# Supplemental Figures

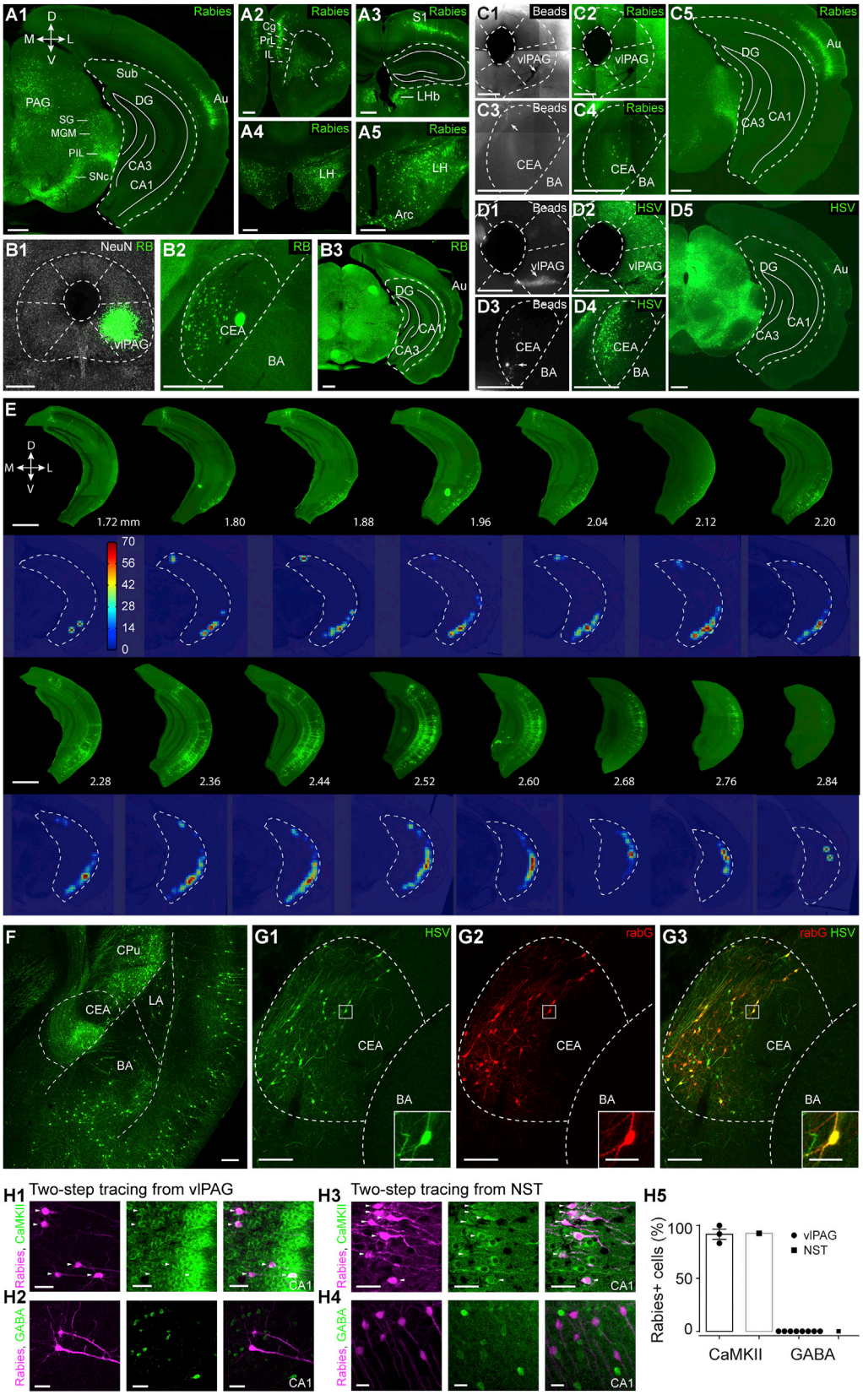


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**Figure S1. Related to Figures 1 and 2**

(A–D) Non *trans*-synaptic rabies tracing from vlPAG. Scale bars, 0.5 mm.

(A1–A5) Examples showing rabiesΔG-mCherry-labeled cells in various brain areas (in addition to CEA shown in Figure 1B) by non *trans*-synaptic retrograde rabies tracing from vlPAG (local injection of rabiesΔG-mCherry and beads into vlPAG).

(B1–B3) Examples showing local injections of retrobeads (RB) into vlPAG (B1) and bead-labeled cells in CEA (B2) other brain areas (B3). Consistent with non *trans*-synaptic rabies tracing (A1), no cells in vHC were labeled (B3).

(C1–C5) Examples showing local injections of rabiesΔG-mCherry and beads (arrow) into vlPAG (C1 – C2), AAV-DIO-G and beads into CEA (C3, arrow) and rabies-labeled cells in CEA (C4) and other brain areas (C5). No cells in vHC were labeled.

(D1–D5) Examples showing local injections of HSV-Cre-Venus and beads (arrow) into vlPAG (D1 – D2), AAV-DIO-G and beads into CEA (D3, arrow) and HSV-labeled cells in CEA (D4) and other brain areas (D5). No cells in vHC were labeled.

Abbreviations: vlPAG, ventrolateral periaqueductal gray; SG, supragenicular thalamic nucleus; MGM, medial geniculate nucleus, medial part; PIL, posterior intralaminar thalamic nucleus; SNc, substantia nigra, pars compacta; DG, dentate gyrus; CA3, field CA3 of hippocampus; CA1, field CA1 of hippocampus; Au, auditory cortex; Cg, cingulate cortex; PrL, prelimbic cortex; IL, infralimbic cortex; S1, primary somatosensory cortex; LHb, lateral habenular nucleus; LH, lateral hypothalamic area; Arc, arcuate hypothalamic nucleus. M: median; L: lateral; D: dorsal; V: ventral.

(E) Example pictures showing vHC cells labeled by *trans*-synaptic rabies tracing from vlPAG-projecting CEA output neurons. Consecutive coronal sections are sorted from anterior to posterior coordinates. Each section is 80 μm thick and its relative distance to the anterior tip of the dorsal hippocampus (Bregma −0.94 mm) is indicated. The corresponding cell density map is shown below each section. Scale bars, 1 mm.

(F) Example pictures showing that cells in BLA and caudate putamen (CPu) were labeled by *trans*-synaptic rabies tracing from vlPAG-projecting CEA output neurons. Scale bar, 0.1 mm.

(G1–G3) To validate Cre-dependent expression of rabies glycoprotein (rabG), HSV-Cre-Venus was injected into vlPAG and AAV-DIO-G was injected into CEA. Examples pictures showing HSV-Cre-Venus-labeled cells (G1) and rabG immunostaining (G2) in CEA. 97% of rabG-stained cells are co-labeled by HSV, suggesting faithful expression of rabG (G3). Insets are the magnification of boxed areas. Scale bars, 200 μm. Scale bars in insets: 50 μm.

(H) Immunohistochemical analysis of rabies-labeled cells in vHC. Scale bars, 50 μm.

(H1) Example pictures showing immunostaining for CaMKII and rabies labeling in vHC upon *trans*-synaptic tracing from vlPAG-projecting neurons in CEA. Arrows indicate co-localization of CaMKII and rabies labeling.

(H2) Example pictures showing immunostaining for GABA and rabies labeling in vHC upon *trans*-synaptic retrograde tracing from vlPAG-projecting neurons in CEA.

(H3) Examples of immunostaining for CaMKII and rabies labeling in vHC upon *trans*-synaptic retrograde tracing from CEA output neurons targeting the nucleus of the solitary tract (NST). Arrows indicate co-localization of CaMKII and rabies labeling.

(H4) Example pictures showing immunostaining for GABA and rabies labeling in vHC upon *trans*-synaptic retrograde tracing from NST-projecting neurons in CEA.

(H5) Summary plot (mean ± SEM) illustrating the percentage of rabies-labeled cells co-staining for CaMKII or GABA in the hippocampus. Two-step tracing from vlPAG: circles, CaMKII, 91.78% ± 4.81%, n = 3 animals; GABA, 0% ± 0%. n = 8 animals. Two-step tracing from NST: squares, CaMKII, 92.59%, n = 1 animal; GABA, 0%, n = 1 animal.

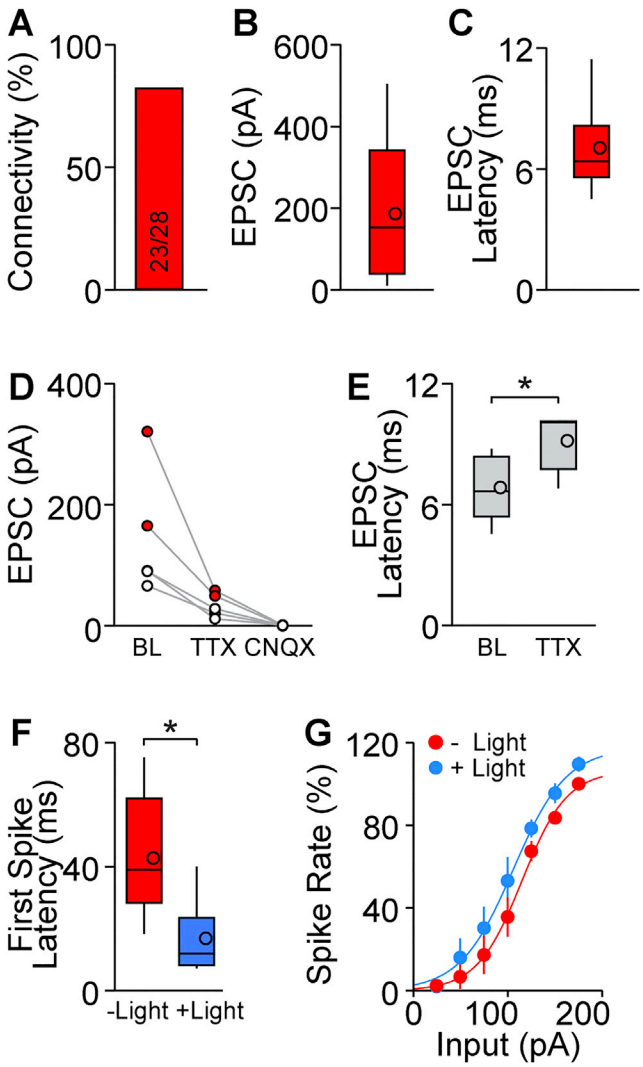



**Figure S2. Recordings from RB+ Neurons in CEA, Related to Figure 3**

(A) Connectivity from vHC afferents to RB+ CEA neurons was 82.1% (23 out of 28 cells).

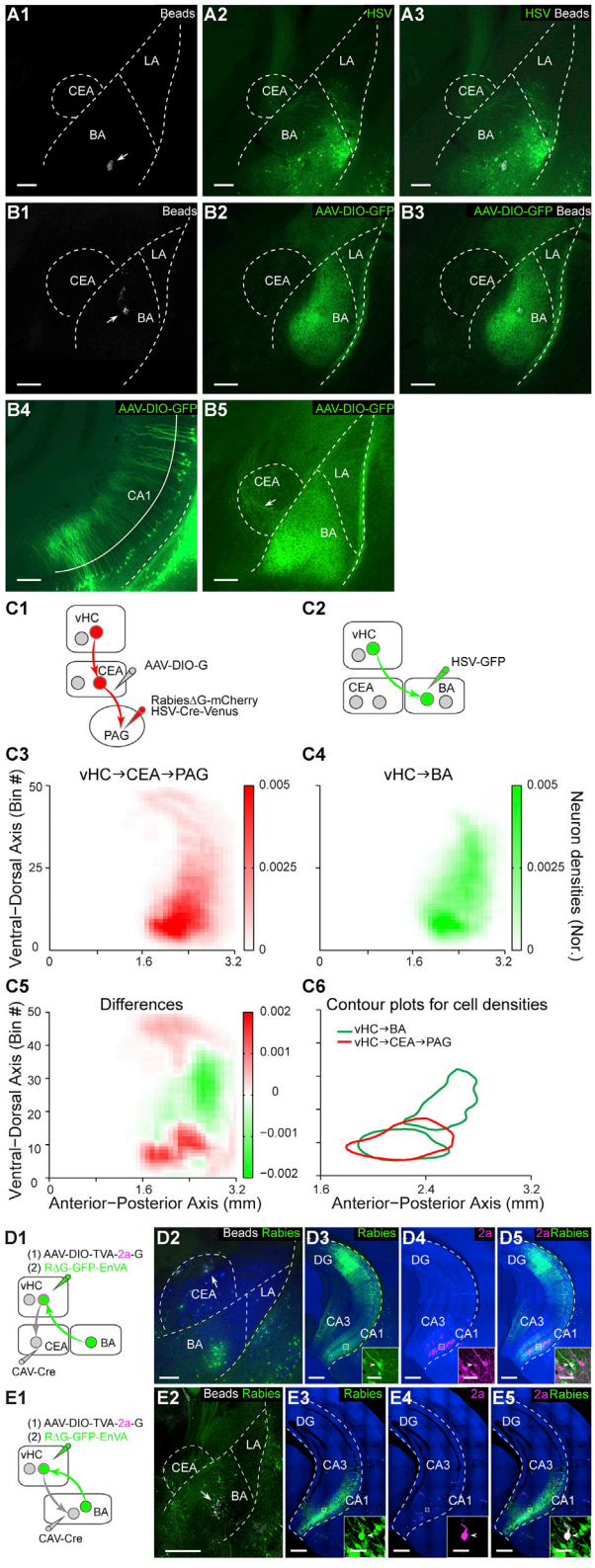
(B and C) Summary of EPSC amplitudes and EPSC peak latencies in RB+ neurons recorded in CEA. Data are presented as median with 25/75 percentile (box) and 10-90 percentile (whiskers). The circle indicates mean; n = 14 cells.

(D) Light-evoked EPSC amplitudes recorded from CEA neurons during baseline condition (BL), bath application of TTX and CNQX. Red circles are from RB+ neurons. The normalized results are shown in Figure 3I.

(E) The EPSC peak latencies recorded from CEA neurons (same neurons as in D) during BL and bath application of TTX.

(F) Latency to the first action potential at a current injection of +100 pA is significantly shorter with vHC fiber activation. (n = 7 cells; Wilcoxon matched-pairs signed rank test, p = 0.0156).

(G) vHC fiber photostimulation increases the excitability of CEA neurons. Spike rate is normalized to the maximum frequency in baseline condition of each cell. Input-output curves are shifted to the left by −11.9 pA with vHC fiber activation ($I_{half}$ baseline 106.1 pA, $I_{half}$ light 94.3 pA; paired t test, p = 0.0697; n = 7 cells). Data are presented as mean ± SEM, n = 7 cells.




**Xu et al. Figure S3**

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**Figure S3. Related to Figure 4**

(A and B) En-passant BA-projecting axons in CEA. Scale bars, 200 μm.

(A1–A3) Example pictures showing BA-projecting axons passing through CEA. The retrograde axon tracing from BA was done by co-injection of blue beads (A1, arrow) and HSV-GFP (A2) in BA. The overlay is shown in (A3).

(B1–B5) Example pictures showing BA-projecting axons passing through CEA (B5, arrow). The BA-projecting axons from vHC were labeled by co-injection of blue beads (B1, arrow) and CAV-Cre (canine adenovirus type 2 expressing Cre-recombinase) into BA (B2 and B3) and AAV2/1-CAG-DIO-GFP into vHC (B4).

(C) Spatial distribution of CEA- and BA-projecting neurons in hippocampus.

(C1 and C2) Scheme illustrating the experimental design used for retrograde tracing of the vHC → CEA pathway (C1) and the vHC → BA pathway (C2).

(C3 and C4) Density maps for CEA-projecting and BA-projecting vHC neurons along the anterior-posterior hippocampal axis. Each coronal section of hippocampus is divided into 50 bins along the ventral-dorsal axis (y axis) of the hippocampus. Cell numbers in each bin were normalized to the total number of vHC neurons labeled in each brain sample and were color-coded to reflect cell density. Density maps were averaged across different animals (vHC → CEA pathway, 92% ± 1% in CA1 and 8% ± 1% in Subiculum; n = 8; vHC → BA pathway, 95% ± 1% in CA1; 5% ± 1% in Subiculum; n = 5; Mann Whitney U test for ratios of CA1 neurons between two pathways, p = 0.09). No cells were labeled in dorsal HC.

(C5) Plot illustrating difference (C3 minus C4) between density maps shown in (C3) and (C4). vHC neurons projecting to CEA are located more ventrally than BA-projecting neurons.

(C6) Contour plots displaying isolines based on highest density in maps shown in (C3) and (C4).

(D and E) *Trans*-synaptic rabies tracing from BA- or CEA-projecting vHC neurons.

(D1) Injection scheme illustrating experimental design for *trans*-synaptic retrograde tracing from vHC neurons projecting to CEA. CAV-Cre and beads were co-injected into CEA and AAV-DIO-TVA-2a-G is injected into vHC for Cre-dependent expression of TVA, G (glycoprotein) and the 2a tag (Yonehara et al., 2013). Two weeks later, RabiesΔG-GFP-EnVA was injected into vHC.

(D2) Example picture illustrating labeling in the BA after *trans*-synaptic rabies tracing from CEA-projecting vHC neurons (342 and 186 cells, n = 2 animal). The injection site in CEA is indicated by beads (arrow).

(D3–D5) Example pictures showing rabies-labeled cells (green) and 2a immunostaining (magenta, rabbit anti-2a, Millipore ABS31, 1:500; Donkey anti-Rabbit 647, Invitrogen A31573, 1:500) in vHC. Blue background is the transmission image. Insets are magnifications of the boxed area in which a starter cell (arrow) was identified.

(E1) Injection scheme illustrating experimental design for *trans*-synaptic retrograde tracing from vHC neurons projecting to BA. CAV-Cre and beads are co-injected into BA and AAV-DIO-TVA-2a-G is injected into vHC. Two weeks later, RabiesΔG-GFP-EnVA is injected into vHC.

(E2) Example picture illustrating sparse labeling in the BA after *trans*-synaptic rabies tracing from BA-projecting vHC neurons (65 ± 35 cells, n = 4 animals). The injection site in the BA is indicated by beads (arrow).

(E3–E5) Example pictures showing rabies-labeled cells and 2a immunostaining in vHC. Insets are magnifications of the boxed area in which a starter cell (arrow) was identified.

Scale bars, 0.5 mm; insets, 0.02 mm.

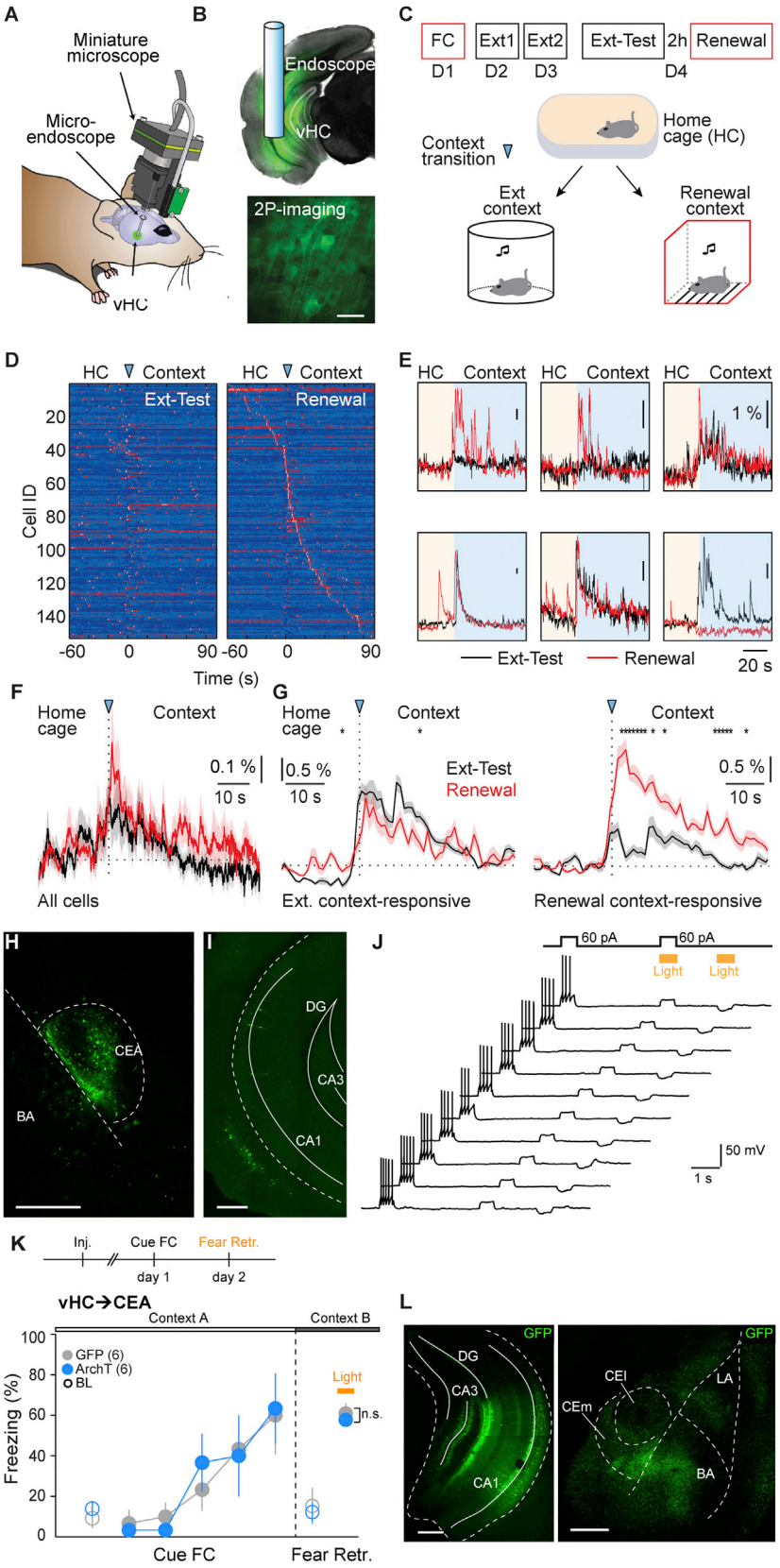


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**Figure S4. Related to Figures 5 and 6**

(A–G) Deep brain $Ca^{2+}$ imaging of vHC activity upon home cage to context transition.

(A) Scheme showing combined miniature microscope and endoscope imaging of vHC in freely moving animals.

(B) Top, AAV2/5-CaMKII-GCaMP6f expression and microendoscope (GRIN) lens position in vHC. Bottom, two-photon microscopy in vivo image of GCaMP6f-expressing neurons in vHC via the GRIN lens. Scale bar, 20 μm.

(C) Experimental paradigm to measure context activation of vHC. Animals were trained with a similar fear renewal paradigm as shown in Figure 6A. vHC activity was analyzed on Day 4 upon context transition from home cage (HC) to extinction test context or HC to renewal context.

(D) Normalized population activity before and after context transition for extinction test and renewal (session-to-session matched neurons, n = 153 out of 3 animals). Cells were sorted based on the timing of peak $Ca^{2+}$ fluorescence during renewal, demonstrating a context-specific temporal ensemble pattern in vHC. Time 0 s (blue triangle) indicates the time point at which the animal first touches the context.

(E) $Ca^{2+}$ traces of six example neurons upon the context transition from HC to the extinction test context (black) and from HC to the renewal context (red).

(F) Averaged population response of session-matched cells (same as in D) upon context transition.

(G) Population $Ca^{2+}$ responses (mean ± SEM, 1 s time bin) of context-responsive neurons before and after context transition. Context-responsive neurons were defined by a significantly increased mean $Ca^{2+}$ response before and after context transition (1 s bins, 60 s before versus after context transition; Wilcoxon signed-rank test). Left: On average, extinction context-responsive neurons show similar context transition-evoked $Ca^{2+}$ responses in the renewal context compared to the extinction context (n = 45 cells, 30% ± 2% activated neurons/animal, N = 3). Right: Renewal context-responsive neurons show larger $Ca^{2+}$ responses upon the transition in the renewal context compared to the extinction context (n = 50 cells, 38% ± 9% activated neurons/animal, N = 3). Significance is determined by paired Wilcoxon signed-rank test with Benjamini-Hochberg correction for repeated-measures; * = $p < 0.05$.

(H and I) Examples of rabiesΔG-ArchT-GFP infections in CEA (A) and vHC (B) after local injection into CEA. RabiesΔG-ArchT-GFP infects local neurons in CEA, but very few neurons in vHC. Scale bars, 400 μm.

(J) Top, scheme showing the whole-cell recording protocol and light stimulation. Bottom, raw traces of whole-cell recording for the example recording shown in Figure 5B. Three tests were performed in each trial. The first test is that the current injection (−60 pA) evoked a train of spikes, the second test is that the current-evoked spikes were reliably inhibited by light stimulation, and the last test is that the light stimulation only elicited hyperpolarization.

(K) vHC→CEA pathway is not necessary for the retrieval of non-extinguished CS fear. Top, scheme illustrating the behavioral paradigm used to test the functional role of the vHC→CEA pathway in the retrieval of non-extinguished CS fear. During the fear retrieval session (Fear Retr.), the entire CS (tone) was paired with yellow light stimulation (yellow bar) targeted to the vHC. Bottom, summary data (mean ± SEM) of CS-induced freezing during tone fear conditioning (Cue FC) and subsequent CS fear retrieval (Fear Retr.). Freezing in all sessions is shown for single CSs. Open circles indicate 2 min baseline (BL) freezing for each behavioral session. Inhibition of the vHC→CEA pathway does not affect CS fear retrieval without extinction (control, gray; 62.2% ± 5.1%, n = 6; ArchT, blue; 58.9% ± 3.2%, n = 6; Mann-Whitney U test, $p = 0.426$).

(L) vHC axonal distribution in amygdala. Left, example picture showing AAV2/9-CMV-GFP injection in vHC. Right, example picture showing axonal tracings in amygdala. Scale bars, 400 μm.

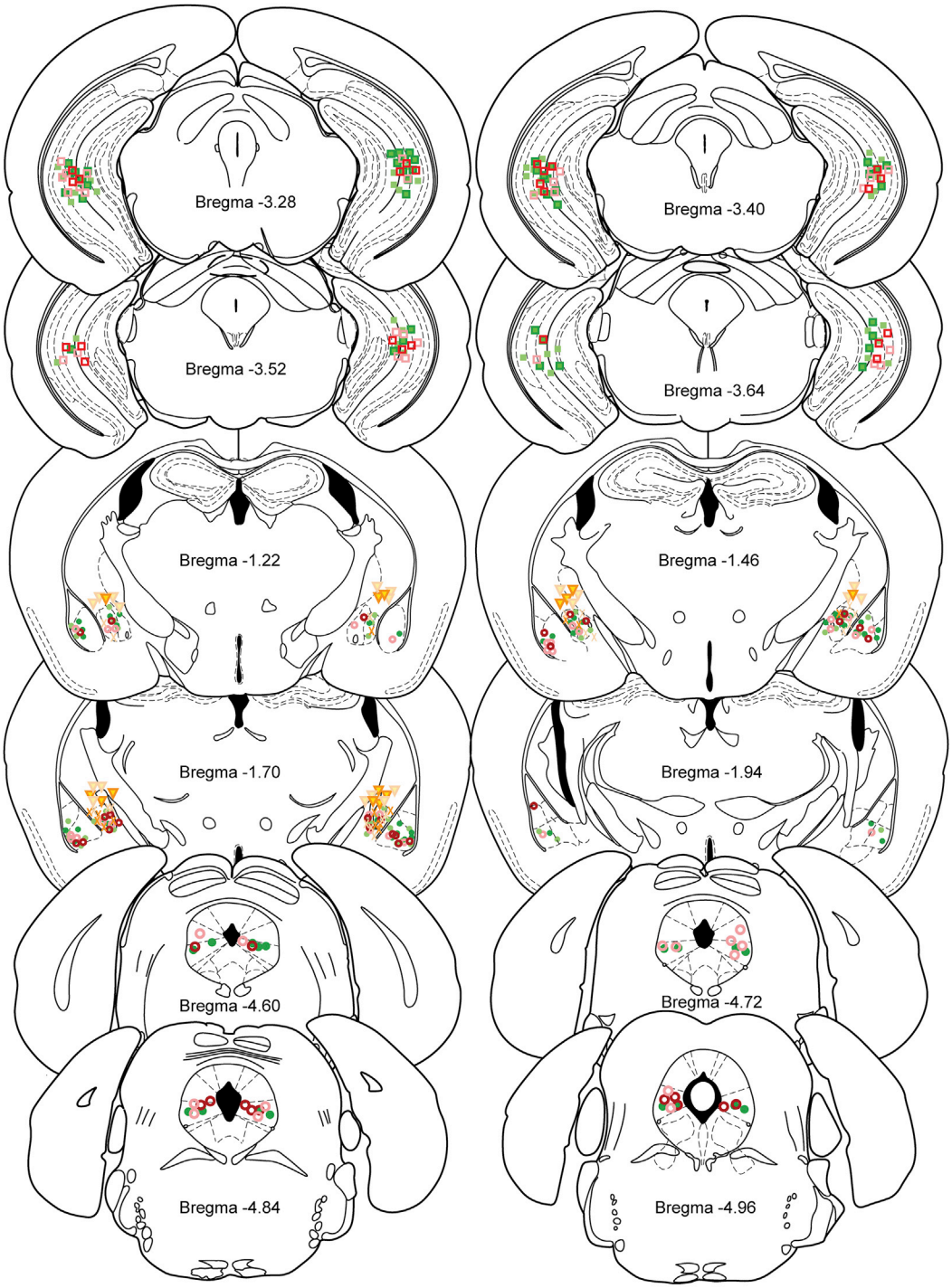


**Figure S5. Optical Fiber Placements and Injection Sites for Behavioral Experiments, Related to Figures 5 and 6**

Positions of implanted optical fiber tips (squares and triangles) and injection sites (circles and crosses) in animals with optogenetic manipulations during fear renewal are shown in green and yellow, and those in animals with optogenetic manipulations during contextual fear retrieval are shown in red.

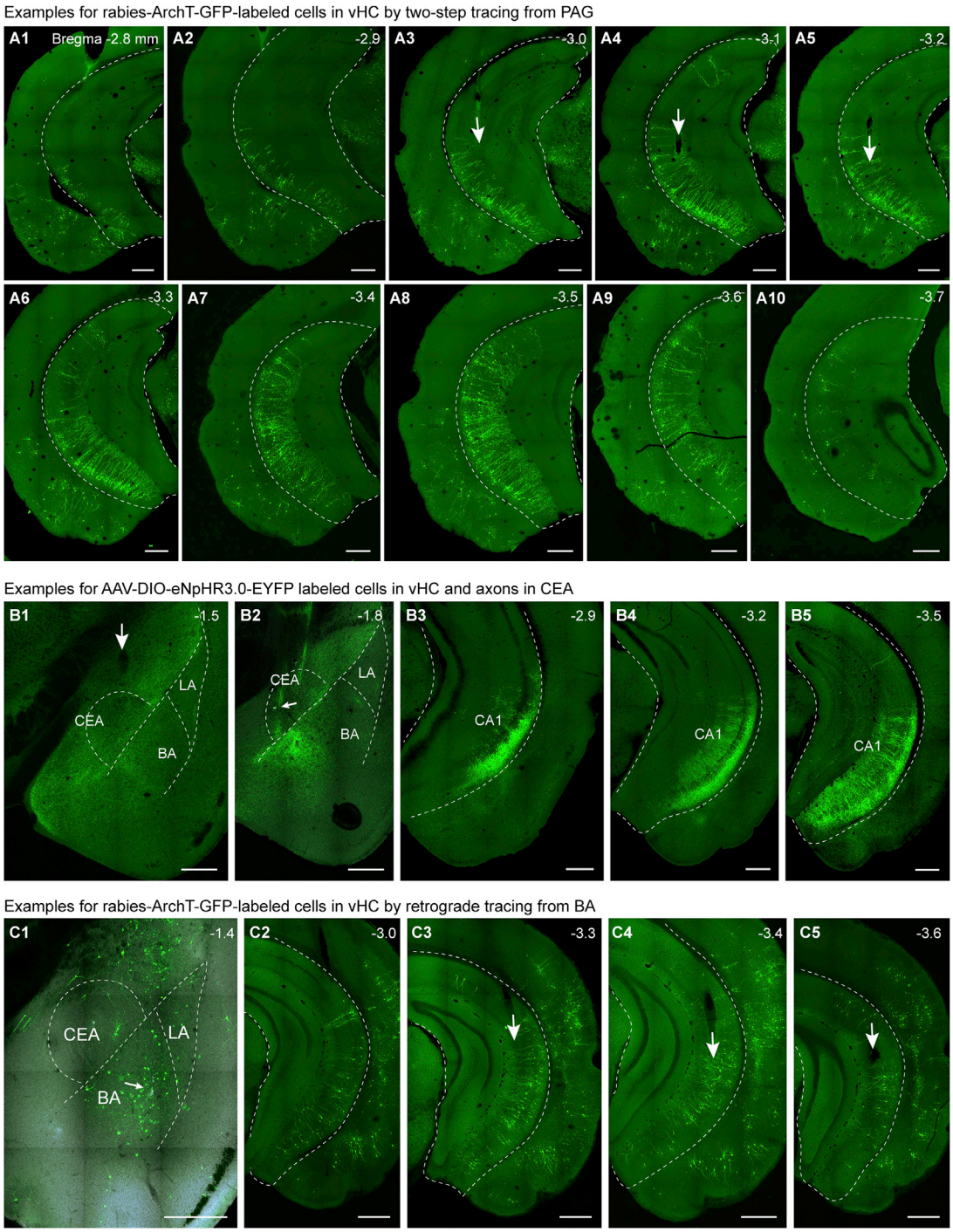


**Figure S6. Histology Examples for Behavior Experiments with Optogenetics, Related to Figures 5 and 6**

(A1–A10) Examples for vHC cells labeled by rabiesΔG-ArchT-GFP after transsynaptic tracing from PAG-projecting CEA neurons. The arrows indicate optical fiber tracks. Unlike rabiesΔG-mCherry and rabiesΔG-GFP, rabiesΔG-ArchT-GFP primarily label vHC neurons but very few APir neurons (also see C1 – C5).

(B1–B5) Examples for eNpHR3.0-EYFP labeled cells in vHC and axons in CEA. The arrow in B1 indicates optical fiber track. The CAV-Cre and blue beads were co-injected into CEA (arrow in B2, beads shown in gray). Scale bars, 0.5 mm.

(C1–C5) Examples for vHC cells labeled by rabiesΔG-ArchT-GFP after transsynaptic tracing from the BA. The arrow in C1 indicates co-injected beads (gray). The arrows in C3 – C5 indicate optical fiber tracks.