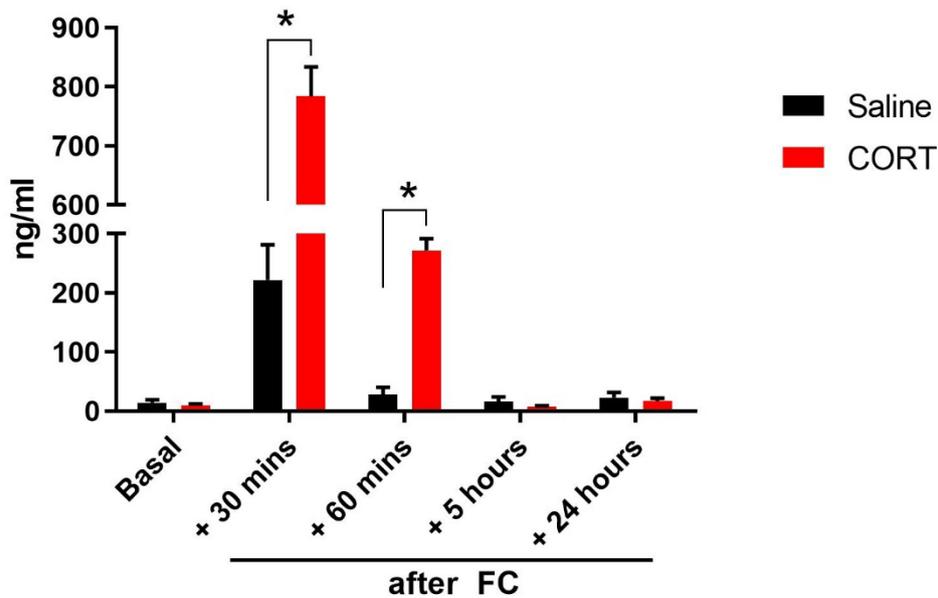


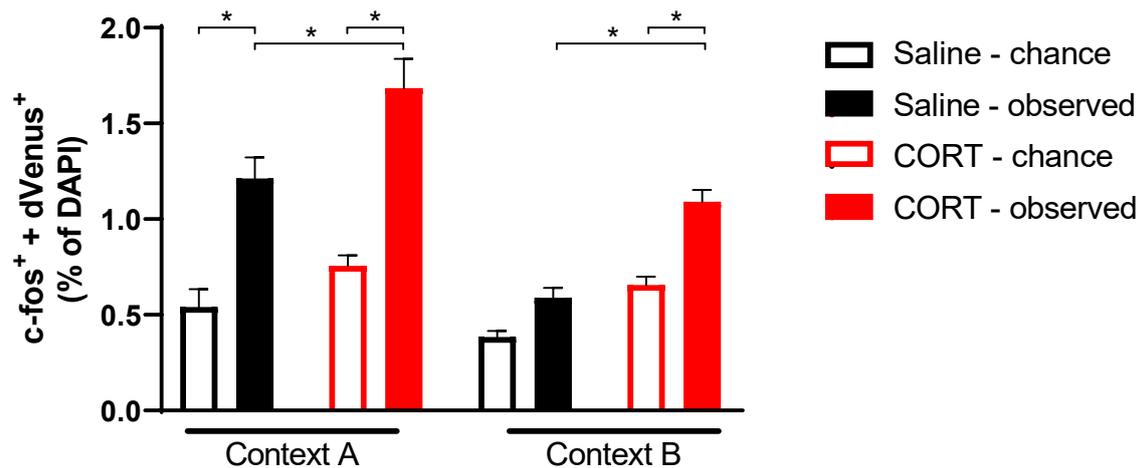
Glucocorticoids Promote Fear Generalization by Increasing the Size of a Dentate Gyrus Engram Cell Population

Supplemental Information

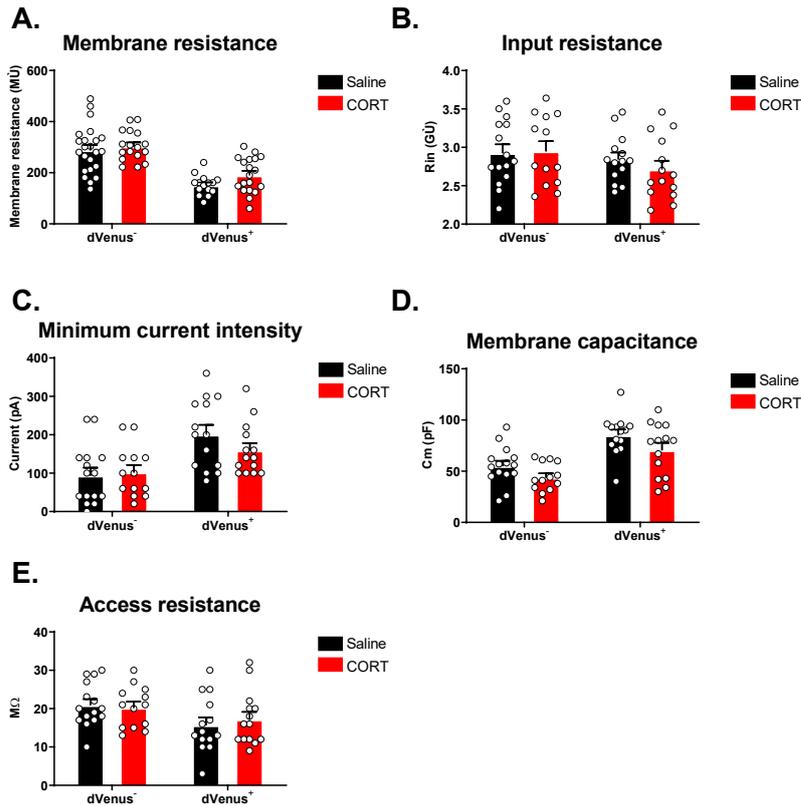
Supplementary Figures



Suppl. Fig. S1: Plasma corticosterone levels after fear conditioning (FC) and corticosterone treatment. Corticosterone injection resulted in increased circulating corticosterone levels at 30 and 60 minutes after FC. N = 4-8 mice/experimental group. Graph shows means + s.e.m.



Suppl. Fig. S2: Chance level vs. observed colocalization between c-fos⁺ and dVenus⁺ cells. We found increased colocalization between c-fos⁺ and dVenus⁺ cells following CORT treatment in context A and B, whereas in saline treated mice this was only enhanced in context A. Three-way ANOVA with Context and Treatment as between factors, and Chance/Observed as within factor, revealed a significant Chance/Observed x Treatment interaction effect: $F(1,24)=6.91$, $p=0.01$) and significant Chance/Observed x Context effect: $F(1,24)=26.90$, $p<0.0001$). Post hoc Sidak's multiple comparisons test: Chance: Saline-Context A vs. Observed: Saline-Context A: $p<0.0001$; Chance: CORT-Context A vs. Observed: CORT-Context A: $p>0.0001$; Chance: Saline-Context B vs. Observed: Saline-Context B: $p=0.26$; Chance: CORT-Context B vs. Observed: CORT-Context B: $p<0.001$; Observed: Saline-Context A vs. Observed: CORT-Context A: $p=0.003$; Observed: Saline-Context B vs. Observed: CORT-Context B: $p=0.001$). Saline: N=8 mice/group; CORT: N=6 mice/group. Graph shows means + s.e.m.



Suppl. Fig. S3. Membrane properties of dVenus⁻ and dVenus⁺ neurons at 1 hour after training. **A.** Membrane resistance in dVenus⁻ and dVenus⁺ was not affected by CORT treatment (interaction effect cell type x treatment: $F(1,70)=0.53$, $p=0.47$), although it was lower in dVenus⁺ cells compared to dVenus⁻ cells (main cell type effect: $F(1,70)=56.96$, $p<0.0001$). dVenus⁻-Saline: $n=23$; dVenus⁻-CORT: $n=17$; dVenus⁺-Saline: $n=15$; dVenus⁺-CORT: $n=19$ neurons from $n = 5$ mice/experimental group. **B.** Input resistance in dVenus⁻ and dVenus⁺ was not affected by CORT treatment ($F(1,52)=0.53$, $p=0.47$). dVenus⁻-Saline: $n=15$; dVenus⁻-CORT: $n=13$; dVenus⁺-Saline: $n=14$; dVenus⁺-CORT: $n=14$ neurons from $n = 5$ mice/experimental group. **C.** Minimum current intensity in dVenus⁻ and dVenus⁺ was not affected by CORT treatment ($F(1,52)=1.50$, $p=0.23$). dVenus⁻-Saline: $n=15$; dVenus⁻-CORT: $n=13$; dVenus⁺-Saline: $n=14$; dVenus⁺-CORT: $n=14$ neurons from $n = 5$ mice/experimental group. **D.** Membrane capacitance in dVenus⁻ and dVenus⁺ was not affected by CORT treatment ($F(1,52)=0.09$, $p=0.77$). dVenus⁻-Saline: $n=15$; dVenus⁻-CORT: $n=13$; dVenus⁺-Saline: $n=14$; dVenus⁺-CORT: $n=14$ neurons from $n = 5$ mice/experimental group. **E.** Access resistance in dVenus⁻ and dVenus⁺ was not affected by CORT treatment ($F(1,52)=0.41$, $p=0.52$). dVenus⁻-Saline: $n=15$; dVenus⁻-CORT: $n=13$; dVenus⁺-Saline: $n=14$; dVenus⁺-CORT: $n=14$ neurons from $n = 5$ mice/experimental group. Data are means + s.e.m. Statistical analysis was done with a two-way ANOVA on **A-E**. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

Supplementary Methods and Materials

Fear conditioning

Fear conditioning was performed using a standard fear conditioning chamber with a stainless steel grid floor connected to a shock generator. Mouse behavior was recorded by a camera connected to a computer with Ethovision software (version 13.0, Noldus, The Netherlands), which automatically scored freezing behavior. Mice were placed in the conditioning chamber (square chamber with steel grid floor, 17x17x25 cm, cleaned with 25% EtOH) for 180 s, followed by a series of three co-terminating presentations of a tone conditioned stimulus (CS) (30 s, 2.8 kHz, 82 dB) and a foot shock unconditioned stimulus (US) (2s, 0.2 mA). Discrimination ratio for Context (**Fig. 1D,J**) was calculated by dividing freezing percentages during the first 180 s in Context B by freezing in Context A. Discrimination ratio for tone (**Fig. 1E,K**) was calculated by dividing freezing percentage during the tone in Context B by freezing percentage prior to tone onset in Context B.

The inter-stimulus interval between tone-shock presentations was 60 s (1). CS-evoked freezing was tested 24 hours after conditioning in the same (180 s), or a completely novel context B (180 s baseline, 30 s tone) (circular chamber with bedding material on the floor, 30 cm diameter, cleaned with 1% acetic acid). Naïve mice remained in their standard housing conditions until immediately prior to behavioral testing for context- or tone-evoked freezing.

Immunohistochemistry

Mice were sacrificed by decapitation, and brains were dissected and post-fixed in 4% paraformaldehyde in phosphate buffer (0.1 M PB, pH 7.4) at 4°C. Left hemispheres were collected in light of the different Arc expression between the hemispheres (2), and were cryo-protected overnight in 30% sucrose/0.1 M PB. 40 µm thick coronal sections were collected using a freezing microtome. All stainings were performed on parallel series from the same brains within a cohort. Sections were incubated with Fab Fragments (1:200, Affinipure Fab Fragment Goat anti-mouse IgG, Lot # 121337, Jackson ImmunoResearch) for mouse

antibodies. Sections were pre-incubated with a blocking mix (5% Normal Donkey Serum; 1% Bovine Serum Albumin) for 30 mins on a shaker at room temperature. They were then incubated with primary antibodies in blocking mix and stored overnight at 4°C after 1h at room temperature. Primary antibodies used were: Rabbit anti c-fos (sc-52, Lot # A3014, Santa Cruz Biotechnology, 1:1000); Mouse monoclonal ARC (sc-17839, Lot # J3116, Santa Cruz Biotechnology, 1:500); and Chicken anti-GFP (Lot # ab13970, Abcam, 1:750). Sections were incubated with corresponding secondary antibodies in blocking mix for 2 h at room temperature. Secondary antibodies used: Alexa Fluor Dn anti-Rb 647 (Lot # 1626613, Life Technologies, 1:500); Alexa Fluor Gt anti-Ms 594 (Lot # 1107474, Life Technologies, 1:500); Alexa Fluor Gt anti-Ch 594 (Lot # A21468, Invitrogen, 1:500); and Alexa Fluor Gt anti-Ms 594 (Lot # 1920483, Invitrogen, 1:500). Vectashield with DAPI (Vectashield Mounting Medium with DAPI, H-1200, Vector Laboratories Inc.) was used for cover slipping.

Quantitative analysis of dVenus, Arc, c-fos, and DAPI positive nuclei was performed on coronal sections of the DG of the dorsal hippocampus (bregma -2.0 mm to -3.2 mm) across at least six sections per animal of matched anatomical levels along the rostral-caudal axis. Values from different sections were averaged per animal. Using a Nikon DS-Ri2 microscope, representative images of 20x magnification were systematically captured, and cells were manually counted by an experimenter blind to the experimental conditions of the mice. To assess colocalization, cells (as assessed by DAPI⁺) were assigned to one of the following categories: 1) c-fos⁺ within Arc⁻/dVenus⁻/mCherry⁻ or 2) c-fos⁺ within Arc⁺/dVenus⁺/mCherry⁺, and the percentage of cells colocalizing was calculated per animal. For Supplemental Fig. 2, the chance of co-localization of c-fos⁺ cells and dVenus⁺ cells was calculated by: $((c-fos^+/DAPI) \times (dVenus^+/DAPI)) \times 100$. The observed co-localization was calculated by: $(c-fos^+ \& dVenus^+ / DAPI) \times 100$.

Recording Action Potential (AP) Frequency

Mice were left undisturbed for 1 hour after the fear conditioning paradigm and the acute corticosterone or vehicle injections. Subsequently, mice were sacrificed by acute decapitation

and brains were quickly removed and prepared for whole cell recordings in current clamp as described in the supplementary materials and methods section. to be placed in ice-cold Artificial Cerebral Spinal Fluid (aCSF). The aCSF contained (in mM): NaCl (118.1), KCl (2.5), NaHCO₃ (26.2), NaH₂PO₄ (1), CaCl₂ (2), MgCl₂ (1) and D-glucose (22.2), saturated with 95% O₂ – 5% CO₂. The left hemisphere was dissected and coronal slices (350 μm) were made in ice-cold slicing aCSF using a vibratome (VT1000S, Leica). The slicing aCSF contained (in mM): C₅H₁₄CINO (139), KCl (3.5), CaCl₂ (0.5), MgSO₄ (6), NaH₂PO₄ (1.25), NaHCO₃ (25), and D-glucose (10) saturated with 95% O₂ – 5% CO₂. For recovery, slices were incubated for 20 minutes in warm (32°C) oxygenated aCSF followed by a 1-hour incubation in oxygenated aCSF at room temperature (20°C). Whole cell recordings in current clamp were performed using a DIC microscope (Axioskop 2 FS Plus, Zeiss) with a water immersion 40x objective (0.8 W), equipped with a CCD Camera (TVCCD 624, Monacor) and a headstage (CV 203BU, Axon Instruments) assembled to a motorized micromanipulator (Scientific). For all the recordings, borosilicate glass pipettes (1.5 mm outer diameter, Hilgerberg, Malsfeld, Germany) were pulled with a Sutter (USA) micropipette puller to establish a pipette resistance of 3-6 mΩ. The pipette solution contained (in mM): K-Gluconate (131.3), KCl (8.8), HEPES (10), EGTA (0.5), Mg ATP (4) and Na-GTP (0.4). The pH of this intracellular solution was 7.3 (adjusted with KOH) and the osmolarity was 290.6 mOsm. Under positive pressure, the electrode was directed towards a neuron in the suprapyramidal blade of the DG. Prior to patching, neurons were identified as either dVenus⁺ cells or dVenus⁻ cells using a HBO100 mercury lamp and a Zeiss excitation/emission filter set (excitation BP: 450-490 nm, emission LP: 515 nm). Once a seal was established on the cell membrane (resistance > 1GΩ), the membrane patch was ruptured by gentle suction and kept at a holding potential of -65 mV. Neurons with access resistance <30 MΩ were used for whole cell recordings in current clamp. Current was injected starting from -100 pA to 250 pA in steps of 20 pA with a duration of 1 second. Recordings were amplified using a Axopatch 200B amplifier and digitized with a Axon Digidata 1550A. Recordings alternated between dVenus⁺ and dVenus⁻ cells within a slice.

Data acquisition was performed in pClamp 10.7 and analyzed off-line with Clampfit 9.0. Neurons with a resting membrane potential >-50 mV were excluded from the data analysis.

Recording miniature excitatory postsynaptic currents (mEPSCs)

Mice were left undisturbed for 5 hours after the fear conditioning paradigm and the acute corticosterone or vehicle injections. Slices were prepared as for action potential recordings. The pipette solution contained (in mM) 120 Cs methane sulfonate; CsCl (17.5); HEPES (10); BAPTA (5); Mg-ATP (2); Na-GTP (0.5); QX-314 (10); pH 7.4, adjusted with CsOH; pipette resistance was between 3–6 M Ω . The cell was kept at a holding potential of -70 mV. The liquid junction potential caused a shift of no more than 10 mV, which was not compensated during mEPSCs recording. Recordings with an uncompensated series resistance of <15 M Ω and <2.5 times of the pipette resistance with a shift of $<20\%$ during the recording, were accepted for analysis. Tetrodotoxin (0.25 μ M, Latoxan, Rosans, France) and bicuculline methobromide (20 μ M, Biomol) were added to the buffer to block action potential induced glutamate release and GABA-A receptor mediated miniature inhibitory postsynaptic currents (mIPSCs), respectively. The events were identified as mEPSCs when the rise time was faster than the decay time. mEPSCs were recorded for 5 min from each cell.

Supplementary References

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2. S. Inberg, A. Elkobi, E. Edri, K. Rosenblum, Taste familiarity is inversely correlated with Arc/Arg3.1 hemispheric lateralization. *J. Neurosci.* **33**, 11734–11743 (2013).