Social Reactivation of Fear Engrams Enhances Recall and Reinstatement

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Summary

The power of one's social environment to bidirectionally modulate cognitive abilities is well documented, but the processes by which social experiences impact the cellular substrates of memory remain unknown. Here we show that social experience and exposure to ambient stimuli emitted by stressed conspecifics, but not individually experienced physical stress, enhances the recall and reinstatement of previously acquired fear memories. Activity-dependent tagging of cells in the dentate gyrus of the hippocampus during fear learning revealed that these ensembles were endogenously reactivated during the social, but not directly stressful, experiences. These reactivated cells were revealed to be functional engrams, as optogenetic stimulation of the cells active during the social experience was sufficient to drive fear-related behaviors only in animals that had previously been fear conditioned. Our findings suggest that social encounters can reactivate pre-existing engrams and thereby strengthen discrete memories.

Keywords: social, engram, reinstatement, recall, dentate gyrus

Introduction

For group-living species such as humans and rodents, conspecific interactions directly impact social learning and memory (Hoppitt and Laland, 2008; Leblanc and Ramirez, 2020), and pervasively shape emotion (Neumann and Strack, 2000; Reavis et al., 2015; Williamson and Austin Williamson), attention (Caplin et al., 2015), and cognitive ability (Dause and Kirby, 2019; Doulames et al., 2014; Heimer-McGinn et al., 2020; Kuhlmann et al., 2005). Higher-order cognitive processes such as memory within a social brain are thus interlaced with social influences. While a traditional laboratory rodent cage offers limited inter-individual experiences compared to the natural world, there still exists a rich multi-modal landscape of communication, including auditory calls (Castellucci et al., 2018; Peleh et al., 2019; Simola and Brudzynski, 2018; Wöhr et al., 2016), chemical signaling (Biasi et al., 2001; Bredy and Barad, 2009), and tactile stimulation (Ebbesen et al., 2019; Hertenstein). Rodents establish social hierarchies even in single-sex cages containing a small number of animals (Barker et al., 2017; Fan et al., 2019; Williamson et al., 2016), and the dominance relationships between rodents can influence how they learn from one another (Jones and Monfils, 2016). The absence of such social encounters (e.g. single housing animals) results in cognitive impairments and depression-like phenotypes (Liu et al., 2020), likely obscuring how the social brain has evolved to function. It is thus important to understand the relationship between social context and how individuals process memories. Recent work suggests that the dentate gyrus (DG) of the hippocampus and the basolateral amygdala (BLA) serve as hubs for the enduring physiological and structural changes that accompany memory, or "engrams."(Davis and Reijmers, 2018; Duvarci and Pare, 2014; Janak and Tye, 2015; Josselyn and Tonegawa, 2020; Sun et al., 2020) As social interaction recruits hippocampal (Montagrin et al., 2018) and amygdalar (Gothard,

2020) circuitry, we hypothesized that pre-existing engrams in these regions can be modulated by social experiences and lead to changes in memory expression.

Results

Social stimuli bidirectionally modulate subsequent fear recall in a sex-dependent manner

To assess how social experiences influence existing memories, male and female mice were subjected to social or non-social salient events in the day between contextual fear conditioning (FC) and memory recall tests (**Fig. 1a**). A socially salient experience was provided in various forms: free interaction with a juvenile intruder placed in the subject's homecage (Defensor et al., 2012a; Felix-Ortiz and Tye, 2014) (Juvenile Intruder group); exposure to a recently shocked cagemate in the homecage through a 1-way mirror that permits unidirectional visual access for the shocked mouse to see the cagemates on the other side (1-Way Mirror group), or through a 1-way mirror with a black covering blocking the shocked mouse's view of the cagemates (Opaque group), or without any obstruction (Full Interaction group). A non-social salient experience was provided in the form of individual restraint stress in a tube (Restraint group) (**Supplementary Methods, Fig. 1a**). Additional control groups were exposed to novel objects including the 1-way mirror (Mirror No Mouse group) and the tubes (Tubes group) but did not undergo the associated social or stressful stimuli (**Supplementary Methods**). Males exhibited higher levels of baseline freezing and froze more than females after the first two shocks of fear conditioning, after which freezing levels were similar (**Fig. 1b**), corroborating tone-cued experiments that report higher levels of freezing in males during acquisition (Clark et al., 2019). For males, Juvenile Intruder groups exhibited amplified context-specific recall compared to Cagemate control groups; 1-Way Mirror groups exhibited amplified context-specific recall compared to Opaque, Full Interaction, and Mirror No Mouse

groups; and Restraint groups exhibited similar recall as Tube controls (**Fig. 1c-d, 1f-g**). Positively valenced socially salient encounters with females did not amplify freezing compared to cagemate controls (**Fig. S2**). For females, 1-Way Mirror induced the opposite effect than for males, lowering context-specific recall compared to Mirror No Mouse, while Restraint had no effect on freezing (**Fig. 1e and 1h**). These results reveal that socially salient, but not directly physical, events alter subsequent contextual fear recall without impacting generalization, in a sex-specific manner.

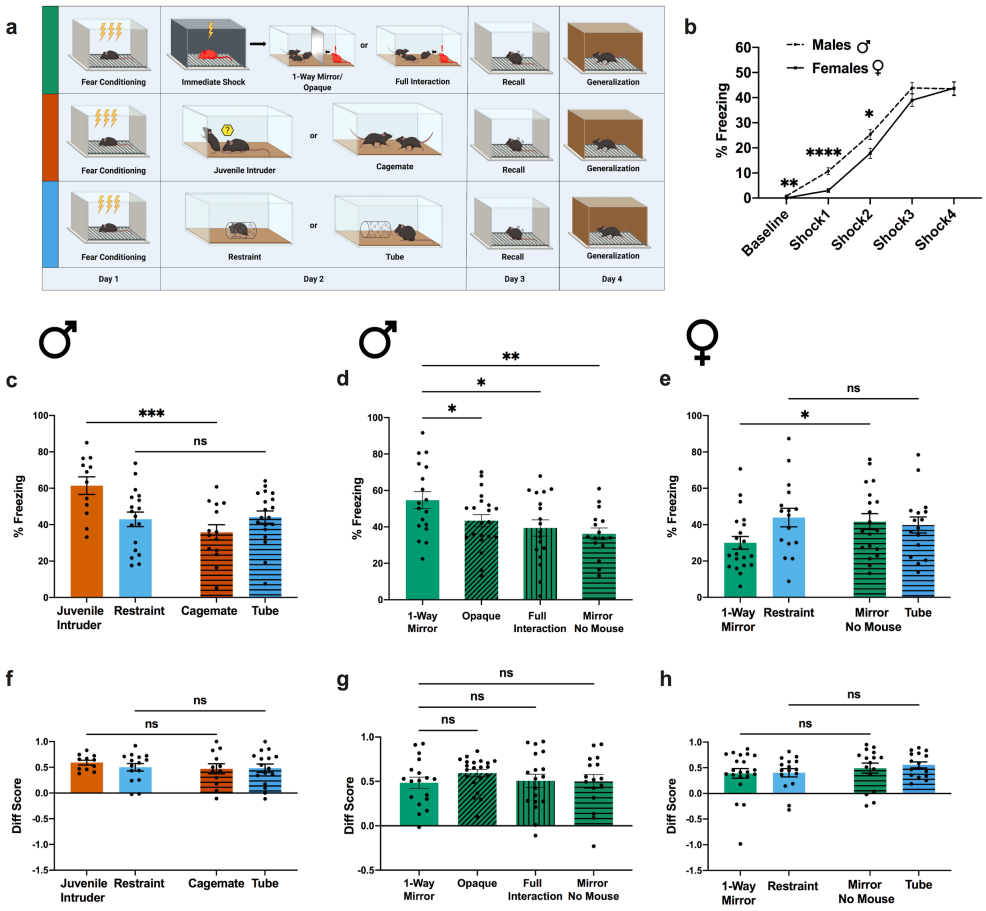

Figure 1. Social stimuli bidirectionally modulate subsequent fear recall in a sex-dependent manner a) Schematic representation of behavioral schedule. **b)** Freezing levels during fear acquisition for males and females (2way ANOVA with Holm-Sidak's multiple comparison test, n=69 males, 63 females). **c, e, f, h)** Freezing levels during fear memory recall (upper) and

generalization (lower) tests (2Way ANOVA with Holm-Sidak's multiple comparison test, males - n=12 Juvenile Intruder, 14 Cagemate control, 18 Restraint**,** 19 Tube control, females - n=21 1-Way Mirror, 19 Mirror No Mouse, 16 Restraint, 16 Tube). **d and g)** Freezing levels during fear memory recall (upper) and generalization (lower) tests (1Way ANOVA with Holm-Sidak's multiple comparison test, n=18 1-Way Mirror, 21 Opaque, 18 Full Interaction, 16 Mirror No Mouse). Other than in B, data shown as mean +/- SEM. n.s. $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, detailed statistics shown in Supplementary Text.

Ambient social emissions of stress reinstate cagemates' extinguished fear memory

Hereafter, we focused on socially-induced memory enhancement in male mice, conjecturing that the enhancement of freezing during recall tests was due to socially-induced reactivation of fear engrams. While outside the scope of our current study, we also speculate that the dampening of recall in females is due to a mechanism involving interference (Chan and LaPaglia, 2013; Forcato et al., 2007; Gisquet-Verrier and Riccio, 2018) by the formation of a social memory, which has been suggested to be longer-lasting and based on different circuitry than in males (Bluthe´ and Dantzer, 1990; Thor, 1980). We next tested whether memory was still susceptible to socially-driven enhancement after extinction learning. Freezing during the reinstatement test was highly correlated with freezing during the first 3 minutes of the second extinction session in groups subjected to experiences that did not affect fear recall (Cagemates, Tube, Opaque, and Full Interaction groups) (**Fig. 2b-d**). This finding indicates that the experience between extinction and reinstatement test had not modulated reinstatement. However, in the Juvenile Intruder and 1-Way Mirror groups (i.e. groups that exhibited enhanced fear recall after social experiences) freezing levels during the reinstatement test were uncorrelated from freezing levels during extinction, indicating that the

social experience had indeed impacted reinstatement (**Fig. 2e**). To quantify the degree of reinstatement, we subtracted the freezing rate during the first three minutes of the second extinction session from the freezing rate during the first 3 minutes of the reinstatement test. We found that, in congruence with the recall results (**Fig. 1d**), 1-Way Mirror groups exhibited a greater degree of reinstatement than the Opaque or Full Interaction groups (**Fig. 2f**), suggesting that visually-perceived social context can bias the recently shocked animal's distressed emissions and that social buffering via physical interaction with cagemates can mitigate these emissions. Here we defined reinstators as mice who froze greater than or equal to 5% higher during the reinstatement test than during the first three minutes of the last extinction session; ~15% of mice were reinstators for 1-Way Mirror, (**Fig. 2g**) and a generalization test showed that their reinstatement was context-specific (Mobbs et al., 2019) (**Fig. 2h**). As social hierarchy status can determine degree of social learning, we also tested whether the dominance status of the stressed mouse, as defined by dominance tube tests, influenced the cagemates' reinstatement behavior (**Fig. S5a**). Cagemates exhibited similar reinstatement scores after exposure to the most dominant vs most subordinate stressed individual, suggesting that dominance role does not impact influence on cagemates' fear memory (**Fig. S5b**). Collectively, these findings demonstrate that exposure to auditory-olfactory stimuli from a stressed cagemate induces context-specific reinstatement of fear.

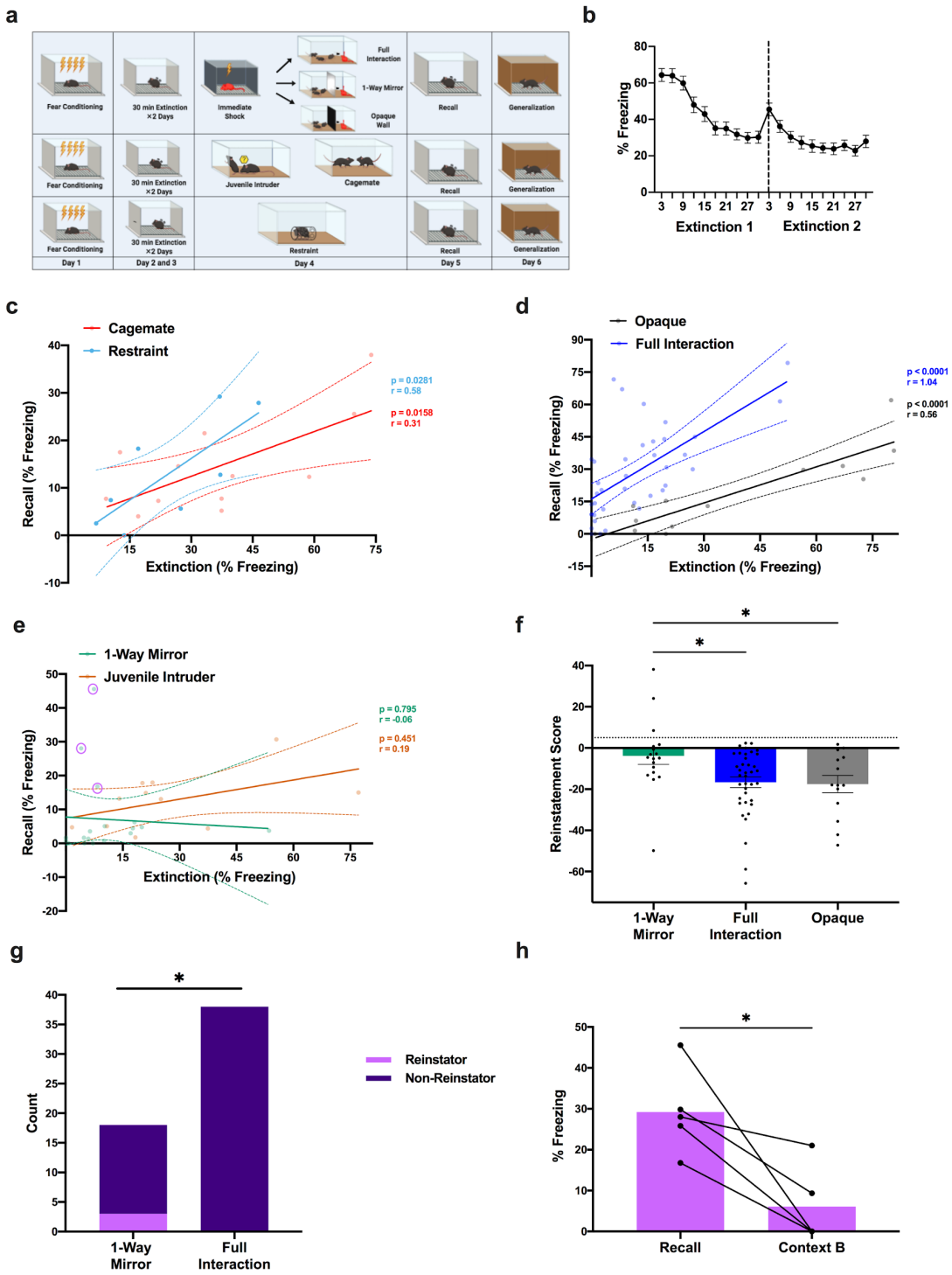

Fig. 2. Ambient social emissions of stress reinstate cagemates' extinguished fear memory. a) Behavioral timeline for the 6 groups shown. **b)** Freezing throughout extinction, dashed line indicates first 3 minutes of last session. **c-e)** The relationship between freezing during the first 3

minutes of the last extinction session and during the first 3 minutes of recall. Solid lines show linear regressions, dotted lines at 95% confidence bands (Pearson correlation tests). **f)** Comparison of the increase in freezing from the first 3 minutes of the last extinction session to the first 3 minutes of recall for the three types of exposure to a recently shocked cagemate; i.e. reinstatement score. (1 Way ANOVA, n=18 1-Way Mirror, n=38 Full Interaction; n=14 Opaque) **g)** Proportion of reinstators (animals who freeze >5% more during recall than extinction) in the 1-Way Mirror and Full Interaction group (Fisher's Exact test, n=18 1-Way Mirror, n=38 Full Interaction). **h)** Freezing during recall test compared to generalization test for reinstators (Student's paired t test, n=5). In e-f, data shown as mean +/- SEM. n.s. $p>0.05$, *$p<0.05$, **$p<0.01$, detailed statistics shown in Supplementary Text).

Socially salient experience reactivates pre-existing fear ensembles

To determine whether fear memories are reactivated during the social experience for males, we used a cFos-based activity-dependent viral strategy (**Fig. 3a, left**) to track fear engrams processed in the DG and BLA post-fear conditioning **(Fig. 3c**). Juvenile Intruder groups had more active cells, as identified by cFos expression, than 1-Way Mirror groups in both the DG and BLA (**Fig. 3d**). Both Juvenile Intruder and 1-Way Mirror groups exhibited reactivation of fear ensemble cells in the DG, and this effect was not observed in Restraint or Immediate Shock groups, nor does it occur in a neutral context (Tanaka et al., 2014), indicating the socially-specific nature of this reactivation. (**Fig. 3e, left**). Conversely, in the BLA, both Juvenile Intruder and Restraint groups displayed reactivation of tagged cells (**Fig. 3e, right**). We observed a significant correlation between the number of reactivated cells in the DG and in the BLA in only the Restraint groups (**Fig. 2h, Table S2**). To test whether the reactivation of the fear memory was specific to events happening close in

time to when engram cells were active, we tracked engram cells' reactivation in 1-Way Mirror and Restraint groups following a two day extinction protocol (**Fig. 3a**), after which mice no longer expressed fear in the original context (**Fig. 3b**). The number of cells active during the two types of experience did not differ in either brain region (**Fig. 3f**). Reactivation of fear ensemble cells occurred in the BLA during both social and non-social stress, and in the DG only during the social experience (**Fig. 3g**). Collectively, these data suggest that specifically social situations reactivate subsets of fear engrams in the DG.

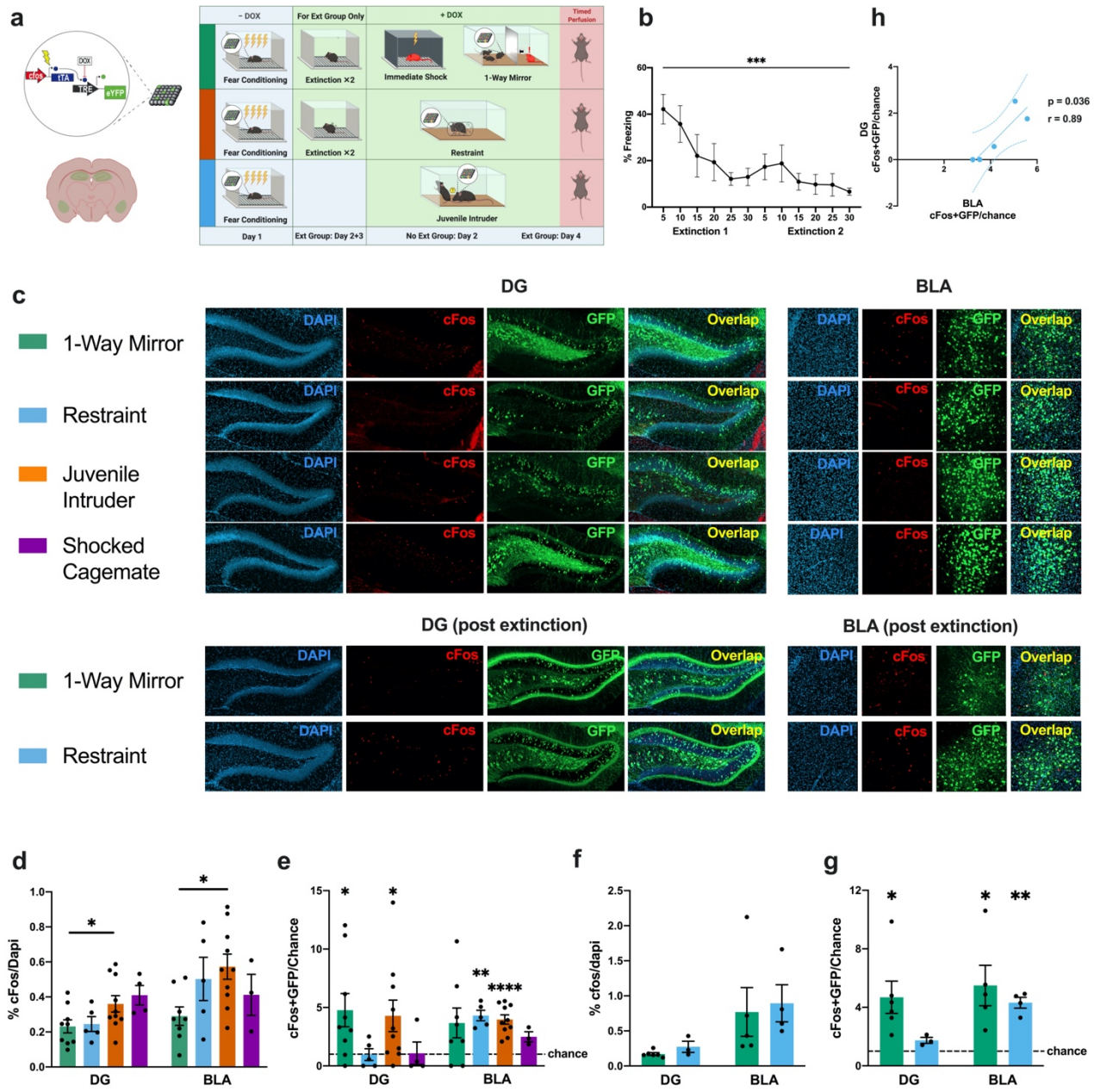

Figure 3. Socially salient experience reactivates pre-existing fear ensembles. a) Schematic representation of the doxycycline mediated viral tagging construct (left) and experimental design (right). **b)** Freezing throughout extinction training (RM 1Way ANOVA, Holm-Sidak's multiple comparisons, n=9). **c)** Confocal visualization of DG and BLA histology showing cFos-tTa+Tre-EYFP labelled cells (active during FC, green) and cFos+ cells (active during the different types of stress exposure, red). Left - without fear extinction, Right - following fear extinction. **d, f)**

Proportion of cFos+ cells per DAPI in the DG and BLA (1way ANOVA with Holm-Sidak's multiple comparisons test), d - without extinction, f - with extinction. **e, g)** Amount of overlap between the two labeled populations normalized over chance (1-sample t tests compared to chance=1), e - without extinction, g - with extinction. **h)** Correlation between overlap in the DG and BLA for the restraint stress group (Pearson's R, n=6). Other than in h, data shown as mean +/- SEM. n.s. $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, detailed statistics shown in ST3.

We next used an activity-dependent chemogenetic strategy to test whether DG fear ensembles active during the social experience were necessary for the subsequent enhancement in memory recall (**Fig. S4a**). Intriguingly, the EYFP control group, which received intraperitoneal CNO (**Supplementary Methods**) prior to the social experience, froze significantly less than non-injected animals (**Fig. S4c**), ostensibly due to a combination of injection-related stress and CNO metabolites (Mahler and Aston-Jones, 2018). The EYFP group did not freeze significantly less than saline controls, supporting a role of injection-related stress in blocking the effect (**Fig. S4c**). While the impact of CNO injection occludes our ability to determine the impact of hM4Di inhibition in the experimental group, subsequent generalization tests revealed that the EYFP and hM4Di groups exhibited significantly different variances in context-specificity of fear: while the EYFP group exhibited a normal distribution, the hM4di group did not pass normality tests and was skewed toward lower difference scores (i.e. high generalization) (**Fig. S4d-e**). This experiment indicates that preventing activation of the fear engram during the social encounter peturbs the specificity of subsequent fear expression.

Reactivated hippocampal ensembles are functional fear engrams

Next, we wanted to investigate the functional properties of the cells which were active during exposure to a stressed cagemate in previously fear-conditioned animals. To this end, we tagged DG or BLA cells with channelrhodopsin-2 during our 1-way mirror paradigm (**Fig. 4a-d;** See Methods). The following day, these cells were stimulated in a novel context to test their capacity to drive fearful behavior. Importantly, the size of the labeled ensembles did not differ between fear conditioned and non-fear conditioned animals (**Fig. 4e**), and although stimulation of cells active in a neutral setting after fear conditioning does not drive freezing (Liu et al., 2012), 20Hz stimulation of the DG ensembles active during the social experience drove significantly higher levels of freezing during the light-on sessions as well as de-arousing self-grooming (Kalueff et al., 2016) in the light-off period following stimulation, specifically in previously fear conditioned animals (**Fig. 4j, 4k**). Although cells previously active during FC were also reactivated in the BLA during both the 1-way mirror and restraint stress groups (**Fig. 4h**), counterbalanced 20Hz and 4Hz stimulation of cells tagged during 1-way mirror in the BLA did not drive fear compared to non-fear conditioned animals (**Fig. 4f, 4g**). A subset of the animals subsequently reinstated the extinguished fear memory (**Fig. 4h, 4l**). These results demonstrate that optogenetic stimulation of DG cells active during exposure to a stressed cagemate through a 1-way mirror drives fear only if mice have previously been fear conditioned.

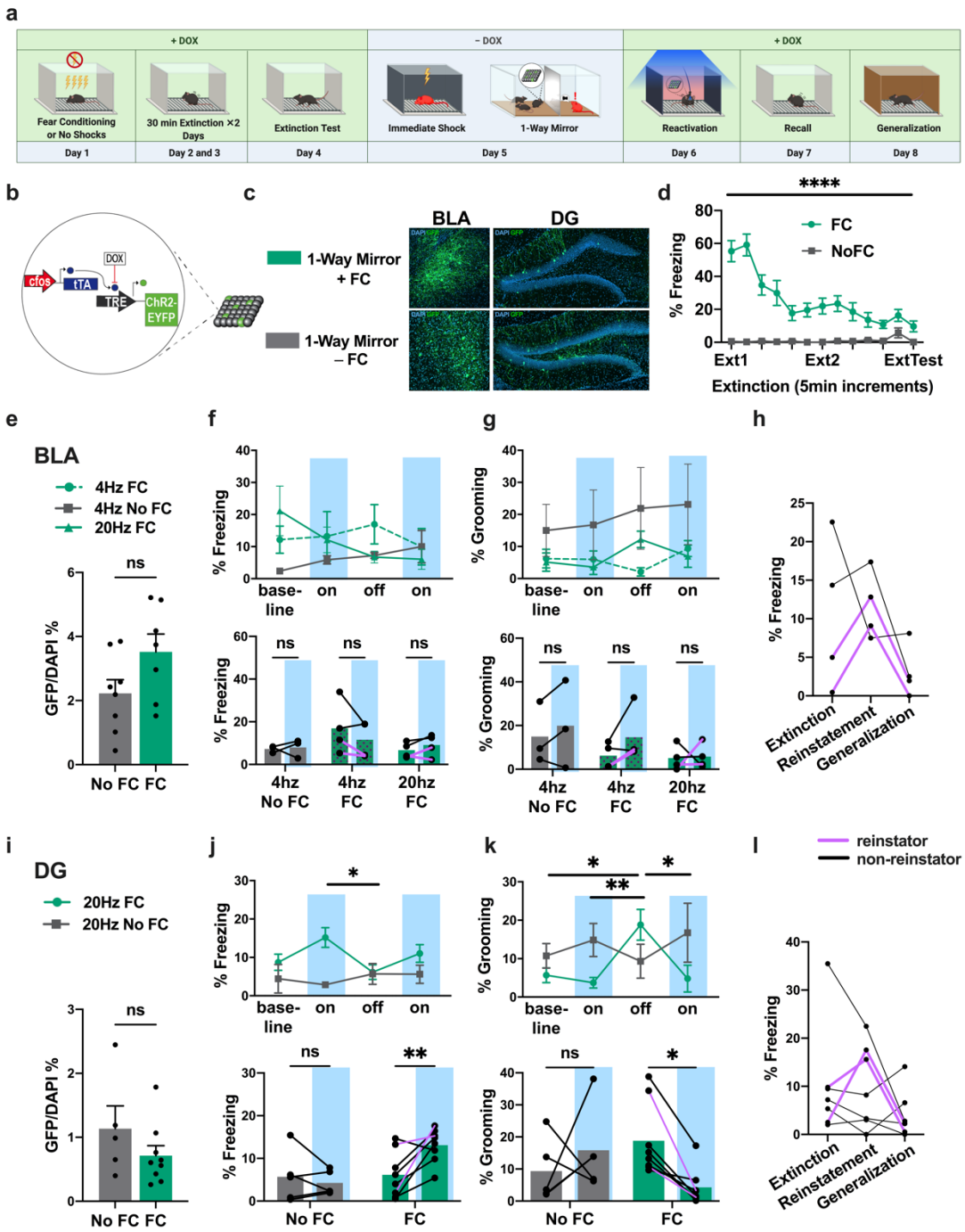

Figure 4. Reactivated hippocampal ensembles are functional fear engrams. a) Behavioral schedule. **b)** Schematic representation of activity-dependent engram tagging strategy. **c)** Representative images of DAPI+ cells (blue) and Chr2-EYFP+ cells (green) in the BLA (left) and DG (right). **d)** Freezing levels throughout extinction (n=11 FC, n=7 NoFC). **e and i)** Proportion of

cells tagged during 1-way mirror or resident intruder in the BLA (E) and DG (I) in FC vs No FC groups (student's t-test, DG - n=5 NoFC, n=9 FC, BLA - n=4 NoFC, n=7 FC). **f and j)** top - freezing levels throughout optogenetic stimulation of cells active during 1-way mirror in BLA (f) and DG (j) in FC vs non FC groups, bottom - freezing levels for the light-off and the averaged light-on sessions (2-way ANOVA, Sidak-Holm's multiple comparison's test, DG: n=8 FC, n=5 NoFC, BLA: n=3 NoFC, n=4 FC). **g and k)** top - grooming levels throughout optogenetic stimulation of cells active during 1-way mirror in BLA (g) and DG (k) in FC vs non FC groups, bottom - grooming levels for the light-off and the averaged light-on sessions (2-way ANOVA, Sidak-Holm's multiple comparison's test, DG: n=8 FC, n=5 NoFC, BLA: n=3 NoFC, n=4 FC). **h and l)** Freezing levels during extinction test, reinstatement test, and generalization test. BLA on top, DG on bottom. Reinstators are defined by >5% increase in freezing between extinction and reinstatement (purple lines). Data shown as mean +/- SEM. n.s. $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, detailed statistics shown in Supplemental Text.

Discussion

Memory is a malleable phenomenon that can be altered by intervening experiences, as associated context or cues can reactivate engrams and thus make them labile during incubation (Inda et al., 2011; Kim et al., 2014; Pickens et al., 2009; Sara, 2000). Our findings here reveal that social encounters have the capacity to reactivate and modify hippocampal engrams even in the absence of such external reminders. We show that in male mice, socially salient, but not physically stressful, events reactivate memory traces in the DG and potentiate subsequent memory expression, supporting the theory that endogenous reactivation can strengthen recall (Sara, 2000). The impact of exposure to a stressed conspecific through the 1-way mirror compared to the opaque wall

suggests that distressed mice emit different auditory-olfactory stimuli depending on their perceived social context. We speculate that the lack of a memory enhancing effect as a result of full interaction with a recently shocked cagemate could be due to social buffering (Leblanc and Ramirez, 2020) of the distressed mouse's emissions, or to the intensity of direct, physical social contact with the distressed cagemate.

Our findings provide evidence that socially-induced reactivation of memory engrams can strengthen the behavioral expression of the memory. Consistent with this notion, the reactivation of fear engrams is thought to be the mechanism by which multiple exposures to the contexts (Inda et al., 2011) or cues (Kim et al., 2014) present during conditioning can strengthen memories in the days after learning. We demonstrate that a single social encounter can reactivate a subset of the DG fear engram (**Fig. 3**) and produce similar enhancement of memory expression (**Fig. 1**). Strikingly, the degree of cellular reactivation during the social encounter was similar to previously reported results in animals re-exposed to the original fear context (Chen et al., 2019). Given the malleability of engrams(Ramirez et al., 2013; Redondo et al., 2014), the natural reactivation of a fear memory ensemble during a stressful social experience might be expected to add additional negative valence from the current experience to the representation of the former, contributing to the observed enhancement of fear (**Fig. 1**) beyond the effects of reactivation in a neutral context by associated cues or context.

Concurrent tracking of fear engrams in the DG and BLA during varied experiences revealed distinct patterns of reactivation. During restraint, there is non-significant reactivation in the DG, and this small amount of reactivation is potentially driven by the correlated significant level of reactivation in the BLA (**Fig. 3e, 3h**). However, DG fear engram reactivation during social experience with a stressed conspecific is discorrelated with BLA engram reactivation (**Table S3**),

suggesting that social inputs to the DG induce an engram excitation process distinct from non-social stress. The increased level of DG activity during exposure to a juvenile intruder compared to a stressed cagemate behind a 1-way mirror, (**Fig. 3d**) despite both experiences occurring in the animal's homecage, highlights the DG's role in encoding experiences beyond physical contexts per se (Stefanini et al., 2020). We posit that the higher level of activity in the BLA and DG during exposure to a juvenile intruder reflects the richness of direct social interaction during the juvenile intruder's presence that is not permitted in the 1-way mirror paradigm.

Our data suggests that during salient social experiences, the DG is concurrently reactivating a pre-existing fear engram and processing the current social stimuli. The coactivation of ensembles leads to co-allocation and the linking of memories, as shown in both mouse (Cai et al., 2016) and human (Yetton et al., 2019) studies. We therefore posit that the reactivated subset of the fear ensemble was co-allocated to form part of the ensemble encoding the social encounter, perhaps explaining why the total number of cells active during the social exposure was similar regardless of prior fear conditioning (**Fig. 4i**) and why stimulation drove fearful behaviors only in the previously fear conditioned animals (**Fig. 4j and 4k**). In the context of the social experience, this functional capacity of the active ensemble to drive freezing was quiescent (example **Movie S1** and **Movie S2**), however, we suggest that the documented deficits in social interaction following trauma (Cisler et al., 2015; Dutton et al., 2016; LaMotte et al., 2017; Siegmund and Wotjak, 2007) could in part be driven by hippocampal fear engrams activation during social events.

We propose that the capacity of social experiences to reactivate memories contributes to the enriching effects of a social environment on cognition (Heimer-McGinn et al., 2020; Shafto et al., 2019). From an evolutionary perspective, a stressed conspecific or their ambient auditory-olfactory emissions might signal a dangerous situation in which it is adaptive to hone one's own memories to

guide decision making. Together, our findings illuminate how the mnemonic contents of an organism's brain may be modulated throughout the course of social interactions to bias future behavioral responses.

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Supplementary Materials for

Social Reactivation of Fear Engrams Enhances Recall and Reinstatement

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This PDF file includes:

Materials and Methods
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Captions for Data S1 to S5

Other Supplementary Materials for this manuscript include the following:

Movies S1 to S2

Materials and Methods

Surgery

For all surgeries, mice were initially anesthetized under 3.5% isoflurane inhalation and then maintained during surgery at 1.0-2.0% isoflurane inhalation through stereotaxic nosecone delivery. Ophthalmic ointment was applied to both eyes to provide adequate lubrication and prevent corneal damage. The hair above the surgical site was removed with scissors and subsequently cleaned with alternating applications of betadine solution and 70% ethanol. 2.0% Lidocaine HCl was injected subcutaneously as local analgesia prior to 10-15mm midsagittal incision of the skin. For optogenetic implant surgeries, two bone anchor screws were secured into the cranium, one anterior and one posterior to the target injection and fiber placement sites. All animals then received bilateral craniotomies with a 0.6 mm drill-bit for dorsal dentate gyrus (dDG) and basolateral amygdala (BLA) injections. For all dDG and BLA surgeries, a 10µL air-tight Hamilton syringe with attached 33-gage beveled needle was lowered to the coordinates for DG of -2.2mm anteroposterior (AP), ±1.3mm mediolateral (ML), -2.0mm dorsoventral (DV), and for BLA of -1.35mm (AP), ±3.25mm mediolateral (ML), and -5.0mm (DV). All coordinates are given relative to bregma (mm). For overlap surgeries, a volume of 300 nL of AAV9-cFos-tTa + TRE-EYFP viral cocktail was bilaterally injected at 200 nL/min using a micro-infusion pump for each coordinate (2x300 nL for dDG and 2x300 nL for BLA) (UMP3; World Precision Instruments). After injection was complete, the needle was left in place 2 minutes prior to incremental retraction of the needle from the brain. For dDG chemogenetic surgeries, a 300nL viral cocktail of AAV9-cFos-tTa + AAV9-TRE-hM4Di was bilaterally injected into the dDG at the coordinates listed above. For dDG **or** BLA optogenetic surgeries, a 300nL viral cocktail of AAV9-cFos-tTa + AAV9-TRE-ChR2-EYFP was bilaterally injected into the dDG **or** BLA (separate surgeries and animals for each brain

region; see overlap surgeries for coordinates and procedure for injection). Following viral injection, bilateral optical fibers (200µm core diameter; Doric Lenses) were placed 0.4mm above the injection sites (dDG: -1.6mm DV; BLA: -4.6mm DV). The implants were secured to the skull with a layer of adhesive cement (C&M Metabond) followed by multiple layers of dental cement (Stoelting). Mice were injected with a 0.1 mg/kg intraperitoneal dose of buprenorphine (volume dependent on weight of animal) and placed in a recovery cage atop a heating pad until recovered from anesthesia. Viral targeting was confirmed by histological study and only animals with proper viral expression were utilized for data analysis.

Immunohistochemistry

Mice were overdosed with 3% isoflurane and perfused transcardially with cold (4°C) phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. Brains were extracted and kept in PFA at 4°C for 24-48 hours and then transferred to PBS solution. Brains were sectioned into 50µm thick coronal sections with a vibratome and collected in cold PBS. Sections were blocked for 1-2 hours at room temperature in PBS combined with 0.2% triton (PBST) and 5% normal goat serum (NGS) on a shaker. Sections were incubated in primary antibody (1:1000 rabbit anti-c-Fos [SySy]; 1:5000 chicken anti-GFP [Invitrogen]) made in PBST-NGS at 4°C for 48 hours. Sections then underwent three washes in PBST for 10 minutes each, followed by a 2 hour incubation period at room temperature with secondary antibody (1:200 Alexa 555 anti-rabbit [Invitrogen]; 1:200 Alexa 488 anti-chicken [Invitrogen]) made in PBST-NGS. Sections then underwent three more wash cycles in PBST. Sections were mounted onto microscope slides (VWR International, LLC). Vectashield HardSet Mounting Medium with DAPI (Vector Laboratories, Inc) was applied and slides were coverslipped and allowed to dry overnight. Once dry, slides were

sealed with clear nail polish on each edge and stored in a slide box in the fridge. If not mounted immediately, sections were stored in PBS at 4°C.

Behavior

Fear Conditioning - Fear conditioning (FC) for all experiments took place in a 18.5 × 18 × 21.5 cm chamber with aluminum side walls and plexiglass front and rear walls (Context A). Each cage animal was placed in a separate chamber (4) and received shocks in parallel during the session. The session consisted of 4 shocks over a time span of 500s (shocks at 198s, 278s, 358s, 438s). Depending on the experiment, the length and strength of the shock varied. For the experiments without extinction training (recall 24hrs after social session), the four shocks were 1 second each at 1mA. For the experiments with extinction, in order to obtain homogenous and reliable fear responses at a more remote time point, more intense fear conditioning was utilized, with shocks at 1.5mA for 2 seconds. After the session, animals were placed back into their home cage and in an isolated holding area until all animals in a cohort had been fear conditioned.

Extinction Training - Animals were placed into Context A (see above) for a duration of 30 minutes on the day following fear conditioning. A second extinction training session occurred on the next day (30 minutes). All animals in a cage of four underwent extinction training simultaneously. After the session, animals were placed back into their home cage and in an isolated holding area until all animals in a cohort had undergone extinction training.

Extinction Test - For the extinction test, animals were placed into Context A for a duration of 5 minutes. Depending on the experiment, the five minute extinction test was used to assess successful extinction and occured on the day following the second session of extinction training.

As in fear conditioning and extinction training, all animals in a cage of four underwent testing simultaneously and were placed back into their home cage and in an isolated holding area until all animals in a cohort were tested.

Recall Test - For the recall test, animals were placed into Context A for a duration of 5 minutes. As in fear conditioning and extinction training, all animals in a cage of four underwent testing simultaneously and were placed back into their home cage and in an isolated holding area until all animals in a cohort were tested.

Generalization - To assess generalization, 24 hours after the recall session animals were placed into Context B (see above for description) for a duration of 5 minutes. All animals in a cage of four underwent testing simultaneously and were placed back into their home cage and in an isolated holding area until all animals in a cohort were tested.

Juvenile Intruder/Cagemate Control - Animals were removed from the home cage and placed into a separate, clean cage with access to food and water. The lid of the cage and the feeder were removed from the home cage, which served as the interaction chamber. An experimental mouse was placed back into the home cage and allowed 1 minute to acclimate. An unfamiliar younger (p43-49) intruder mouse was placed into the home cage for ten minutes of interaction with the experimental resident mouse; younger mice were used to avoid confounding effects of mutual aggression(Defensor et al., 2012b; Felix-Ortiz and Tye, 2014). A clear acrylic top was placed over the home cage during interaction. This was repeated for each experimental cage animal. A different intruder mouse was used in each session. After each session, both mice were removed from the

home cage and placed into a separate holding cage for their respective group. For cagemate control experiments, a cagemate of the resident mouse was used for interaction in lieu of the unfamiliar intruder. The social session was conducted with a procedure otherwise identical to that used for RIT.

Immediate Shock - Immediate shock was administered in a 18.5 × 18 × 21.5 cm chamber with vertical black and white striped (3 cm width) sides, a plastic container holding gauze soaked in almond extract under the chamber floor, and red light as room illumination (Context B). The animal received a single 1.5mA shock if after extinction or 1mA shock if without extinction at the 2 second time point over a 60s session.

Full Interaction - The lid of the cage and the feeder were removed for social interaction and a clear acrylic top was placed over the home cage. The recently shocked cagemate was then placed into the cage and all animals were left in the testing room for one hour of social interaction. After one hour, the home cage was returned to its normal condition.

1-Way Mirror/Mirror No Mouse Control/Opaque Control - Mirror inserts were created out of laminated cardboard and one way mirror material (OuBay). Wooden dowels were placed on the bottom to support the structure. Dimensions were based to fit tightly into the cage (7” x 11” x 5”) to separate the container into two sections (a smaller area for the recently shocked cagemate and larger area for the rest of the cagemates). The lid of the cage and the feeder were removed for social interaction and a clear acrylic top was placed over the home cage. In order to enhance the effect of the mirror by creating a large light difference, black covers were created to cover the section of the

cage with the recently shocked cagemate. Refer to **Figure S1** for a visual representation of the set-up. After Immediate Shock, the recently shocked cagemate was immediately placed into the section opposite of and separate from the other cagemates. Animals were left in the testing room for one hour of social interaction except in the chemogenetic inhibition experiment, in which they were weighed and injected 15 minutes prior to Immediate Shock and then left to experience the 1-Way Mirror manipulation for 15 minutes to ensure efficacy of CNO in brain tissue throughout the entire experience(Jendryka et al., 2019). After one hour, or 15 minutes for the chemogenetic experiment, the recently shocked cagemate was removed and immediately euthanized by overdose with sodium pentobarbital to ensure any subsequent effects were due to the manipulation rather than any social interaction afterward, and the home cage was returned to its normal condition. For the Mirror No Mouse Control, all procedural steps were identical to the one-way mirror experiments, except that there was no shocked cagemate placed on the other side of the mirror. For opaque control experiments, the one way mirror insert was altered through the addition of a black insert on the side of the shocked cagemate so that the wall was bidirectionally blocking visual input from the other side. Social interaction was conducted with a procedure otherwise identical to that used for 1-way mirror experiments.

Restraint/Tube Control - One at a time, animals were removed from the home cage and enclosed in a plastic restraint tube containing holes to permit air flow. The tube was then placed in the center of a clean cage for a duration of two minutes. At the end of the two minutes, the animal was released from the tube into a separate clean cage. For the tube control test, all animals remained in the home cage and a clean restraint tube was placed into the home cage for a duration of two minutes.

Dominance Tube Test- 30mm (width) x 30cm (length) Plexiglass tubes were wide enough for only one mouse to pass through at a time. Protocol for the dominance test was adapted from(Fan et al., 2019). In short, cages of mice were trained for two days to walk through the small acrylic tube in one, forward direction. Following training, each mouse was paired against one another to test dominance. Each mouse in the pair was placed on either side of the tube so that they met in opposite directions inside the tube. Dominance was defined as one of the mice pushing the other out or forcing them to retreat back. After all mice were tested against one another in one day, they were ranked in a 1 to 4 fashion. This was repeated each day until the hierarchy was kept stable at least 4 days in a row. Once the hierarchy determined, the most dominant or least dominant mouse was chosen to become the recently shocked cagemate in the 1-Way Mirror paradigm to determine if hierarchy could predict reinstatement of the fear memory.

Open Field Test- An open 24in x 24in arena with black plastic walls was used for the test, with a red-taped area of 12.5in x 12.5 in the middle delineating "center" from "outside". The lights in the room were adjusted to minimize unwanted shadows. A camera was placed above the open field area in order to record the session. The mouse was taken out of its cage and immediately placed in one corner of the arena. The mouse was then left to explore the space for 10 minutes. Once 10 minutes had passed, the mouse was placed in a separate cage until all of the cagemates had also gone through the behavioral test. They were then all placed back in their homecage.

Optogenetic Stimulation

The optogenetic stimulation session occurred 24 hours after the tagging experience (1-Way Mirror). Prior to the start of the session, optogenetic patch cords were tested to ensure a minimum

10mA laser output. Mice were given stimulation in a separate room than Context A or B. For stimulation, the mice were attached to the optogenetic cords in the palm of ABF's hand and placed into a striped acrylic chamber with either white or dimmed white + red light and either almond or orange scent (Context C). The session lasted 8 minutes and consisted of 4 alternating 2 minute periods of laser stimulation (off/on/off/on; dDG: 450 nm, 20 Hz, 10 ms pulse width(Liu et al., 2012); BLA: 450 nm, 4 Hz and 20 Hz, 10 ms pulse width(Roy et al., 2019)). The BLA group received 4 Hz and 20 Hz stimulation counterbalanced in different contexts (orange vs almond scent, white vs red light) separated by about 1.5hrs.

Behavior Scoring

FreezeFrame - Videos of behavioral sessions were obtained using cameras secured to the chamber either above or to the side of the subject. For extinction, extinction test, and recall sessions, FreezeFrame/View (Coulbourn Instruments; Whitehall, PA) was used to score freezing behavior, defined as 1.25 seconds of animal immobility.

ezTrack - Videos of behavioral sessions were obtained using cameras secured to the chamber either above or to the side of the subject. Videos of the open field test were obtained using a webcam secured above for an aerial view. ezTrack(Pennington et al., 2019) was used to analyze all open field videos. An ROI of the center was defined manually according to the lines present on the floor of the open field arena. The software tracked the movement of the mouse and calculated the amount of frames in which the mouse moved less than specified parameters (both pixel change and distance traveled). The software then tracked and calculated the amount of frames the mouse spent in the center, which was converted to the amount of time spent in the center according to video parameters.

Manual scoring - For optogenetic sessions, due to cord movement and lighting conditions interfering with automated scoring, all freezing and grooming quantification was done manually. This was then converted to a percentage of time that the mouse spent freezing within the bins of stimulation in the session. Each video was scored for grooming by HL, and for freezing by 2 separate observers (ABF and HL or ABF and RC) whose scores were averaged to mitigate variation in bout length perception. Grooming was defined as any syntactic-chain cephalocaudal grooming, scratching, licking, or other observable forms of non-chain self-grooming performed by the animal(Kalueff et al., 2016). Freezing was defined as any observable complete cessation of movement, other than breathing, by the animal.

Imaging and Cell Counting

All coronal brain slices were imaged through a Zeiss LSM 800 epifluorescence microscope with a 20x/0.8NA objective using the Zen2.3 software. Images of the basolateral amygdala were captured in a 2x2 tile (640x640 microns) Z-stack. Images of the dentate gyrus were captured in a 4x2 tile (1280x640 microns) Z-stack. DAPI and GFP were imaged as separate channels for target verification and ensemble size quantification. For overlap counts, DAPI, cFos, and GFP were imaged (DAPI and cFos simultaneously and GFP as a separate channel). 3-4 different slices were imaged for each animal for averaging.

FIJI Software - Images were processed for greater clarity before quantification. The Subtract Background tool was used to enhance contrast of cells to background, and the Despeckle was used to minimize noise that may interfere with quantification. ROIs were selected using Freehand Selection so that only cells within the brain region would be analyzed. The DAPI channel was

segmented using a custom pipeline involving 3D Object Counter. The cFos and GFP channels were initially segmented using a custom pipeline utilizing the 3D Iterative Thresholding tool of 3D ImageSuite (Ollion et al., 2013). Once cells segmented, the z-stacks were Z-Projected into a single slice image and saved.

Cell Profiler - Segmented images of DAPI, cFos, and GFP were loaded into CellProfiler and run through a pipeline that identified cells with more stringent parameters of size and shape. For overlap quantification, the final step in the pipeline counted all identified objects between the cFos and GFP images that had an overlap of greater than 80%.

Cell Quantification and Analysis - Once cells were counted, the relative amounts of $cFos^+$ only, GFP^+ only, and $cFos^+GFP^+$ (overlap) cells in each slice was normalized over the total amount of DAPI cells present ($cFos^+$/DAPI, GFP^+/DAPI, Overlap/DAPI). Chance of an overlap was defined as ($cFos^+$/DAPI)x (GFP^+/DAPI). Overlap over Chance was then calculated by dividing Overlap/DAPI by the Chance Overlap calculated in the previous step.

Supplementary Text

Statistical Analysis Details

<u>Sex differences in fear acquisition</u>

2 Way ANOVA with Sidak's multiple comparison tests of fear acquisition for males vs females, n=69 males, n=63 females; Time x Sex $F(4, 520)=2.910$, $p=0.0212$; Time $F(2.717, 353.3)=323.8$, $p<0.0001$; Sex $F(1, 130)=4.895$, $p=0.0287$; Subject $F(130, 520)=3.594$, $p<0.0001$;

Comparing males to females - Baseline - t=3.336, p=0.0063; Shock1 - t=4.930, p<0.0001; Shock2 - t=2.626, p=0.0475; Shock3 - t=1.464, p=0.5452; Shock4 -t=0.07762, p>0.9999.

Impact of salient experiences on fear recall

Males -2 Way ANOVA with Sidak's multiple comparison tests. n=12 Juvenile Intruder; n=14 Cagemate control; n=18 Restraint; n=19 Tube control, Type of Experience x Control F(1, 59)=10.87, p=0.0017; Type of Experience F(1, 59)=1.525, p=0.2217; Control F(1, 59)=8.949, p=0.0040. Comparing experimentals to controls: Juvenile Intruder t=4.098, p=0.0003; Restraint t=0.2381, p=0.8126.

1 Way ANOVA with Sidak-Holm's multiple comparison tests. n=18 Full Interaction; n=18 1-Way Mirror; n=21 Opaque; n=16 Mirror No Mouse; F=3.949, p=0.0117. Comparing 1-Way Mirror vs Opaque: t=2.074, p=0.0418; 1-Way Mirror vs Full Interaction: t=2.704, p=0.0172; 1-Way Mirror vs Mirror No Mouse: t=3.173, p=0.0068.

Females - 2 Way Anova with Fisher's LSD tests. n=21 1-Way Mirror; n=19 Mirror No Mouse; n=16 Restraint; n=16 Tube control; Type of experience x Control F(1, 68)=3.332, p=0.0723; Type of experience F(1, 68)=1.942, p=0.1680; Control F(1, 68)=0.7553, p=0.3879. Comparing experimentals to controls: 1-Way Mirror t=2.019, p=0.0474; Restraint t=0.6418, p=0.5232.

Impact of salient experiences on fear generalization

Males - 2 Way ANOVA with Sidak's multiple comparison tests. n=11 juvenile intruder; n=12 cagemate control; n=15 restraint; n=16 tube control; Type of Experience F(1, 51)=0.2352,

p=0.6297; Control F(1, 51)=0.6279, p=0.4318. Comparing experimentals to controls; juvenile intruder t=1.002, p=0.5391; restraint t=0.1767, p=0.9805.

1 Way ANOVA with Sidak-Holm's multiple comparison tests. n=18 Full Interaction; n=18 1-Way Mirror; n=21 Opaque; n=16 Mirror No Mouse; F=0.6545, p=0.5828. 1-Way Mirror vs Opaque: t=1.249, p=0.5177; 1-Way Mirror vs Full Interaction: t=0.2355, p=0.9656; 1-Way Mirror vs Mirror No Mouse: t=0.1631, p=0.9656..

Females - 2 Way Anova with Fisher's LSD tests since no multiple comparisons were made, n=21 1-way mirror; n=16 1-way mirror control; n=16 restraint; n=16 tube control; Type of experience F(1, 66)=0.1702, p=0.6813; Control F(1, 66)=2.140, p=0.1483. Comparing experimentals to controls: 1-way mirror t=0.8561, p=0.3951; restraint t=1.210, p=0.2305.

<u>Reinstatement of fear following exposure to stressed cagemate</u>

Reinstatement after three types of exposure - 1 Way ANOVA with Holm-Sidak's multiple comparisons test. n=18 1-way mirror; n=38 full interaction; n=14 opaque; F=4.263, p=0.0181. Comparing 1-way mirror to full interaction, t=2.736, p=0.0158; comparing 1-way mirror to opaque, t=2.345, p=0.0220.

Comparing counts of reinstators - Fisher's exact test. n=18 1-way mirror, n=38 full interaction; 1-way mirror 16.67% reinstators, full interaction 0% reinstators; p=0.0294.

Reinstators' generalization of fear - paired t test, n=5, t=3.616, p=0.0224.

Reactivation of DG and BLA fear ensembles during various experiences

Extinction of fear - Repeated Measures 1 Way ANOVA with Holm-Sidak's multiple comparisons test, n=9. Comparing the first five minutes to the last, t=5.688, p=0.0005.

Number of cFos+ cells during different experiences -

No Extinction - 1 Way ANOVA with Holm-Sidak's multiple comparisons test:

DG: n=10 Juvenile Intruder, 9 1-Way Mirror, 4 Shocked Cagemate, 5 Restraint; F=2.825, p=0.0820. Comparing Juvenile Intruder vs 1-Way Mirror, t=2.249, p=0.0339; **BLA**: n=10 Juvenile Intruder, 8 1-Way Mirror, 5 Restraint, 3 Immediate Shock, F=3.961, p=0.0356. Comparing Juvenile Intruder vs 1-Way Mirror, t=2.782, p=0.0118.

Extinction - **DG:** Welch's t test (due to different variances, F=8.788, p=0.0462), n=6 1-Way Mirror, 3 Restraint, t=1.261, p=0.3232. **BLA**: two-tailed, unpaired t test, n=4 Restraint, 5 1-Way Mirror, t=0.2716, p=0.7938.

Reactivation of fear ensemble during different experiences -

One sample t test comparing to chance overlap of 1:

No Extinction - **DG**: n=10 Juvenile Intruder, t=2.436, p=0.0376; n=9 1-Way Mirror, t=2.656, p=0.0290; n=5 Restraint, t=0.06673, p=0.9500; n=4 Immediate Shock, t=0.08473, p=0.9378. **BLA:** n=10 Juvenile Intruder, t=7.411, p<0.0001; n=8 1-Way Mirror, t=2.101, p=0.0737; n=5 Restraint, t=7.462, p=0.0017; n=3 Immediate Shock, t=3.492, p=0.0731.

Extinction: **DG**: n=6 1-Way Mirror, t=3.340, p=0.0206; n=3 Restraint, t=3.575, p=0.0701. **BLA:** n=4 1-Way Mirror, t=3.257, p=0.0312; n=4 Restraint, t=9.031, p=0.0029.

Optogenetic stimulation of cells active during social experience

Extinction training - Mixed-effects model, n=11 FC, n=7 No FC, time x FC F(12, 186)=8.201, p<0.0001, time F(12, 186)=7.487, p<0.0001 FC F(1, 16)=40.22, p<0.0001. Holm Sidak's multiple comparisons, first five minutes of extinction vs extinction test, t=5.054, p<0.0001.

Ensemble size -

DG: two tailed, unpaired t test, n=9 Fear Conditioned, 5 Non Fear Conditioned, t=1.264, p=0.2300.

BLA: two tailed, unpaired t test, n=7 Fear Conditioned, 8 Non Fear Conditioned, t=1.873, p=0.0838.

Effect of optogenetic stimulation - 2 Way Repeated Measures ANOVA for all

DG (n=8 FC, n=5 No FC)

Freezing:

For individual sessions -light x FC F(3, 33)=2.093, p=0.1201; light F(3,33)=0.7303; FC F(1,11)=8.695, p=0.0132; Subject F(11, 33)=1.254, p=0.2931; Holm-Sidak's multiple comparisons test, FC first light-on vs light-off t=3.021, p=0.0145.

For averaged light-on sessions - light x FC F(1, 11)=7.532, p=0.0191; FC F(1,11)=4.318, p=0.0619; Light F(1,11)=3.257, p=0.0986; Subject F(11,11)=2.168, p=0.1076. Holm-Sidak's multiple comparisons, Light On vs Light Off: No FC- t=0.5991, p=0.5612, FC - t=3.668, p=0.0074.

Grooming:

For individual sessions - light x FC $F(3, 33)=4.111$, $p=0.0139$; light $F(3,33)=1.092$, $p=0.3661$; FC $F(1,11)=1.829$, $p=0.2034$; Subject $F(11, 33)=1.986$, $p=0.0632$; Holm-Sidak's multiple comparisons test, baseline vs light-off $t=3.063$, $p=0.01720$; first light-on vs light-off $t=3.520$, $p=0.0077$; second light-on vs light-off $t=3.268$, $p=0.0126$.

For averaged light-on sessions - light x FC $F(1, 11)=6.915$, $p=0.0234$; FC $F(1,11)=0.06426$, $p=0.8046$; Light $F(1,11)=1.028$, $p=0.3324$; Subject $F(11,11)=1.026$, $p=0.4835$. Holm-Sidak's multiple comparisons, Light On vs Light Off: No FC- $t=1.030$, $p=0.3252$, FC - $t=2.938$, $p=0.0268$.

BLA (n=4 FC, n=3 No FC)

Freezing:

For individual sessions - light x FC $F(6, 24)=1.651$, $p=0.1766$; light $F(3, 24)=0.2742$, $p=0.8434$; FC $F(2, 8)=0.9582$, $p=0.4236$; Subject $F(8, 24)=2.471$, $p=0.0413$.

For averaged light-on sessions - light x FC $F(2, 8)=2.081$, $p=1.873$; FC $F(2, 8)=1.213$, $p=0.3466$; Light $F(1, 8)=0.02020$, $p=0.6651$; Subject $F(8, 8)=5.667$, $p=0.0121$.

Grooming:

For individual sessions - light x FC $F(6, 24)=1.146$, $p=0.3671$; Light $F(3, 24)=1.401$, $p=0.2669$; FC $F(2, 8)=2.084$, $p=0.1869$; Subject $F(8, 24)=8.737$, $p<0.0001$.

For averaged light-on sessions - light x FC $F(2, 8)=0.7235$, $p=0.5142$; FC $F(2, 8)=1.238$, $p=0.3402$; Light $F(1, 8)=2.809$, $p=0.1322$; Subject $F(8, 8)=4.779$, $p=0.0121$.

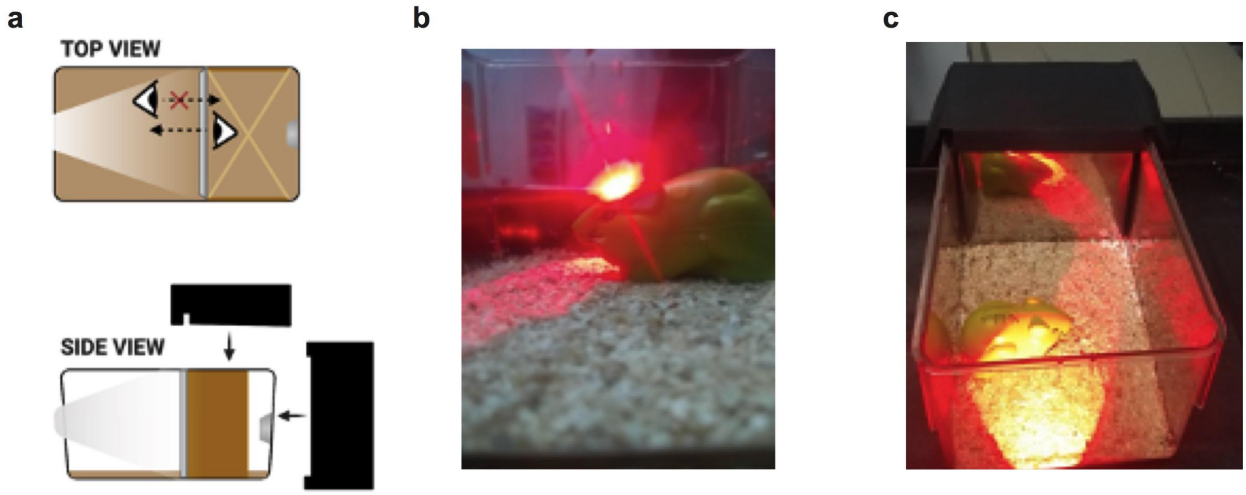

Fig. S1. 1-Way Mirror Prototype. **a)** Unidirectional visual access allows the recently shocked mouse in the darkened compartment to see the cagemates on the other side of the insert, in the bright compartment. **b)** The cagemates do not see the shocked cagemate in the dark compartment but rather see their own reflections. Auditory-olfactory stimuli are exchanged over the top and sides of the 7” x 11” x 5” insert.

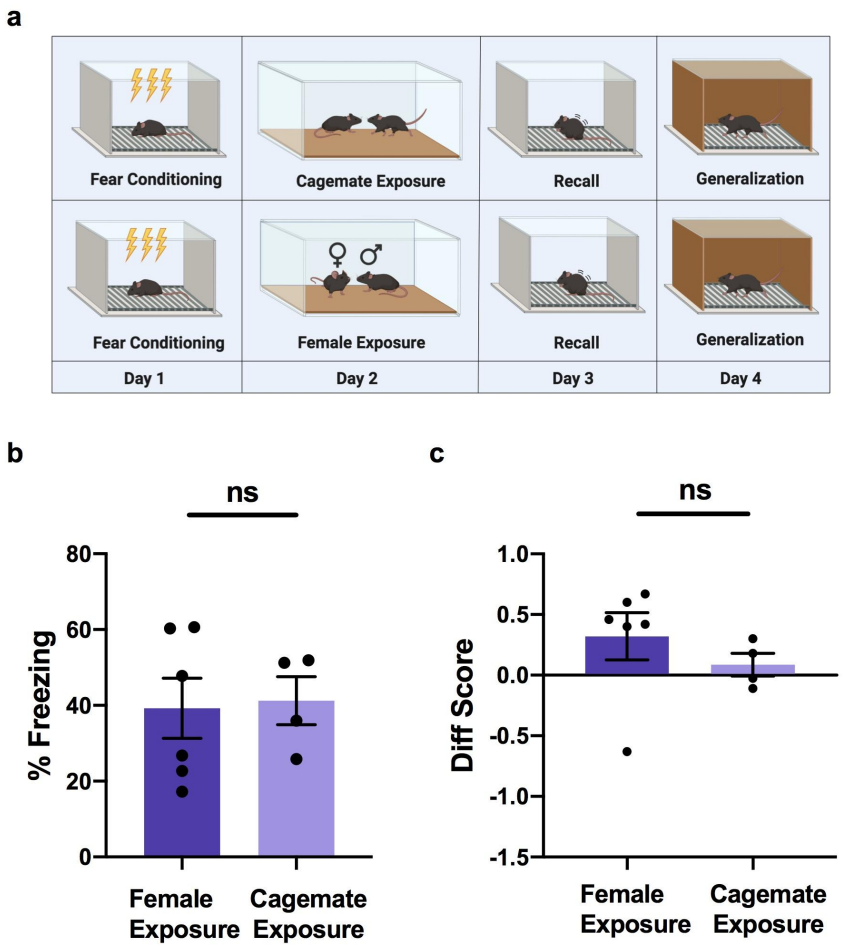

Fig. S2. Exposure to females does not impact males' fear memory recall compared to cagemate controls. a) Schematic behavioral paradigm. **b)** Freezing during recall (two tailed, unpaired t test, n=6 female exposure, 4 cagemate controls, t=0.1799, p=0.8617). **c)** Generalization represented by difference scores between freezing in FC context and a different context (two tailed, unpaired t test, n=6 female exposure, 4 cagemate controls, t=0.9189, p=0.3850).

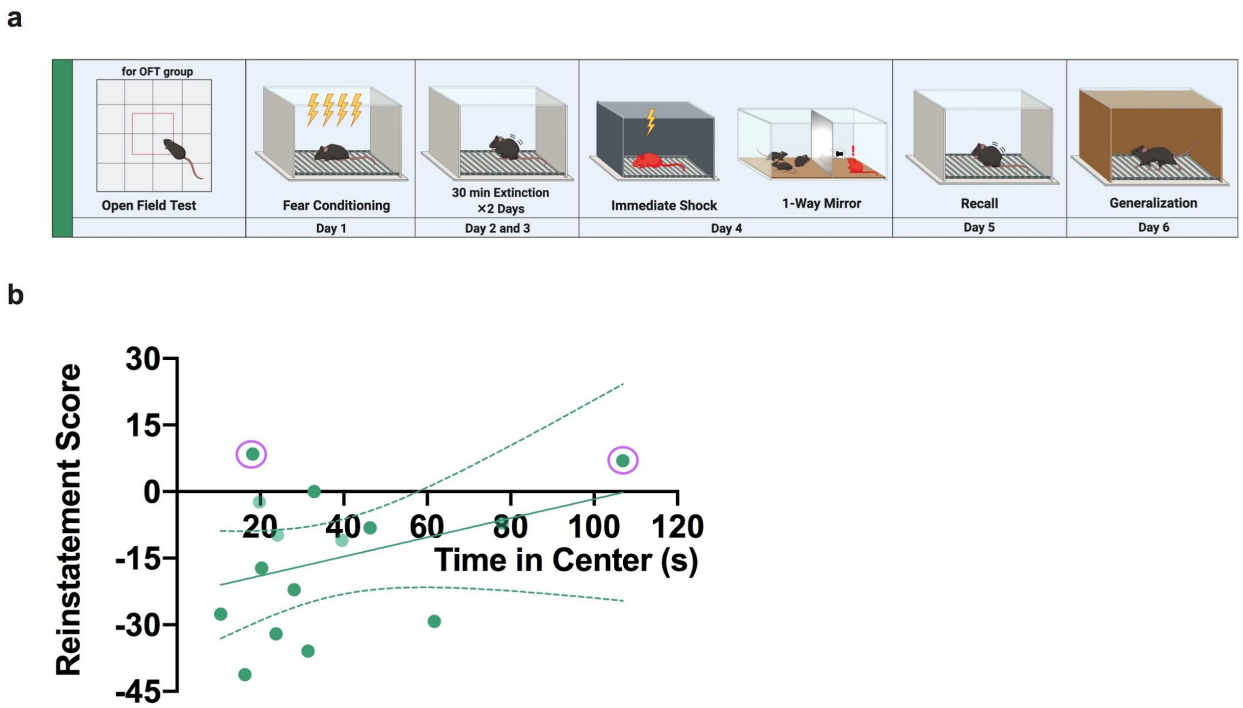

Fig. S3. Open Field Test performance does not predict socially-induced reinstatement of fear. Time in the center of the Open Field plotted on the x-axis, freezing during the first 3 minutes of last extinction session subtracted from freezing during the first 3 minutes of recall plotted on the y-axis. Purple circles indicate reinstators, defined as animals with y-axis scores above 5%. Simple linear regression, n=15, R squared=0.1342, p=0.18.

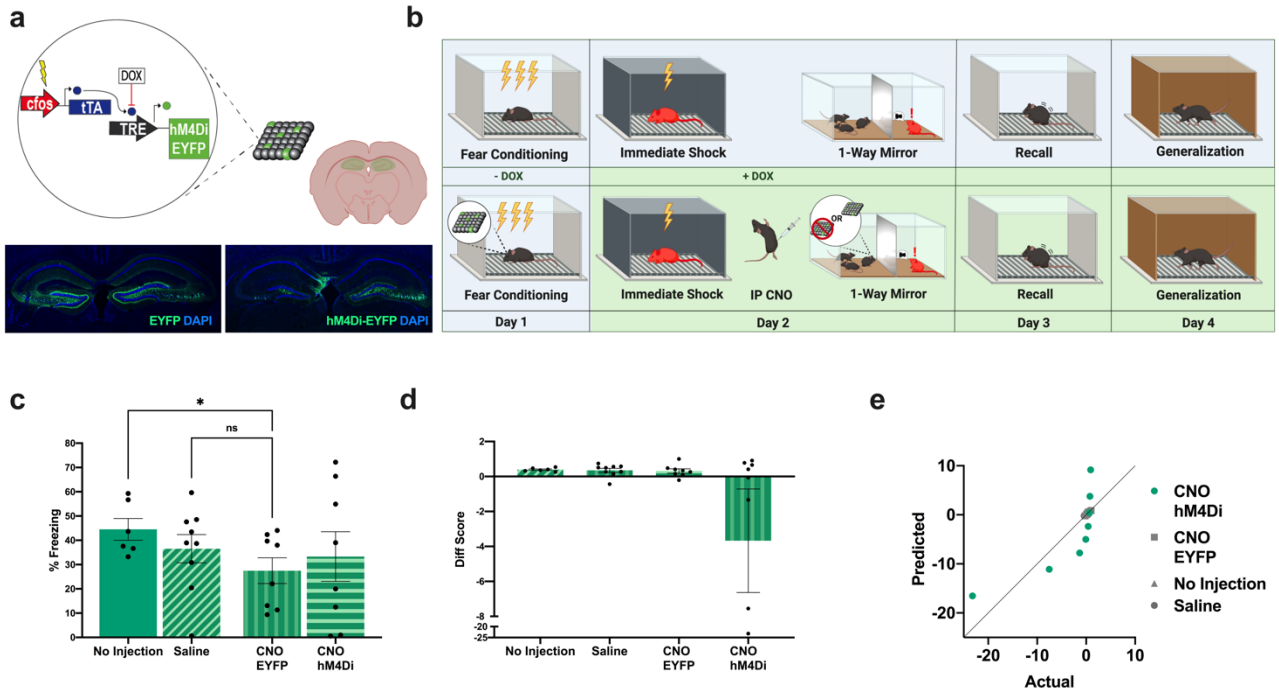

Fig. S4. CNO and injection-related stress blocks social enhancement of memory, thereby occluding the impact of chemogenetic inhibition. a) Schematic of viral construct (upper) and representative histology (lower). **b)** Behavioral timeline. **c)** Freezing during recall, n=8 CNO EYFP, 8 hM4Di, 6 No Injection, 9 Saline, Brown-Forsythe and Welch ANOVA test, F=0.9445, p=0.4396; unpaired t with Welch's correction, CNO EYFP vs No Injection, t=2.451, p=0.0306; CNO EYFP vs Saline, t=1.148, p=0.2690. **d)** Generalization score, Shapiro-Wilk test of normality, No Injection W=0.9854, p=0.9749; Saline W=0.8636, p=0.1048; CNO EYFP W=0.9498, p=0.7089; CNO hM4Di W=0.6306, p=0.0004. **e)** Normal QQ Plot demonstrating skew of CNO hM4Di group (green circles) in contrast to all other groups, in reference to solid black identity line.

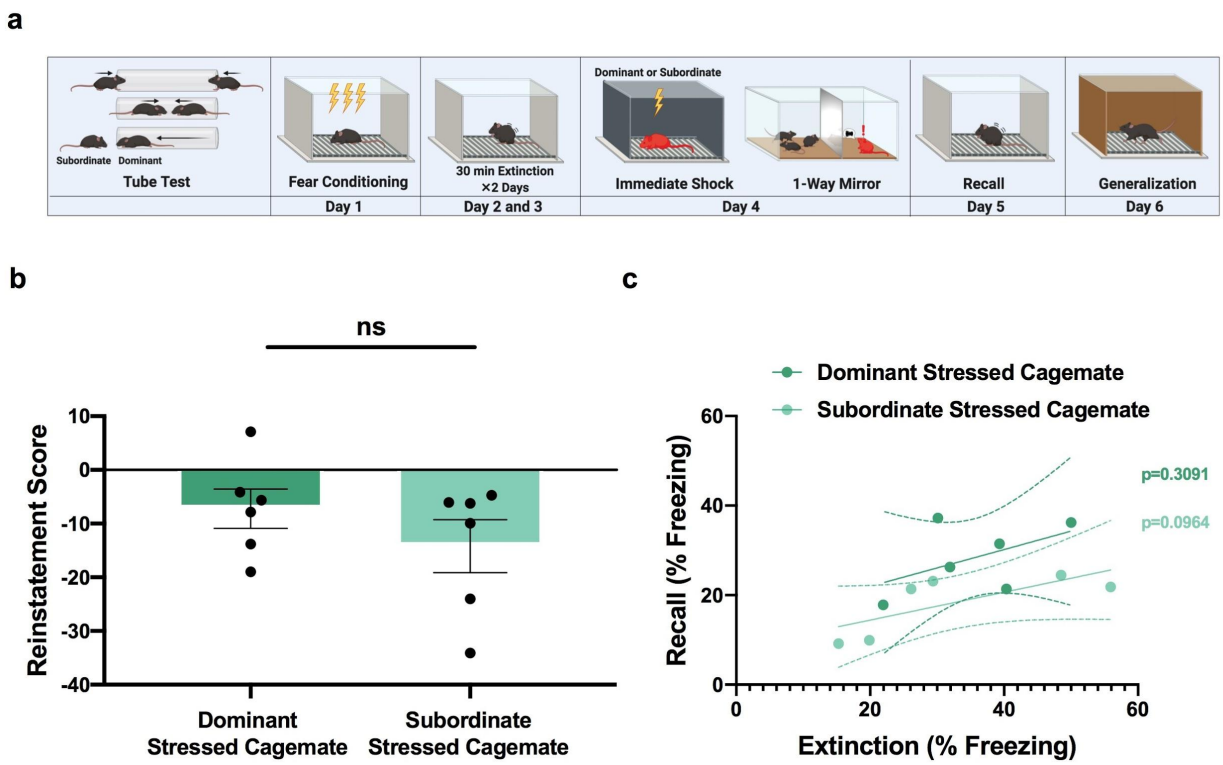

Fig. S5. Hierarchical role of the stressed animal does not impact cagemates' reinstatement. a) Behavioral timeline. **b)** Reinstatement scores, calculated as (%freezing first three minutes of recall) - (%freezing first three minutes of second extinction session). n=6 subordinate stressed cagemate, n=6 dominant stressed cagemate, unpaired two-tailed t test, t= 1.135, p= 0.2828, **c)** Linear regressions of freezing during first three minutes of recall vs first three minutes of second extinction session. Subordinate stressed cagemate: R squared= 0.5394, dominant stressed cagemate, R squared= 0.2530.

	1-Way Mirror	Juvenile Intruder	Opaque	Full Interaction	Restraint	Cagemates
n	18	11	13	38	8	12
R^2	0.00433	0.2401	0.7932	0.4111	0.5799	0.4568
F	0.06955	2.844	42.19	25.13	8.282	8.409
P	0.7954	0.1260	<0.0001	<0.0001	0.0281	0.0158

Table S1.

Simple linear regressions of percentage freezing during the first 3 minutes of the last extinction session against freezing during the first 3 minutes of recall.

Movie S1.

Representative video of 1-way mirror paradigm for fear conditioned cagemates.

Movie S2.

Representative video of juvenile intruder paradigm for fear conditioned cagemates. The experimental resident mouse has two marks at the base of his tail, the intruder has a long solid line from the base to the middle of his tail.