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

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ABSTRACT

BACKGROUND: Traumatic experiences, such as conditioned threat, are coded as enduring memories that are frequently subject to generalization, which is characterized by (re-) expression of fear in safe environments. However, the neurobiological mechanisms underlying threat generalization after a traumatic experience and the role of stress hormones in this process remain poorly understood.

METHODS: We examined the influence of glucocorticoid hormones on the strength and specificity of conditioned fear memory at the level of sparsely distributed dentate gyrus (DG) engram cells in male mice.

RESULTS: We found that elevating glucocorticoid hormones after fear conditioning induces a generalized contextual fear response. This was accompanied by a selective and persistent increase in the excitability and number of activated DG granule cells. Selective chemogenetic suppression of these sparse cells in the DG prevented glucocorticoid-induced fear generalization and restored contextual memory specificity, while leaving expression of auditory fear memory unaffected.

CONCLUSIONS: These results implicate the sparse ensemble of DG engram cells as a critical cellular substrate underlying fear generalization induced by glucocorticoid stress hormones.

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Stressful and emotionally arousing events are generally remembered well (1). Robust memory retention is highly adaptive but comes at increasing cost when memories lose their specificity. Memory encoding and/or processing under stressful circumstances can result in generalization of fear expression in which memories are expressed promiscuously, even in safe situations and/or in the absence of predictive cues, as exemplified by post-traumatic stress disorder or generalized anxiety disorder (1–3).

Stressful experiences activate the hypothalamic-pituitary-adrenal axis and the release of glucocorticoid hormones (cortisol in humans, corticosterone in rodents) from the adrenal cortex (4). Glucocorticoid hormones alter neuronal function by activating high-affinity brain mineralocorticoid receptors and lower affinity glucocorticoid receptors (GRs) (5,6). Through these receptors, glucocorticoid hormones rapidly and persistently increase glutamatergic synaptic transmission, which is critical for synaptic plasticity and learning and memory (6–9). Behaviorally, glucocorticoid hormones facilitate memory consolidation, extinction, and habitual learning (1,10,11). In addition, glucocorticoid hormones have also been reported to modify memory contextualization, which may confer memory generalization (12,13).

Specific memories are encoded by small populations of cells, also known as engram cells, that are activated during specific learning epochs (14–16). The hippocampal dentate gyrus (DG) is strongly involved in spatial aspects of memory processing, and learning-activated engram populations in this region support the consolidation and expression of contextual fear memories (17–19). Here, we examined whether glucocorticoid levels affect the specificity of contextual fear memory through changes in the size and physiological properties of the hippocampal DG engram cell population (20).

METHODS AND MATERIALS

Study Design

Wild-type (C57BL/6J) mice (Harlan) and Arc::dVenus mice that were backcrossed for more than 10 generations into a C57BL/6J background (21) were used. Experiments were performed during the light phase, using male adult mice (8–12 weeks). Mice were individually housed for 14 days before the experiments. All experiments were conducted under the European Union directive 2010/63/EU for animal experiments and were approved by the animal welfare committee of the University of Amsterdam. Mice were maintained under standard housing conditions (temperature 20–22 °C, 40%–60% humidity) on a

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12-hour light/dark cycle with standard chow and water available ad libitum. Assignment to treatment condition, data collection, and data processing was conducted at random by an experimenter blinded to the experimental condition. Sample size was determined a priori by conducting a power analysis. All experimental procedures were performed using independent cohorts of mice.

Fear Conditioning

Fear training involved placing mice into a conditioning chamber and 3 minutes later, presenting three coterminating presentations of a tone-conditioned stimulus (30 s, 2.8 kHz, 82 dB) and a foot shock unconditioned stimulus (2 s, 0.2 mA). This mild conditioning paradigm was selected to avoid potential ceiling effects on behavior after administration of corticosterone injections (22,23). During testing, mice were placed in the same context (context A) and/or a novel context (context B) and freezing was assessed. See [Supplemental Methods and Materials](#) for details.

Drug Treatment

Corticosterone (Sigma-Aldrich) (dissolved in 5% ethanol, final dose: 2 mg/kg, injection volume: 5 μ L/g body weight) was injected intraperitoneally immediately following fear conditioning. RU486 (Sigma-Aldrich) (dissolved in 10% ethanol in peanut oil, final dose: 10 mg/kg, injection volume: 5 μ L/g body weight) was injected intraperitoneally immediately following conditioning. The corresponding vehicle solution was administered to the control group accordingly (22).

Immunohistochemistry

Sections were stained for Arc and c-fos as described in [Supplemental Methods and Materials](#).

Patch Clamp Recordings

Mice were left undisturbed for 1 hour (action potential frequency) or 5 hours (miniature excitatory postsynaptic current recordings) after fear conditioning and injections. Mice were sacrificed by acute decapitation, and brains were quickly removed and prepared for whole-cell recordings in the DG in current clamp mode as described in [Supplemental Methods and Materials](#).

Stereotactic Microinjections for Viral Targeted Recombination in Activated Populations

AAV-Fos::CreER^{T2} (titer: 1.2×10^{12}) and Cre-dependent AAV-hSyn::DIO-hM4Di-mCherry and AAV-hSyn::DIO-mCherry (titer: $5\text{--}6 \times 10^{12}$) were packaged as serotype 5 virus. A virus mixture of AAV5-Fos::CreER^{T2} and Cre-dependent AAV (adeno-associated virus) (ratio, 1:500; AAV-Fos-CreER^{T2} was injected at a final titer of 2.4×10^9) (24,25) was infused bilaterally in the DG using microinjection glass needles (0.5 μ L; flow rate: 0.1 μ L/min; anteroposterior = -2.2; mediolateral = ± 1.2 ; dorsoventral = -2.2, relative to bregma).

4-Hydroxytamoxifen Treatment

A solution of 50 mg/mL 4OHT (H6278; Sigma-Aldrich Chemie N.V.) dissolved in DMSO (D8148; Sigma-Aldrich Chemie N.V.)

and diluted 10 \times in saline containing 2% Tween80 (P1754; Sigma-Aldrich Chemie N.V.) and 10 \times in saline (final concentration: 2.5 mg/mL 4OHT, 5% DMSO, and 1% Tween80 in saline) was injected 2 hours after training in hM4Di-mCherry and control mice. The final dose was 25 mg/kg.

Chemogenetic Intervention

Clozapine N-oxide (CNO) (BML-NS105; Enzo LifeSciences) dissolved in saline (final dose: 5 mg/kg, injection volume 10 μ L/g body weight) was injected intraperitoneally 30 minutes before a retrieval session in both mCherry and hM4Di-mCherry mice.

Statistical Analysis

Data were analyzed using SPSS version 22.0 (IBM Corp., Armonk, NY). All data were expressed as mean + SEM. Data were considered statistically significant when *p* values were < .05 and were tested two sided. Outliers were determined using a Grubb's test, removing a maximum of 1 data point. Independent-samples *t* tests were performed to assess differences between saline- and corticosterone-treated mice. When assumption of normality was not met (based on Shapiro-Wilk test), Mann-Whitney *U* test was conducted. A 2 \times 2 analysis of variance was performed to assess the interaction between two factors (treatment \times context or treatment \times cell type), with post hoc Tukey testing.

RESULTS

Corticosterone-Induced Fear Memory Generalization

Mice underwent fear conditioning by three mild foot shocks (0.2 mA) paired with a discrete tone, immediately followed by systemic administration of corticosterone (2 mg/kg) or saline (Figure 1A). As expected, we observed a conditioning-induced increase of plasma corticosterone levels, which was even further elevated and prolonged in mice receiving post-training corticosterone administration (Figure S1).

Freezing was comparable between saline- and corticosterone-injected mice during re-exposure to the training context 24 hours after conditioning (Figure 1B) ($t_{14} = 0.24$, $p = .82$). In contrast, mice receiving corticosterone immediately following conditioning exhibited significantly higher freezing to a distinct context (neutral context B), in both the presence and absence of the conditioned tone (Figure 1C) ($F_{1,28} = 77.13$, $p < .0001$; post hoc: saline-baseline vs. corticosterone-baseline: $p = .02$; saline-tone vs. corticosterone-tone: $p = .002$). Moreover, corticosterone injection significantly increased contextual fear generalization, which was quantified as the ratio of context-evoked freezing in context B (novel context) relative to context A (training context) (Figure 1D) ($t_{14} = 2.84$, $p = .013$). Although absolute freezing to the tone was higher in corticosterone-treated mice, the relative increase in freezing during the tone compared with baseline freezing in context B was similar between saline- and corticosterone-treated mice (Figure 1E) ($t_{14} = 1.61$, $p = .13$), indicating that the increase during the tone was caused by a generalized fear response in the novel context.

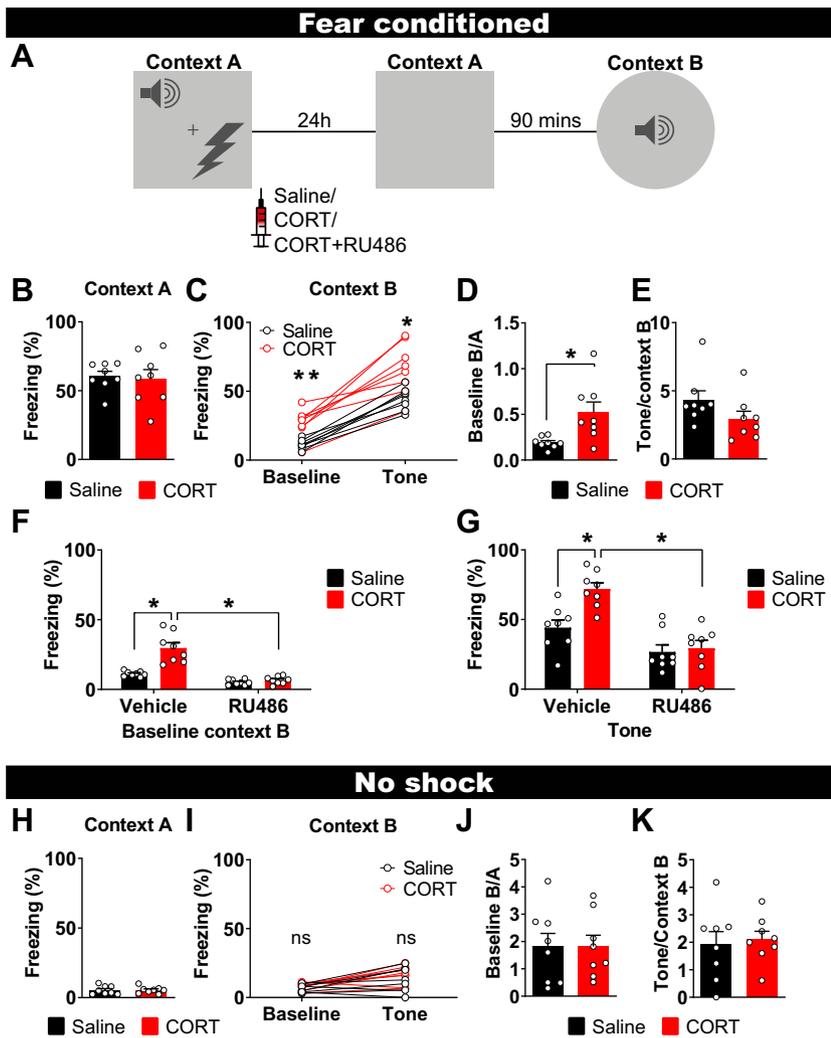


Figure 1. Corticosterone-induced contextual fear memory generalization. **(A)** Schematic overview of the fear conditioning paradigm. Mice received corticosterone (2 mg/kg) and/or RU486 (40 mg/kg) immediately after training. **(B)** Following paired training, freezing in conditioning context A was comparable between experimental groups ($n = 8$ mice/group). **(C)** Baseline freezing responses in neutral context B and to the tone were enhanced after corticosterone treatment ($n = 8$ mice/group). **(D)** Corticosterone-treated mice displayed more freezing in context B relative to context A. **(E)** The ratio of freezing to the tone over baseline freezing in context B was not affected by corticosterone. **(F)** Blocking the glucocorticoid receptor by RU486 treatment reduced corticosterone-induced freezing in context B ($n = 8$ mice/group). **(G)** Blocking the glucocorticoid receptor by RU486 treatment reduced corticosterone-induced freezing to the tone. **(H)** Response to context A of mice treated with saline or corticosterone after training in context A in the absence of foot shocks. No difference in freezing levels was observed ($n = 8$ mice/group). **(I)** Baseline freezing responses in neutral context B and to the tone were not different between saline- and corticosterone-treated mice ($n = 8$ mice/group). **(J)** No differences in the ratio of freezing between context A and B were present. **(K)** No differences in the ratio between context B and tone freezing were present. Data are mean + SEM. Statistical analysis was performed using a one-way repeated-measures analysis of variance with post hoc Tukey test in panels **(C)** and **(I)**, a Student's unpaired t test in panels **(B)**, **(D)**, **(E)**, **(H)**, **(J)**, and **(K)**, and a two-way analysis of variance with post hoc Tukey test in panels **(F)** and **(G)**. * $p < .05$; ** $p < .01$. CORT, corticosterone; ns, not significant.

We next examined whether the effect of corticosterone on generalization of conditioned fear was mediated by activation of GRs. Co-administration of the GR antagonist RU486 prevented the effect of corticosterone on freezing in a neutral context (Figure 1F) ($F_{1,28} = 17.46, p = .0003$, post hoc: vehicle-saline vs. vehicle-corticosterone: $p < .0001$; vehicle-corticosterone vs. RU486-corticosterone: $p < .0001$) as well as to the tone (Figure 1G) ($F_{1,28} = 5.89, p = .02$, post hoc: vehicle-saline vs. vehicle-corticosterone: $p = .004$; vehicle-corticosterone vs. RU486-corticosterone: $p < .0001$). Furthermore, freezing in context A and the enhanced freezing in neutral context B after corticosterone treatment was absent in mice that underwent the same paradigm, but without receiving foot shocks during exposure to context A (Figure 1H–K). Together, these results show that increased corticosterone levels immediately after fear conditioning subsequently result in a generalized fear response in a neutral context. Moreover, this study showed that the effect of corticosterone is mediated through activation of GRs.

Corticosterone Enhanced the Size of a Fear Engram Cell Population

To identify how corticosterone triggered memory generalization, we examined neuronal activation in the DG, a brain region that is strongly involved in contextual fear memory (17–19). For this, mice underwent fear conditioning followed by a corticosterone or saline injection. At 90 minutes after conditioning, brains were isolated and immunostained for c-fos, an immediate early gene and molecular marker of recent neuronal activation (26) (Figure 2A). The number of neurons expressing endogenous c-fos was increased in the DG of corticosterone-treated mice at 90 minutes after training compared with saline-treated animals (Figure 2B, C) ($t_{10} = 7.8, p < .0001$). To further characterize the neuronal population that was activated by fear conditioning, we used a transgenic mouse with destabilized Venus fluorescent protein (dVenus) expression under control of the activity-regulated cytoskeleton-associated protein (Arc) promoter

(Arc::dVenus mice) (21), which allows for visualization and characterization of activated neurons in the DG for at least 24 hours after conditioning (17). At 90 minutes after fear conditioning, we observed an increase in the number of dVenus-labeled cells in corticosterone-treated animals (Figure 2D, E) ($t_{14} = 3.0, p = .009$). After context exposure alone (without the delivery of a foot shock), corticosterone did not increase the number of c-fos⁻ or dVenus-labeled cells in the DG compared with control mice (c-fos: $t_{14} = 1.8, p = .10$; dVenus: $t_{14} = 0.06, p = .95$) (Figure 2F–J). At 90 minutes after fear conditioning, we observed significant colocalization of cells expressing c-fos and endogenous Arc ($t_{15} = 22.55, p < .0001$), as well as of the cells coexpressing c-fos and dVenus ($t_{14} = 18.6, p < .0001$) (Figure 2K–M), indicating that these molecular markers represent, to a large extent, the same cellular population, consistent with what has been reported previously (17). We found that the number of Arc::dVenus-positive cells was still increased in corticosterone-treated mice at 24 hours after training compared with control animals (Figure 2N–P) ($t_{30} = 4.92, p < .001$), suggesting that elevated corticosterone levels during the first hours after conditioning persistently increased the size of the DG engram population.

On retrieval (Figure 2Q), corticosterone-injected and saline-injected mice showed comparable numbers of c-fos⁺ cells in context A (Figure 2R) ($F_{1,25} = 10.58, p = .003$; post hoc: saline-context A vs. corticosterone-context A: $p = .99$). In contrast, corticosterone-injected mice exhibited a larger number of c-fos⁺ cells than saline-injected mice in context B (Figure 2R) (saline-context B vs. corticosterone-context B: $p < .001$). To assess whether training-activated neurons were reactivated on exposure to context A or B, we used the long-lasting detectability of dVenus in the DG to examine reactivation during memory retrieval. Importantly, at 90 minutes after memory retrieval in either context A or context B (Figure 2Q), the density of dVenus⁺ neurons was equivalent to the density in the absence of a retrieval session (see Figure 2P) (main treatment effect: $F_{1,40} = 26.73, p \leq .0001$; treatment \times test interaction effect: $F_{2,40} = 0.17, p = .84$) (Figure 2S), indicating that retrieval did not alter the size of the training-induced dVenus⁺ population. In contexts A and B, we observed preferential expression of c-fos within the dVenus⁺ populations compared with the dVenus⁻ populations after saline and corticosterone treatment (main effect cell type—context A: $F_{1,24} = 578.8, p < .0001$; context B: $F_{1,24} = 320.5, p < .0001$). However, corticosterone-injected mice exhibited increased colocalization of c-fos⁺ and dVenus⁺ neurons in the DG after exposure to context B compared with saline-treated mice (treatment \times cell type interaction effect: $F_{1,24} = 14.22, p < .001$, post hoc: saline-dVenus⁺ vs. corticosterone-dVenus⁺: $p < .0001$) (Figure 2T, V), whereas this did not differ between groups after exposure to context A (treatment \times cell type interaction effect: $F_{1,24} = 1.03, p = .32$) (Figure 2T, U). Importantly, colocalization between c-fos⁺ and dVenus⁺ cells was significantly higher than chance level after exposure to context A for both groups and higher than chance level after exposure to context B for the corticosterone-treated animals (Figure S2). These findings are consistent with a less precise contextual representation of the memory, resulting in fear generalization from post-training corticosterone.

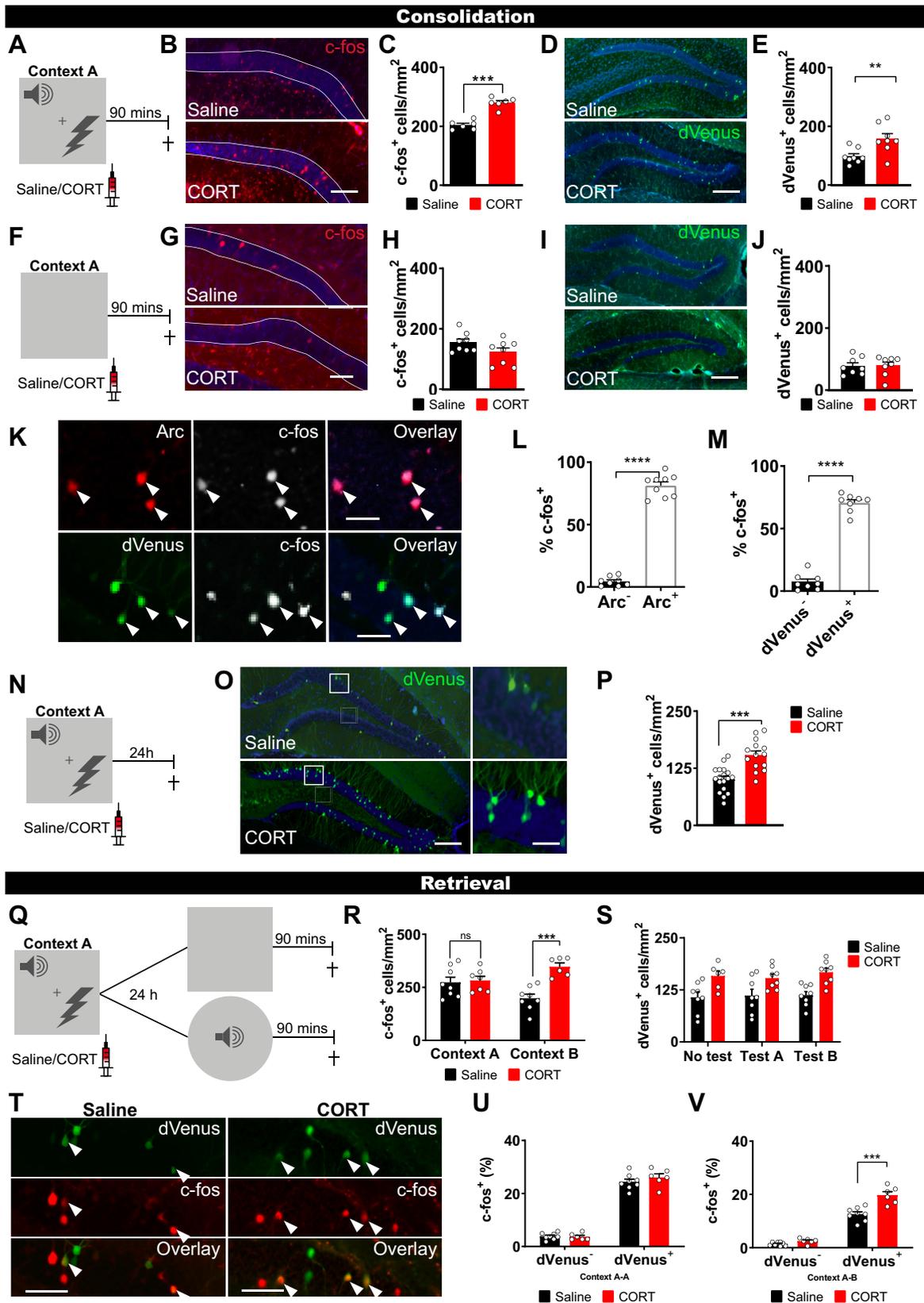
Corticosterone Selectively Enhanced the Excitability of dVenus⁺ Neurons After Fear Conditioning

Because neural activation and excitability determine neuronal selection into a memory engram (17,27–29), we used whole-cell patch clamp recordings to further investigate the effects of corticosterone on the physiological properties of activated dVenus⁺ neurons and nonactivated dVenus⁻ neighboring neurons at 1 hour after conditioning. Corticosterone injection after training did not affect membrane resistance, input resistance, minimum current intensity, membrane capacitance, or access resistance differently in dVenus⁻ and dVenus⁺ neurons (Figure S3). Depolarizing current injections evoked an increase in action potential (AP) frequency, which was significantly reduced in dVenus⁺ neurons compared with dVenus⁻ neurons (Figure 3A, B) (current injected \times treatment \times cell type interaction effect: $F_{75,1225} = 1.93, p < .0001$; saline-dVenus⁺ vs. saline-dVenus⁻: $p = .04$). However, compared with saline-injected animals, corticosterone selectively increased the AP frequency elicited by depolarizing current injections in dVenus⁺, but not in dVenus⁻, neurons in mice that were fear conditioned (Figure 3A–C) (saline-dVenus⁺ vs. corticosterone-dVenus⁺: $p = .026$; corticosterone-dVenus⁺ vs. corticosterone-dVenus⁻: $p = .78$). In naïve home cage mice with no prior fear conditioning, the AP frequency was similarly lower in dVenus⁺ cells compared with dVenus⁻ cells in both groups, but corticosterone injection did not enhance AP frequency in dVenus⁺ or dVenus⁻ neurons (Figure 3D, E) (current injected \times treatment \times cell type interaction effect: $F_{75,1700} = 4.30, p < .0001$; saline-dVenus⁺ vs. saline-dVenus⁻: $p = .01$; saline-dVenus⁺ vs. corticosterone-dVenus⁺: $p = .93$). Hence, corticosterone increased the excitability of activated (dVenus⁺) DG neurons, potentially contributing to the enhanced ensemble size at 90 minutes and 24 hours after conditioning (Figure 2B–E).

At 5 hours after training, we examined the effects of corticosterone injection on synaptic transmission by analyzing the properties of mEPSCs. We found that the frequency of mEPSCs was higher in dVenus⁺ neurons than dVenus⁻ neurons of saline-injected mice (Figure 3F–H) ($F_{1,14} = 10.9, p = .005$). Compared with saline-treated mice, corticosterone injection significantly enhanced the mEPSC frequency selectively in dVenus⁻ neurons (Figure 3F–H) (interaction effect: $F_{1,14} = 8.05, p = .01$, post hoc: $p = .04$) but did not alter the amplitude of mEPSCs in dVenus⁺ and dVenus⁻ neurons (Figure 3G, H) ($F_{1,14} = 2.91, p = .11$). The increase in frequency of mEPSCs in dVenus⁻ neurons after corticosterone injection may reflect altered presynaptic input onto these neurons.

Chemogenetic Suppression of the DG Engram Population Reduced Corticosterone-Induced Generalized Fear

Previous studies have shown that inhibition of fear conditioning-activated DG neurons impairs memory retrieval on re-exposure to the training context (18,27). Based on this, we next investigated whether the DG neurons activated during conditioning paired with corticosterone were responsible for the observed fear generalization in a neutral context. To assess



this, we used a well-established viral targeted recombination in activated populations approach, restricting expression of the inhibitory Gi-DREADD (designer receptor exclusively activated by designer drugs) [hM4Di (30)] to activated neurons under control of the Fos promoter in a Cre recombinase- and 4OHT-dependent manner (24,25) (Figure 4A). After training, both groups were injected with corticosterone and 2 hours later received 4OHT to allow expression of hM4Di-mCherry or mCherry alone (control) in activated DG neurons (Figure 4B). When mice were re-exposed to context A (without CNO), freezing levels were similar between groups (Figure 4C) ($t_{15} = 0.64$, $p = .53$). Thirty minutes before exposure to the neutral context B, all mice received CNO to suppress the activity of DG neurons that were tagged after training. Whereas mCherry control mice showed substantial baseline freezing in context B, suppression of hM4Di⁺ neurons reduced freezing behavior (Figure 4D) (post hoc: baseline-mCherry vs. baseline-hM4d: $p = .0001$) to a level that was comparable with control mice that did not receive post-training corticosterone (Figure 1B). Indeed, contextual fear generalization, quantified as the ratio of context-evoked freezing in context B (novel context) relative to freezing in context A (training context), was reduced following suppression of hM4Di⁺ neurons in corticosterone-treated mice (Figure 4E) ($t_{15} = 3.55$, $p = .003$). Freezing to the tone remained unaffected by CNO (Figure 4D) ($F_{1,30} = 7.57$, $p = .01$, post hoc tone-mCherry vs. tone-hM4Di: $p = .66$). This confirms that inhibition of these DG neurons prevented the expression of contextual memory generalization.

Finally, we determined whether DG neurons that were tagged with viral targeted recombination in activated populations were reactivated on exposure to context B, similar to what we observed in the Arc::dVenus mice. Indeed, control mice expressing mCherry alone showed substantial reactivation of tagged neurons, whereas in mice injected with hM4Di-mCherry, c-fos expression was selectively reduced in mCherry⁺ neurons (Figure 4F, G) ($F_{1,30} = 48.79$, $p < .0001$, post hoc: control-mCherry⁺ vs. hM4Di-mCherry⁺: $p < .0001$; hM4Di-mCherry⁻ vs hM4Di-mCherry⁺: $p = .86$).

This confirms that the neurons tagged during training were indeed reactivated during expression of fear in a neutral context. Together, these data indicate that corticosterone-induced generalized fear is driven by the increased DG engram population and that suppression of this improves contextual discrimination.

DISCUSSION

A delicate balance between memory strength, specificity, and generalization is essential to enable efficient encoding and retrieval of highly salient, stress-related information (31). We report here that corticosterone, the main glucocorticoid stress hormone in mice, alters the size of a fear memory-encoding DG engram population and that this population is functionally responsible for the expression of generalized contextual fear.

Various lines of evidence indicate that GRs are involved in enhancing memory consolidation. Our findings demonstrate that glucocorticoid hormones, via GRs, are also involved in the effects of corticosterone on memory generalization (Figure 1F, G). In line with this, it was recently reported that corticosterone, at levels that activate GRs, reduces memory accuracy (32). Thus, activation of GRs enhances not only contextual memory consolidation but also memory generalization, and we now show that this effect is mediated through an increase in the DG engram cell population. The presently observed contextual memory generalization in a novel context was not simply due to enhanced memory strength, because no differences were observed between saline- and corticosterone-injected mice in fear expression when mice were tested in the conditioning context. In addition, the observed effects required associative learning as corticosterone did not alter the number and excitability of activated DG neurons when mice did not receive aversive foot shocks in the conditioning context. These two findings point toward a specific role of corticosterone in the enhancement of memory generalization.

Figure 2. Corticosterone enhanced the size of a fear conditioning-activated DG ensemble. **(A)** Schematic overview of the fear conditioning paradigm. Mice were sacrificed 90 minutes after conditioning. **(B)** Representative image showing c-fos expression in the DG. Scale bar = 150 μ m. **(C)** Corticosterone treatment increased the number of c-fos⁺ cells in the DG after training ($n = 6$ mice/group). **(D)** Representative image showing dVenus expression in the DG. Scale bar = 250 μ m. **(E)** Corticosterone treatment increased the number of dVenus⁺ cells in the DG after training ($n = 8$ mice/group). **(F)** Schematic overview of the experiment. Mice were put in context A without tone or foot shock and were sacrificed 90 minutes after saline/corticosterone treatment. **(G)** Representative image showing c-fos expression in the DG. Scale bar = 150 μ m. **(H)** Corticosterone treatment did not affect the number of c-fos⁺ cells in the DG ($n = 8$ mice/group). **(I)** Representative image showing dVenus expression in the DG. Scale bar = 250 μ m. **(J)** Corticosterone treatment did not affect the number of dVenus⁺ cells in the DG ($n = 8$ mice/group). **(K)** Representative images showing colocalization between c-fos⁺ and Arc⁺ neurons and between c-fos⁺ and dVenus⁺ neurons. Arrowheads indicate colocalization. Scale bar = 50 μ m. **(L)** Quantification of **(K)** (upper panels) ($n = 9$ mice/group). **(M)** Quantification of **(K)** (lower panels) ($n = 8$ mice/group). **(N)** Schematic overview of the fear conditioning paradigm. Mice were sacrificed 24 hours after conditioning. **(O)** Representative images showing dVenus expression in saline- and corticosterone-treated mice. Scale bar (left) = 250 μ m; scale bar (right) = 50 μ m. **(P)** The number of dVenus⁺ neurons in the DG was increased in corticosterone-treated mice 24 hours after conditioning. Saline: $n = 18$ mice/group; corticosterone: $n = 14$ mice/group. **(Q)** Schematic overview of the fear conditioning paradigm. Mice were killed 90 minutes after exposure to context A or B. **(R)** Post-training corticosterone treatment resulted in an increase in the number of c-fos⁺ cells on exposure to context B, but not to context A. Context A: saline, $n = 8$ mice/group; corticosterone, $n = 7$ mice/group. Context B: saline, $n = 8$ mice/group; corticosterone, $n = 6$ mice/group. **(S)** Testing in either context A or context B did not increase the number of dVenus⁺ compared with untested control subjects ($n = 8$ mice/group). **(T)** Representative image showing c-fos⁺ and dVenus⁺ cells after exposure to context B. Scale bar = 50 μ m. **(U)** Colocalization between dVenus⁺ and c-fos⁺ neurons did not differ between saline- and corticosterone-treated mice after re-exposure to context A. Saline: $n = 8$ mice/group; corticosterone: $n = 6$ mice/group. **(V)** Compared with control mice, corticosterone-treated mice displayed increased colocalization of c-fos⁺ cells in dVenus⁺ neurons after exposure to context B. Saline: $n = 8$ mice/group; corticosterone: $n = 6$ mice/group. Data are mean + SEM. Statistical analysis was done with Student's unpaired t test in panels **(C)**, **(E)**, **(H)**, **(J)**, **(L)**, **(M)**, and **(P)**, and a two-way analysis of variance followed by a Tukey post hoc test was used in panels **(R)**, **(S)**, **(U)**, and **(V)**. ** $p < .01$; *** $p < .001$; **** $p < .0001$. CORT, corticosterone; DG, dentate gyrus; ns, not significant.

We observed that corticosterone increased the sparse number of activated DG cells after training and at 24 hours after training, pointing to a stable change in the fear-encoding DG engram population. Whereas reactivation of learning-activated DG neurons on re-exposure to the conditioned context was not affected by corticosterone treatment, we found enhanced reactivation of the same population on

exposure to a neutral context. Importantly, selective inhibition of DG neurons that were activated by fear conditioning plus elevated corticosterone levels prevented the expression of generalized contextual fear, while leaving memory for the conditioned tone unaffected. Although we did not examine whether activity of the learning-activated DG neurons was required for memory expression on re-exposure to the

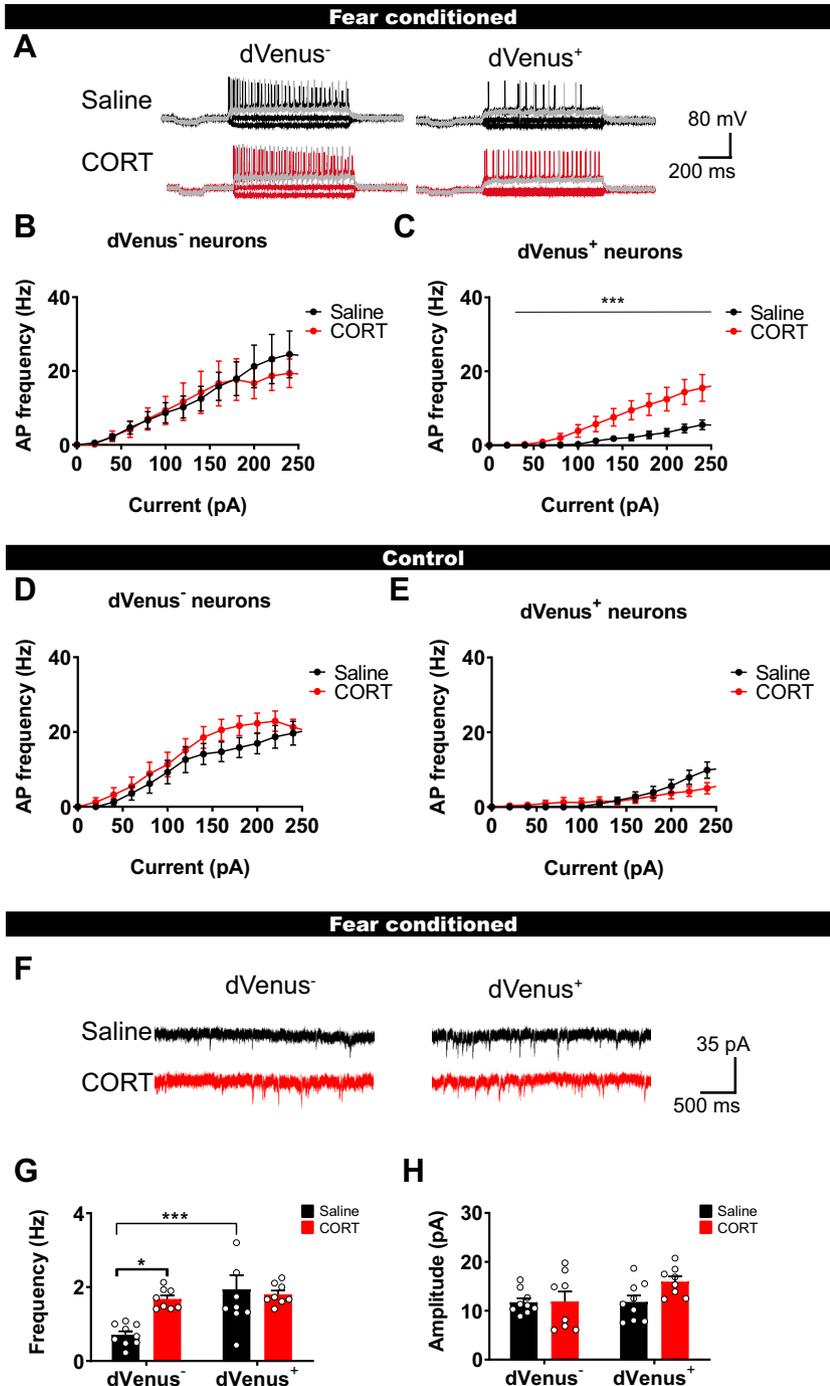


Figure 3. Corticosterone selectively enhanced the excitability of *dVenus*⁺ neurons after fear conditioning. **(A)** Representative traces of excitability measurements. **(B)** Corticosterone did not alter AP frequency (in Hz) in *dVenus*⁻ neurons after training. Saline: *n* = 12 neurons; corticosterone: *n* = 13 neurons from *n* = 5 mice/experimental group. **(C)** Corticosterone enhanced the excitability of *dVenus*⁺ neurons. Saline: *n* = 11 neurons; corticosterone: *n* = 17 neurons from *n* = 5 mice/experimental group. **(D)** Corticosterone without training did not alter AP frequency in *dVenus*⁻ neurons compared with saline-injected mice. Saline: *n* = 18 neurons; corticosterone: *n* = 13 neurons from *n* = 5 mice/experimental group. **(E)** Corticosterone without training did not alter AP frequency in *dVenus*⁺ neurons. Saline: *n* = 15 neurons; corticosterone: *n* = 11 neurons from *n* = 5 mice/experimental group. **(F)** Typical examples of mEPSC traces. **(G)** The frequency of the mEPSCs was enhanced in *dVenus*⁺ neurons compared with *dVenus*⁻ cells, irrespective of treatment. Corticosterone increased mEPSC frequency in *dVenus*⁻ neurons. *dVenus*⁻: saline, *n* = 9 neurons; corticosterone, *n* = 8 neurons from *n* = 4 mice/experimental group. *dVenus*⁺: saline, *n* = 9 neurons; corticosterone, *n* = 8 neurons from *n* = 4 mice/experimental group. **(H)** No effect of treatment was observed on mEPSC amplitude in *dVenus*⁻ and *dVenus*⁺ neurons. *dVenus*⁻: saline, *n* = 9 neurons; corticosterone, *n* = 8 neurons from *n* = 4 mice/experimental group. *dVenus*⁺: saline, *n* = 9; corticosterone, *n* = 8 neurons from *n* = 4 mice/experimental group. Data are mean ± SEM. Statistical analysis was done with a repeated-measures analysis of variance on the combined data in panels **(B)** and **(C)** and on the combined data in panels **(D)** and **(E)**, and a two-way analysis of variance was conducted on the data in panels **(G)** and **(H)**. **p* < .05; ****p* < .001. AP, action potential; CORT, corticosterone; mEPSC, miniature excitatory postsynaptic current.

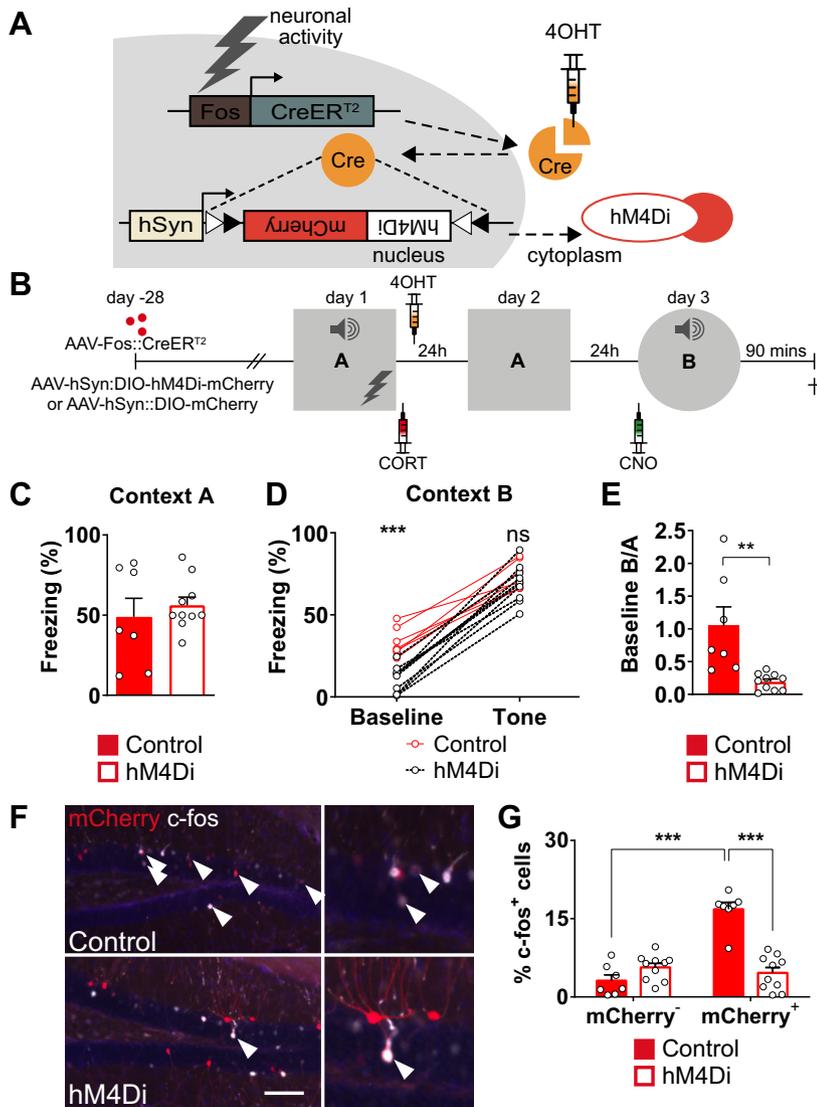


Figure 4. Chemogenetic suppression of the training-activated DG ensemble reduced corticosterone-induced fear generalization. **(A)** Schematic representation of Fos promoter-driven and 4OHT-dependent expression of hM4Di-mCherry in DG neurons. **(B)** Timeline of injection, viral transduction, and behavioral testing paradigm. Freezing in context A and context B (\pm tone) was assessed at 24 hours and 48 hours after training, respectively. **(C)** Freezing in context A did not differ between groups. mCherry: $n = 7$ mice; hM4Di-mCherry: $n = 10$ mice. **(D)** CNO-induced suppression of hM4Di-mCherry⁺ neurons reduced baseline freezing levels in the neutral context. Freezing to the tone was unaffected. mCherry: $n = 7$ mice; hM4Di-mCherry: $n = 10$ mice. **(E)** CNO-induced suppression of hM4Di-mCherry⁺ neurons decreased freezing in context B relative to context A. **(F)** Representative image showing the expression of hM4Di-expressing DG neurons and c-fos colocalization. Blue: DAPI, red: mCherry, white: c-fos. Scale bar = 125 μ m. **(G)** Percentage of c-fos⁺ cells within mCherry⁺ and mCherry⁻ populations of both groups. mCherry: $n = 7$ mice; hM4Di-mCherry: $n = 10$ mice. Data are mean + SEM. Statistical analysis was done with a Student's t test in panels **(C)** and **(E)**, repeated-measures analysis of variance, followed by Sidak post hoc testing in panel **(D)**, and two-way analysis of variance followed by Tukey post hoc testing in panel **(G)**. ** $p < .01$; *** $p < .001$. 4-OHT, 4-hydroxytamoxifen; CNO, clozapine N-oxide; CORT, corticosterone; DG, dentate gyrus; ns, not significant.

conditioning context (i.e., context A), this has previously been shown by others (18,27), and we thus expect that this is not further affected by elevated corticosterone levels. However, we extend these earlier findings by showing here that the corticosterone-induced increase in the size of a DG engram cell population is responsible for a generalized expression of fear also in a neutral context. Our results support previous observations that memory specificity is related to the size of neuronal ensembles in the DG (33–35), but, to our knowledge, this is the first study to causally demonstrate that glucocorticoid hormones have a critical role in modulating the incorporation of neurons in an engram population.

Alterations in neuronal excitability have been implicated in recruitment of neurons in a memory ensemble in the lateral amygdala (29,36) and after retrieval in the DG (27), possibly via regulation of potassium channels (17,37). We found that fear conditioning-activated DG neurons have lower excitability than nonactivated neurons, independent of elevated glucocorticoid

hormone levels. However, we demonstrate that glucocorticoid hormones specifically enhance excitability of dVenus⁺ neurons after fear conditioning, while leaving excitability unaffected in the absence of aversive associative learning. The reduced excitability of the dVenus⁺ cells that we observed in control mice after learning contrasts with enhanced excitability of dVenus⁺ cells in the lateral amygdala after fear conditioning (36) and with a transient increase in excitability of DG engram cells after memory retrieval (37). We speculate that decreased dVenus⁺ cell excitability shortly after learning functions to protect the integrity of the information encoded in the DG engram cell population by avoiding subsequent recruitment of these neurons during a different experience shortly thereafter and thereby the linking of memories (38,39). This would be consistent with the pattern separation function of the DG (40). At the cellular level, the reduced excitability is likely due to a decrease in the membrane resistance in dVenus⁺ cells (Figure S3A). What the molecular mechanism is that underlies

the change in membrane resistance is an important topic for future research but is likely driven by posttranslational modifications or trafficking of membrane channels given the rapid induction after learning (within 1 hour). Changes in potassium channels may contribute to conditioning-induced reduction in excitability (41) because these proteins are thought to modulate the excitability of fear memory–encoding DG granule cells (17,42). As corticosterone selectively enhanced excitability of dVenus⁺ neurons, glucocorticoid hormones may act as a switch to enhance excitability of conditioning-activated DG neurons and thereby the stable incorporation of neurons in a fear memory–encoding engram cell population.

Persistent alterations in synaptic strength in engram cells have been implicated in memory consolidation, and this is crucial for subsequent memory expression (36,43). In control animals, we observed that fear conditioning elicited an increase in the mEPSC frequency, specifically in activated DG neurons. This is in line with enhanced input of projections from the medial entorhinal cortex onto Fos-expressing DG neurons after fear conditioning (44). Although our recordings were performed on neurons that expressed dVenus driven by the Arc promoter, we found that the Arc⁺ and Fos⁺ population overlap to a large extent. Moreover, Fos-expressing DG neurons drive generalized fear in a neutral context (44), similar to what we observed with our Fos promoter–based viral targeted recombination in activated populations approach. We also found that glucocorticoid hormones enhanced the frequency of mEPSCs in nonactivated DG neurons to the level detected in activated neurons 5 hours after training. Thus, glucocorticoids seem to increase general synaptic transmission in DG neurons after training, possibly via increased input from the medial entorhinal cortex (44), which may contribute to aberrant processing of contextual information (45).

In line with our observations, it has been reported that stress elicits memory generalization and that glucocorticoid hormones impair the ability to correctly predict threat (3,12,13). Although some level of memory generalization is adaptive because it allows a learned response to transfer to other relevant situations and stimuli, overgeneralization of fear is often maladaptive. Indeed, stressful learning experiences contribute to fear sensitization, resulting in exaggerated startle responses to trauma-unrelated stimuli (46), particularly in relation to the contextual component of the memory trace. This impairment is similar to symptoms observed in patients with posttraumatic stress disorder, who often forget contextual peritraumatic cues, whereas salient but irrelevant stimuli are strongly remembered. These salient cues, and other similar cues, can then induce strong fear responses in contexts that differ from the traumatic environment (2). Interestingly, noradrenaline, another key mediator of the stress response, has been shown to contribute to memory specificity (47). Potentially, a closely governed balance in the release of noradrenaline (specificity) and glucocorticoids (generalization), which have been reported to interact at the level of the neuronal structure (48), electrophysiological functioning (10,49), and behavior (50), is essential for accurate memory consolidation and retrieval.

This study demonstrates that a transient postlearning increase in glucocorticoid hormone levels in male mice reduces memory specificity, thereby promoting fear memory

generalization. We further reveal that glucocorticoids alter the size of the DG neuronal population that is activated by an aversive experience. Selectively suppressing this neuronal ensemble restores memory specificity. Given that stress-related disorders are often sex dependent and that glucocorticoids regulate fear memory formation in a sex-dependent manner (22), further investigation is warranted regarding the role of these hormones on memory generalization in females.

Moreover, understanding how glucocorticoids modify the delicate balance between memory strength and specificity through changes in the size of the engram cell population opens novel avenues for the development of treatments for stress-related disorders that are characterized by maladaptive aversive memories. In this respect, our findings may be particularly relevant in relation to anxiety disorder and posttraumatic stress disorder, which have been linked to memory generalization and impaired pattern separation (51).

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SLL, MCvdO, and HJK contributed to conception and design of the study; SLL organized the database; SLL, RJvdL, PB, NB, MM, and NI conducted the experiments; SLL performed the statistical analysis; SLL wrote the first draft of the manuscript; SLL, MCvdO, PB, CPF, NI, MM, P.JL, and HJK contributed to manuscript revision and read and approved the submitted version.

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