Stress and memory in health and disease

*Impact on Alzheimer's disease and memory mechanisms*

Lesuis, S.L.

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Glucocorticoids promote fear generalisation by increasing the size of memory-encoding neuronal ensembles

Sylvie L. Lesuis1, Michel C. van den Oever2, Nathalie Immerzeel1, Rolinka J. van der Loo2, Paul J. Lucassen1, Harm J. Krugers1

Submitted
Abstract

Glucocorticoids help to remember threatening and stressful experiences. However, we show that high levels of these hormones after an aversive experience induces fear memory generalisation. This is accompanied by alterations in the electrophysiological properties and increase in size of neuronal ensembles in the dentate gyrus of the mouse hippocampus. Selective chemogenetic suppression of these ensembles prevents the expression of generalised fear memory induced by glucocorticoids.

Keywords: stress, memory, fear, generalisation, corticosterone, memory specificity, dentate gyrus.
1. Introduction

Stressful and emotionally arousing events are generally remembered very well. Enhanced memory retention clearly has adaptive and survival value, but only when memories remain specific for the arousing cue or context. If memory encoding and/or processing is aberrant, this can trigger inappropriately expressed, yet intrusive and vivid fear memories, even in safe situations and/or in the absence of predictive cues, such as seen in posttraumatic stress disorder (PTSD) or generalised anxiety disorders.

Glucocorticoid hormones (GCs) (cortisol in humans, corticosterone in rodents) are released from the adrenal cortex in response to stressful experiences. They enhance memory processing including response selection and memory consolidation by activating brain mineralocorticoid (MRs) and glucocorticoid receptors (GRs). Considering that stress can also induce memory impairments and fear generalisation in a time- and dose-dependent manner, we here tested whether GCs also modify specificity of a fear memory. Since recent studies demonstrate that a memory is encoded by small populations of cells (neuronal ensembles) that are activated during specific learning epochs, also known as ‘engram cells’, we examined whether GCs affect the formation of memory-encoding neuronal ensembles in the hippocampal dentate gyrus, and whether these engram cells are responsible for GC-induced fear generalisation.

We employed a specific experimental design to restrict fear responses to an appropriate predictor of an aversive stimulus. Mice were first exposed to an aversive situation (3 mild (0.2 mA) foot shocks that were paired with a discrete tone, in a specific context (conditioning context A)), immediately followed by saline or corticosterone (2 mg/kg) treatment (Figure 1a). This fear conditioning procedure increased plasma corticosterone levels that were even further increased and prolonged by post-training corticosterone administration. Plasma corticosterone levels returned to baseline levels at 5 hours after training (Supplementary Figure 1).

Exposure to context A, but not to the neutral context B, induced freezing behaviour in saline treated mice 24 hours after training (black bold line, Figure 1b). Exposing animals to the conditioned tone in context B evoked robust freezing, indicating that animals had learned the tone-shock association (Figure 1b). Corticosterone (dashed red lines) did not alter freezing in context A compared to control mice, but enhanced freezing behaviour in neutral context B, as well as to the tone in context B. Freezing in context B relative to context A was increased in corticosterone-treated mice (Figure 1c), while Freezing to the
tone relative to baseline freezing in context B was not affected (Figure 1d). This effect of corticosterone was mediated by GRs since the GR-antagonist RU486 prevented the corticosterone-induced enhanced freezing (Figure 1e).
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Figure 1 (continued): f Response to context A and B and tone of naïve mice treated with saline or corticosterone 24 h earlier. No difference in freezing levels was observed (Context A: t(14)=0.04, p=0.97; Context B: t(14)=1.03, p=0.32; tone: t(14)=1.03, p=0.32). g No differences in the ratio of freezing between context A and B (t(14)=0.01, p=0.99) was present. h No differences in the ratio between context B and tone freezing (t(14)=0.32, p=0.75) was present. Data are means ± s.e.m. Statistical analysis was performed using Student’s unpaired t-test in b-d and f-h, and with a one-way repeated measures ANOVA with post hoc Tukey test in e. Grey box in b and f indicates context B. *: p<0.05; **: p<0.01; ***: p<0.001.

Enhanced freezing in neutral context B was absent in mice that received corticosterone but did not undergo fear conditioning (Figure 1f-h), indicating that generalised freezing only occurs after an aversive experience. Together, this shows that increased corticosterone levels immediately after training elicit a generalised fear response in a neutral context, pointing to reduced specificity of the memory. Notably, the presence and predictability of the tone during training was required for this corticosterone-induced impairment in memory specificity, as following an unpaired training paradigm no effects of corticosterone were observed (Supplementary Figure 2).

To identify how corticosterone induced memory generalisation, we first examined neuronal activation in the DG, a region implicated in contextual memory and fear generalisation. We studied endogenous expression of the immediate early genes Arc and c-fos 90 minutes after training and corticosterone treatment, when both immediate early genes peak (Figure 2a). Corticosterone increased the number of c-fos+ cells, while Arc+ cell numbers were not affected (Figure 2b). Colocalisation of Arc+ cells with c-fos, which is induced in highly activated neurons, may indicate that within the Arc+ populations, the proportion of neurons with high activity patterns increased as a result of corticosterone treatment (Figure 2c). To determine whether corticosterone had a lasting effect on the size of the neuronal ensembles activated after fear conditioning, we used a transgenic mouse with destabilised
Figure 2. **a** Representative microscopic image showing Arc and c-fos expression 90 mins after training and corticosterone injection. Scale bar = 25 µm. **b** Corticosterone treatment increased the number of c-fos* cells in the DG (t(4)=3.44, p=0.03), while the number of Arc* cells was unaltered (t(4)=0.85, p=0.44). **c** Of the total Arc* population, more cells became c-fos* (t(4)=4.72, p=0.009). **d** Representative microscopic image showing dVenus expression in saline and corticosterone-treated mice. Scale bar = 250 µm. **e** The number of dVenus* neurons in the dorsal DG was increased in corticosterone-treated mice in the DG 24 hours after conditioning (t(30)=4.92, p<0.001). **f** Typical examples of mEPSC traces. **g** The frequency of the mEPSCs was enhanced in dVenus* neurons irrespective of treatment (interaction effect: F(1,13)=6.84, p=0.02). Corticosterone increased the mEPSC frequency in dVenus* neurons (p=0.04). **h** No effect of dVenus or treatment was observed in the mEPSC amplitude (main effect dVenus: F(1,13)=2.45, p=0.14).

*Figure continues on next page.*
Venus fluorescent protein (dVenus) expression under control of the Arc promotor (Arc::dVenus mice) \(^7\) (Figure 2d) to monitor activated neurons in the DG at 24 hours after conditioning \(^8\). We found that the number of dVenus+ neurons in the dorsal DG were significantly increased in corticosterone-treated mice 24 hours after training (Figure 2e). This is in line with earlier findings that later waves in Arc induction after training are crucial in the perpetuation of fear memories \(^6,9\). Together, these data indicate that enhanced corticosterone after training increased the size of activated DG neuronal ensembles. Since neural activation and excitability is relevant for the recruitment of neurons into a memory engram \(^10,11\), we used whole-cell patch clamp recordings to investigate the effects of corticosterone on the physiological properties of dVenus+ neurons and non-activated (dVenus-) neighbouring neurons after fear conditioning (Figure 2f). Irrespective of treatment, the frequency of mEPSCs was enhanced in dVenus+ neurons compared with dVenus- neurons, but corticosterone significantly enhanced the mEPSC frequency in dVenus+ neurons (Figure 2g). Corticosterone did not affect the mEPSC amplitude in
both cell populations (Figure 2h). Together with the increased population of c-fos+ neurons after corticosterone treatment (Figure 2b), the data suggest that corticosterone enhances overall activity of DG neurons, thereby biasing recruitment of neurons into the memory engram.

Next, the prolonged detectability of dVenus was used to investigate whether neurons that were recruited during training, were re-activated during exposure to context A and B. Arc::dVenus mice treated with corticosterone showed similar levels of c-fos+ cells in the DG in context A, but an increase in c-fos+ cells in context B (Figure 2i). In parallel, corticosterone-treated mice showed increased colocalisation of c-fos and dVenus+ neurons (Figure 2j), indicating that corticosterone increased reactivation of DG neurons in a neutral context B.

We next investigated whether the neurons that were activated by training and corticosterone treatment were causally involved in the
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expression of generalised fear. For this, we used a dual-virus variant of Targeted Recombination in Active Populations (TRAP)\(^9\), which enabled us to express an inhibitory G\(_i\)-DREADD (hM4Di\(^{12}\)) in dentate gyrus neurons under control of the Fos promoter in a Cre recombinase and 4-hydroxytamoxifen (4OHT) dependent manner (Figure 3a,b). After training plus corticosterone treatment, mice received 4OHT to allow expression of hM4Di-mCherry or mCherry alone (control) in c-Fos\(^+\) neurons in the dentate gyrus (Figure 3c,d). Groups did not differ in freezing behaviour during training (Supplementary Figure 3), nor when mice were re-exposed to context A (without CNO; Figure 3e). Thirty minutes before exposure to neutral context B, mice received CNO to suppress the activity of neurons that were tagged after training. Whereas corticosterone-treated control mice showed substantial freezing in context B, suppression of hM4Di\(^+\) neurons reduced freezing behaviour (Figure 3e) to a level that equalled control mice (comparable with Figure 1b). Importantly, freezing to the tone remained unaffected by CNO (Figure 3e), indicating that

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**Figure 3 (continued).** c. The centre of virus expression is depicted for all the animals in schematic coronal sections. d. Representative microscopic image showing the expression of hM4Di-tagged neurons. Scale bar = 125 \(\mu\)m. e. Freezing in context A did not differ between groups (\(t(15)=0.39, p=0.70\)). Silencing hM4Di-tagged neurons with CNO reduced freezing levels in the neutral context B (\(t(15)=4.97, p<0.001\)). Freezing to the tone was unaffected by silencing these neurons (\(t(15)=1.06, p=0.31\)). mCherry: \(n = 7\); hM4Di-mCherry: \(n = 10\). Data are means ± s.e.m. Statistical analysis was done with Student’s unpaired t-test. *: \(p<0.05\); **: \(p<0.01\); ***: \(p<0.001\).
DG ensembles were not involved in auditory fear memory, but were necessary for the expression of generalised contextual fear memory.

Our present data show that corticosterone reduces memory specificity, in line with an earlier study demonstrating that corticosterone elicits memory impairments and inappropriate fear responses\(^4\). Memory specificity is related to the size (i.e. the number of neurons) of neuronal ensembles\(^14\). In line with this, we find that glucocorticoid effects on memory generalisation were accompanied by an increase in the number of activated DG neurons within the first 24 h after conditioning and an increased percentage of reactivated DG neurons upon memory expression in a safe context. Thus, the increase in neuronal ensemble size may result from a corticosterone-evoked increase in excitation of DG neurons through enhanced synaptic transmission, thereby promoting recruitment of additional cells into the fear memory trace. Alterations in the excitation of engram cells may therefore affect the size of a fear-encoding ensemble and induce fear generalisation. In line with this, we found that suppression of DG neuronal ensembles that were activated by fear conditioning and corticosterone prevented the expression of generalised contextual fear memory (Figure 3e).

The present study demonstrates that GCs reduce memory specificity, thereby promoting fear memory generalisation. We further show for the first time that GCs alter the size of distinct neuronal ensembles in the DG. Selectively suppressing these neuronal ensembles restores memory specificity. Understanding how glucocorticoids modify the delicate balance between memory strength and fear generalisation on the one hand and activity in neural ensembles on the other, may open novel avenues for the development of treatments for stress-related disorders such as PTSD.

2. Materials and Methods

2.1. Mice and breeding

WT mice (Harlan, The Netherlands) and Arc::dVenus mice (kindly provided by prof. dr. Steven Kushner, Erasmus University Rotterdam) that were backcrossed for more than 10 generations into C57BL/6J mice\(^7\), were used. Mice were maintained under standard housing conditions (temperature 20-22 °C, 40-60% humidity) on a 12 h light/dark cycle with standard chow and water available ad libitum. A radio provided background noise. All experiments were performed during the light phase, using male adult mice (postnatal weeks 8–12). Mice were individually housed for 14 days prior to the start of experiments. Experimenter
blinding was performed whenever possible. All experiments were conducted under the EU directive 2010/63/EU for animal experiments and were approved by the animal welfare committee of the University of Amsterdam.

2.2. Fear conditioning

Fear conditioning was performed using a standard fear conditioning chamber with a stainless steel grid floor connected to a shock generator\textsuperscript{15-18}. Mouse behaviour was recorded by a camera connected to a computer with Ethovision software (version 13.0, Noldus, The Netherlands), which automatically scored freezing behaviour. On the day of fear conditioning, mice receiving paired training were placed in the conditioning chamber 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) (2 s, 0.2 mA). The inter-stimulus interval between tone-shock presentations was 60 s. In the unpaired training group, mice received the identical CS and US stimuli, but in an explicitly unpaired sequence. The first shock was delivered 180 s after introducing mice in the conditioning chamber, followed by 2 tones 30 s later, 20 s apart, followed 30 s later by a shock, and 30 s later another tone, followed 60 s later by the final shock. CS-evoked freezing was tested 24 hours after conditioning in the same (180 s), or a completely novel context (180 s baseline, 30 s tone). Naïve mice remained in their standard housing conditions until immediately prior to behavioural testing for context- or tone-evoked freezing.

2.3. Drug treatment

Corticosterone (Sigma) (16 mg/ml dissolved in 99.9% EtOH and diluted 40x in saline; final dose: 2 mg/kg, injection volume: 5 µl/g body weight) or both corticosterone and RU486 (Sigma) (40 mg/ml dissolved in 99.9% EtOH and diluted 20x in arachide oil; final dose: 10 mg/kg, injection volume 5 µl/g body weight) was injected intraperitoneally (i.p.) immediately following fear conditioning. The appropriate vehicle solution was administered to control mice accordingly.

2.4. 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\textsuperscript{19}, and were cryo-protected overnight in 30%
sucrose/0.1 M PB. Coronal sections of 40 µm, rostral to caudal, were collected using a freezing microtome and stored in antifreeze solution (30% ethylene glycol, 20% glycerol, 50% 0.05M PBS) at -20 °C until staining. All stainings were performed on parallel series from the same brains within a breeding cohort. Sections were incubated with Fab Fragments (1:200, Affinipure Fab Fragment Goat anti-mouse IgG, Lot # 121337, Jackson ImmunoResearch) in case a mouse antibody was used. 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.

The primary antibodies that were 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 washed with 0.1 M PBS (pH 7.4), and incubated with corresponding secondary antibodies in blocking mix for 2 h at room temperature. As secondary antibodies we 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). They were washed with 0.1 M PBS, placed in 0.01 M PB and mounted on slides. 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 dentate gyrus 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 rostro-caudal axis. Using a Nikon DS-Ri2 microscope, representative images of 10x magnification were systematically captured, and cells were manually counted. To assess colocalisation, cells (as assessed by DAPI) were assigned to one of the following categories: 1) Arc, c-fos, 2) Arc, c-fos$, 3) Arc, c-fos$, 4) Arc, c-fos$, and the portion of cells colocalising was calculated per animal.

### 2.5. Stereotactic microinjections

Before surgery, mice were pretreated with Temgesic (0.1 mg/kg) (RB Pharmaceuticals, UK), anesthetised with isoflurane, and placed in a stereotaxic frame. Lidocaine (2%, Sigma-Aldrich Chemie N.V., The Netherlands) was topically applied to the skull to provide local analgesia. Skin was retracted and holes were drilled in the skull above the dorsal hippocampus. AAV-Fos::CreERT2 (titer: 1.2 x 1012) and Cre-dependent AAV-hSyn:DIO-hM4Di and AAV-hSyn::DIO-
mCherry (titer: 5-6 x 1012) were packaged as serotype 5 virus. A virus mixture of AAV5-Fos::CreERT2 and Cre-dependent AAV (ratio 1:500, AAV-Fos-CreERT2 was injected at a final titer of 2.4 x 109) was infused bilaterally in the dentate gyrus using microinjection glass needles (5 µl; flow rate: 0.1 µl/min; AP = -2.2; ML= ±1.2; DV= -2.2, relative to Bregma). After surgery, mice remained in their home cage for 3 weeks until the start of behavioural experiments.

2.6. 4-Hydroxytamoxifen (4OHT) treatment

4OHT (H6278, Sigma-Aldrich Chemie N.V., The Netherlands) (50 mg/ml 4OHT dissolved in DMSO (D8148, Sigma-Aldrich Chemie N.V., The Netherlands) and diluted 10x in saline containing 2% Tween80 (P1754, Sigma-Aldrich Chemie N.V., The Netherlands) and 10x in saline; final concentration: 2.5 mg/ml 4OHT, 5% DMSO and 1% Tween80 in saline) was injected 2 h after training in hM4Di-mCherry and control mice (final dose: 25 mg/kg).

2.7. Chemogenetic intervention

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

2.8. Electrophysiology

Mice were sacrificed through quick decapitation, followed by rapid removal of the brain in ice-cold oxygenated (95% O2/5% CO2) solution containing (in mM): Cholinechloride (120), glucose (10), NaHCO3 (25), MgSO4 (6), KCl (3.5), NaH2PO4 (1.25), CaCl2 (0.5). Coronal slices (250 µm) were cut using a microtome (Leica VT1000S). For recovery, slices were incubated for 20 minutes in warm (32 °C) oxygenated standard artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl (120), KCl (3.5), MgSO4 (1.3), NaH2PO4 (1.25), CaCl2 (2.5), glucose (10), NaHCO3 (25), after which the sections were maintained at room temperature (20 °C). Sections containing the dorsal hippocampal CA1 area (bregma -2.0 mm to -3.2 mm) of the left hippocampus were placed in a recording chamber with a constant flow of oxygenated aCSF.

Whole cell patch clamp recordings were made using an AXOPATCH 200B amplifier (Axon Instruments, USA), with electrodes from borosilicate glass (1.5 mm outer diameter, Hilgerberg, Malsfeld, Germany). The electrodes were pulled
on a Sutter (USA) micropipette puller. 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 MΩ. Under visual control (40X objective and 10X ocular magnification) the electrode was directed towards a neuron with positive pressure. Once sealed on the cell membrane (resistance above 1 GΩ) the membrane patch under the electrode was ruptured by gentle suction and 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 MΩ and <2.5 times of the pipette resistance with a shift of <20% during the recording, were accepted for analysis. Data acquisition was performed with pClamp 8.2 and analysed off-line with Clampfit 9.0. Miniature excitatory postsynaptic currents (mEPSCs) were recorded at a holding potential of -70 mV. Tetrodotoxin (0.25 µM, Latoxan, Rosans, France) and bicuculline methobromide (20 µM, Biomol) were added to the buffer to block action potential induced glutamate release and GABAA 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 in each cell.

### 2.9. Statistical analysis

Data were analysed using SPSS 22.0 (IBM software). All data are expressed as mean ± standard error of the mean (SEM). Data were considered statistically significant when p<0.05. Outliers were determined using a Grubb's test. Independent-samples t-tests were performed to assess differences between Saline and CORT-treated mice. Appropriate corrections were applied when assumption of homogeneity of variance was not met. When assumption of normality was not met, Mann-Whitney test was conducted. A repeated measure ANOVA was performed to assess freezing behaviour during the different tones. Greenhouse-Geisser correction was applied when the assumption of sphericity was violated.

### 3. Acknowledgements

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4. Author contribution

SLL, MCvdO, and HJK contributed to conception and design of the study; SLL organised the database; SLL, RJvdL and NI conducted the experiments, SLL and NI performed the statistical analysis; SLL wrote the first draft of the manuscript; SLL, MCvdO, NI, PJL and HJK contributed to manuscript revision, read and approved the submitted version.
5. References

1. de Quervain D, Schwabe L, Roozendaal B. Stress, glucocorticoids and memory: implications for treating fear-related disorders. *Nat Rev Neurosci* 2017; **18**: 7–19.


6. Supplementary figures

Supplementary Figure 1: Plasma corticosterone levels measured at different time points after fear conditioning. Corticosterone injection results in increased circulating corticosterone levels 30 and 60 minutes after the fear conditioning. Data are means ± s.e.m.
Supplementary Figure 2: Unpaired or foreground conditioning (no tone present) does not result in differences in freezing after corticosterone injections. a) Schematic of the unpaired conditioning paradigm. b) Response of mice treated with saline or corticosterone following an unpaired fear conditioning paradigm to the context and tone. No difference in freezing levels was observed, c) nor in the ratio of freezing between context A over B, or d) between tone and cue freezing, as measures for memory specificity. n = 8 mice/group. Data are means ± s.e.m. Statistical analysis was done with Student’s unpaired t-test. Black thin lines: individual data points of saline-treated mice; black thick line: average of all saline-treated mice; red dashed thin lines: individual data points of corticosterone-treated mice; red dashed thick line: average of all corticosterone-treated mice.
Supplementary Figure 3. Freezing levels during training were comparable between mCherry and hM4Di-injected mice ($F(1,15)=0.15$, $p=0.71$). mCherry: $n=7$; hM4Di-mCherry: $n=10$. Data are means ± s.e.m. Statistical analysis was done with a repeated measures ANOVA.