red), EGFP or Gabra1-GFP (green), and GABA_AR $\alpha 1$ (cyan) in ENs from the mCherry+EGFP, ICER+EGFP, and ICER+Gabra1 groups. Dashed lines indicate the somatic boundaries of ENs. Scale bar, 5 μm . (F, G) Quantification of mean perisomatic GABA_AR $\alpha 1^+$ punctum size (F) and density (G) in ENs from the mCherry+EGFP ($n = 21$ cells from $N = 3$ mice), ICER+EGFP ($n = 22$ cells from $N = 3$ mice), and ICER+Gabra1 ($n = 30$ cells from $N = 3$ mice) groups. Each dot represents an individual analyzed cell. Data are analyzed using Kruskal-Wallis test followed by Dunn's *post hoc* test, *** $p < 0.001$.

Fig. S18

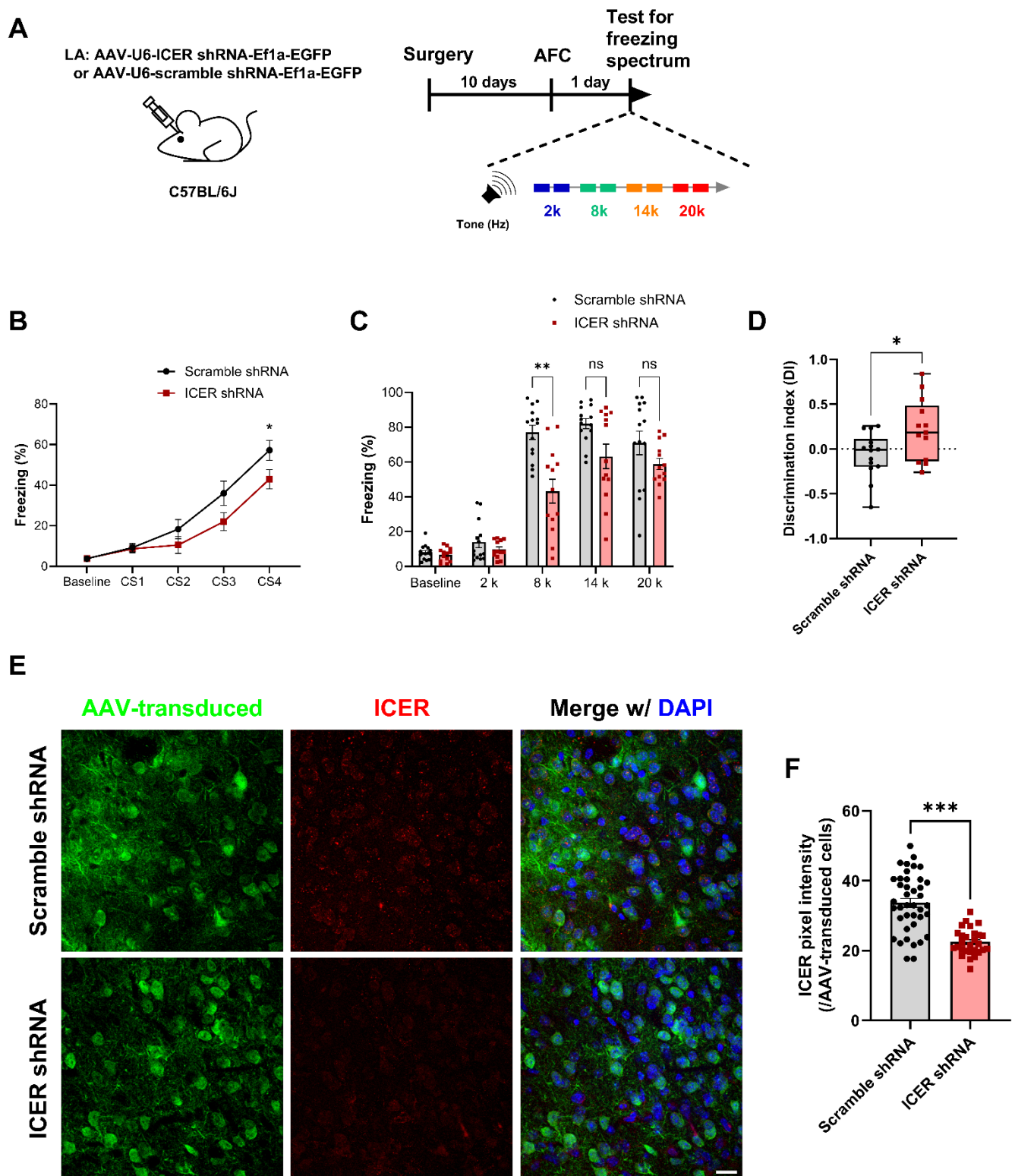


Fig. S18. Depletion of ICER in the LA improves fear discrimination.

(A) Schematic of the AAV injection strategy and experimental timeline. (B) Freezing levels during AFC in mice injected with AAV encoding ICER shRNA ($N = 13$ mice) or scramble control shRNA ($N = 14$ mice). Two-way ANOVA followed by Fisher's *post hoc* test. (C) Freezing levels during baseline and presentations of the 2, 8, 14, and 20 kHz tones in ICER shRNA- or scramble control shRNA-injected mice. Two-way ANOVA followed by Šidák's *post hoc* test. (D) DI between the novel 8 kHz tone and 20 kHz CS in ICER shRNA- or scramble control shRNA-injected mice. Mann-Whitney *U* test. (E) Representative images showing ICER expression (red) in AAV-transduced LA neurons (green) from scramble control shRNA-injected mice

(top) and ICER shRNA-injected mice (bottom). Cell nuclei were stained with DAPI (blue). Scale bar, 20 μm . **(F)** Quantification of ICER pixel intensity in AAV-transduced LA neurons from scramble control shRNA ($n = 40$ cells from $N = 3$ mice) and ICER shRNA ($n = 28$ cells from $N = 3$ mice) groups. Mann-Whitney U test. Data are presented as mean $\pm$ SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Supplementary References

1. Kwon O-B, Lee JH, Kim HJ, Lee S, Lee S, Jeong M-J, et al. Dopamine Regulation of Amygdala Inhibitory Circuits for Expression of Learned Fear. *Neuron*. 2015;88:378–389.
2. Bieganska K, Figiel I, Gieriej D, Kaczmarek L, Klejman A. Silencing of ICERs (Inducible cAMP Early Repressors) results in partial protection of neurons from programmed cell death. *Neurobiol Dis*. 2012;45:701–710.
3. Hochgerner H, Singh S, Tibi M, Skarbianskis N, Admati I, Ophir O, et al. Neuronal types in the mouse amygdala and their transcriptional response to fear conditioning. *Nat Neurosci*. 2023;26:2237–2249.
4. Gomez JL, Bonaventura J, Lesniak W, Mathews WB, Sysa-Shah P, Rodriguez LA, et al. Chemogenetics revealed: DREADD occupancy and activation via converted clozapine. *Science*. 2017;357:503–507.
5. Bonaventura J, Eldridge MAG, Hu F, Gomez JL, Sanchez-Soto M, Abramyan AM, et al. High-potency ligands for DREADD imaging and activation in rodents and monkeys. *Nat Commun*. 2019;10:4627.
6. Piskorowski RA, Chevalayre V. Delta-Opioid Receptors Mediate Unique Plasticity onto Parvalbumin-Expressing Interneurons in Area CA2 of the Hippocampus. *J Neurosci*. 2013;33:14567–14578.
7. Guenther CJ, Miyamichi K, Yang HH, Heller HC, Luo L. Permanent Genetic Access to Transiently Active Neurons via TRAP: Targeted Recombination in Active Populations. *Neuron*. 2013;78:773–784.
8. Kim WB, Cho J-H. Encoding of Discriminative Fear Memory by Input-Specific LTP in the Amygdala. *Neuron*. 2017;95:1129–1146.e5.
9. Grosso A, Santoni G, Manassero E, Renna A, Sacchetti B. A neuronal basis for fear discrimination in the lateral amygdala. *Nat Commun*. 2018;9:1214.
10. Bergstrom HC. Assaying Fear Memory Discrimination and Generalization: Methods and Concepts. *Curr Protoc Neurosci*. 2020;91:e89.
11. Trott JM, Hoffman AN, Zhuravka I, Fanselow MS. Conditional and unconditional components of aversively motivated freezing, flight and darting in mice. *eLife*. 2022;11:e75663.
12. Myagmarsuren S. Exploring the Use of Silhouette Score in K-Means Clustering for Image Segmentation. *Int J Eng Res Technol*. 2024;13.
13. Yulisasih BN, Herman H, Sunardi S, Yuliansyah H. Evaluation of K-Means Clustering Using Silhouette Score Method on Customer Segmentation. *Ilk J Ilm*. 2024;16:330–342.
14. Tang M, Kaymaz Y, Logeman BL, Eichhorn S, Liang ZS, Dulac C, et al. Evaluating single-cell cluster stability using the Jaccard similarity index. *Bioinformatics*. 2021;37:2212–2214.
15. Breiman L. Random Forests. *Mach Learn*. 2001;45:5–32.
16. Simpson JE, Muir MT, Lee M, Naughton C, Gilbert N, Pollard SM, et al. Autophagy supports PDGFRA-dependent brain tumor development by enhancing oncogenic signaling. *Dev Cell*. 2024;59:228–243.e7.
17. Gundersen BB, Briand LA, Onksen JL, LeLay J, Kaestner KH, Blendy JA. Increased Hippocampal Neurogenesis and Accelerated Response to Antidepressants in Mice with Specific Deletion of CREB in the Hippocampus: Role of cAMP Response-Element Modulator τ . *J Neurosci*. 2013;33:13673–13685.
18. Kim SJ, Woo Y, Kim HJ, Goo BS, Nhung TTM, Lee S-A, et al. Retinoic acid-induced protein 14 controls dendritic spine dynamics associated with depressive-like behaviors. *eLife*. 2022;11:e77755.

19. Glazewski S, Bejar R, Mayford M, Fox K. The effect of autonomous alpha-CaMKII expression on sensory responses and experience-dependent plasticity in mouse barrel cortex. *Neuropharmacology*. 2001;41:771–778.
20. Heinz N, Hennig K, Loew R. Graded or threshold response of the tet-controlled gene expression: all depends on the concentration of the transactivator. *BMC Biotechnol*. 2013;13:5.