Social Reactivation of Fear Engrams Enhances Recall and Reinstatement

Authors: Abby Basya Finkelstein[1,2*], Héloïse Leblanc[1,3], Rebecca Cole[1], Troy Gallerani[1,3], Anahita Vieira[1,4], Yosif Zaki[1,5], Steve Ramirez[1*]

Affiliations

[1]Department of Psychological and Brain Sciences, Boston University, Boston, MA, USA.

[2]Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA, USA.

[3]Boston University School of Medicine, Boston, MA.

[4]The Broad Institute of MIT and Harvard, Cambridge, MA, USA.

[5]Icahn School of Medicine at Mount Sinai, NYC, NY, USA.

*Correspondence to: afinkelstein@fas.harvard.edu and dvsteve@bu.edu

Abstract

The power of one's social environment to bidirectionally modulate cognitive abilities is well documented[1–4], but the processes by which social experiences impact the cellular substrates of memory remain unknown. Here we show that social interactions and exposure to ambient stimuli emitted by stressed conspecifics, but not individually experienced physical stress, enhance 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.

Main

For group-living species such as humans and rodents, conspecific interactions directly impact social learning and memory[5,6], and pervasively shape emotion[7–9], attention[10], and cognitive ability[1]. 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[11–14], chemical signaling[15,16], and tactile stimulation[17,18]. Rodents establish social hierarchies even in single-sex cages containing a small number of animals[19–21], and the dominance relationships between rodents can influence how they learn from one another[22]. The absence of such social encounters (e.g. single housing animals) results in cognitive impairments and depression-like phenotypes[23], 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."[2425–28] As social interaction recruits hippocampal[29] and amygdalar[30] circuitry, we hypothesized that pre-existing engrams in these regions can be modulated by social experiences and lead to changes in memory expression.

Social experiences modulate subsequent fear recall

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[31,32] (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[33]. 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.

Social emissions of stress reinstate cagemates' extinguished fear memory

72 Hereafter, we focused on socially-induced memory enhancement in male mice, conjecturing that
73 the enhancement of freezing during recall tests was due to socially-induced reactivation of fear
74 engrams. While outside the scope of our current study, we also speculate that the dampening of
75 recall in females is due to a mechanism involving interference[34–36] by the formation of a social
76 memory, which has been suggested to be longer-lasting and based on different circuitry than in
77 males[37,38]. We next tested whether memory was still susceptible to socially-driven enhancement
78 after extinction learning. Freezing during the reinstatement test was highly correlated with freezing
79 during the first 3 minutes of the second extinction session in groups subjected to experiences that
80 did not affect fear recall (Cagemates, Tube, Opaque, and Full Interaction groups) (**Fig. 2b-d**). This
81 finding indicates that the experience between extinction and reinstatement test had not modulated
82 reinstatement. However, in the Juvenile Intruder and 1-Way Mirror groups (i.e. groups that
83 exhibited enhanced fear recall after social experiences) freezing levels during the reinstatement test
84 were uncorrelated from freezing levels during extinction, indicating that the social experience had
85 indeed impacted reinstatement (**Fig. 2e**). To quantify the degree of reinstatement, we subtracted
86 the freezing rate during the first three minutes of the second extinction session from the freezing
87 rate during the first 3 minutes of the reinstatement test. We found that, in congruence with the
88 recall results (**Fig. 1d**), 1-Way Mirror groups exhibited a greater degree of reinstatement than the
89 Opaque or Full Interaction groups (**Fig. 2f**), suggesting that visually-perceived social context can
90 bias the recently shocked animal's distressed emissions and that social buffering via physical
91 interaction with cagemates can mitigate these emissions. Here we defined reinstators as mice who
92 froze greater than or equal to 5% higher during the reinstatement test than during the first three
93 minutes of the last extinction session; ~15% of mice were reinstators for 1-Way Mirror, (**Fig. 2g**)
94 and a generalization test showed that their reinstatement was context-specific[39] (**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.

Social reactivation of cells active during prior fear conditioning

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[40], 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

118 experience did not differ in either brain region (**Fig. 3f**). Reactivation of fear ensemble cells
119 occurred in the BLA during both social and non-social stress, and in the DG only during the social
120 experience (**Fig. 3g**). Collectively, these data suggest that specifically social situations reactivate
121 subsets of fear engrams in the DG.

122 We next used an activity-dependent chemogenetic strategy to test whether DG fear
123 ensembles active during the social experience were necessary for the subsequent enhancement in
124 memory recall (**Fig. S4a**). Intriguingly, the EYFP control group, which received intraperitoneal
125 CNO (**Supplementary Methods**) prior to the social experience, froze significantly less than non-
126 injected animals (**Fig. S4c**), ostensibly due to a combination of injection-related stress and CNO
127 metabolites[41]. The EYFP group did not freeze significantly less than saline controls, supporting a
128 role of injection-related stress in blocking the effect (**Fig. S4c**). While the impact of CNO injection
129 occludes our ability to determine the impact of hM4Di inhibition in the experimental group,
130 subsequent generalization tests revealed that the EYFP and hM4Di groups exhibited significantly
131 different variances in context-specificity of fear: while the EYFP group exhibited a normal
132 distribution, the hM4di group did not pass normality tests and was skewed toward lower difference
133 scores (i.e. high generalization) (**Fig. S4d-e**). This experiment indicates that preventing activation
134 of the fear engram during the social encounter peturbs the specificity of subsequent fear
135 expression.

136

137 **Functional role of reactivated cells**

138 Next, we wanted to investigate the functional properties of the cells which were active during
139 exposure to a stressed cagemate in previously fear-conditioned animals. To this end, we tagged
140 DG or BLA cells with channelrhodopsin-2 during our 1-way mirror paradigm (**Fig. 4a-d;** See

141 Methods). The following day, these cells were stimulated in a novel context to test their capacity to
142 drive fearful behavior. Importantly, the size of the labeled ensembles did not differ between fear
143 conditioned and non-fear conditioned animals (**Fig. 4e**), and although stimulation of cells active in
144 a neutral setting after fear conditioning does not drive freezing[42], 20Hz stimulation of the DG
145 ensembles active during the social experience drove significantly higher levels of freezing during
146 the light-on sessions as well as de-arousing self-grooming[43] in the light-off period following
147 stimulation, specifically in previously fear conditioned animals (**Fig. 4j, 4k**). Although cells
148 previously active during FC were also reactivated in the BLA during both the 1-way mirror and
149 restraint stress groups (**Fig. 4h**), counterbalanced 20Hz and 4Hz stimulation of cells tagged during
150 1-way mirror in the BLA did not drive fear compared to non-fear conditioned animals (**Fig. 4f,**
151 **4g**). A subset of the animals subsequently reinstated the extinguished fear memory (**Fig. 4h, 4l**).
152 These results demonstrate that optogenetic stimulation of DG cells active during exposure to a
153 stressed cagemate through a 1-way mirror drives fear only if mice have previously been fear
154 conditioned.
155

156 **Discussion**

157 Memory is a malleable phenomenon that can be altered by intervening experiences, as associated
158 context or cues can reactivate engrams and thus make them labile during incubation[44–47]. Our
159 findings here reveal that social encounters have the capacity to reactivate and modify hippocampal
160 engrams even in the absence of such external reminders. We show that in male mice, socially
161 salient, but not physically stressful, events reactivate memory traces in the DG and potentiate
162 subsequent memory expression, supporting the theory that endogenous reactivation can strengthen
163 recall[45]. The impact of exposure to a stressed conspecific through the 1-way mirror compared to

164 the opaque wall suggests that distressed mice emit different auditory-olfactory stimuli depending
165 on their perceived social context. We speculate that the lack of a memory enhancing effect as a
166 result of full interaction with a recently shocked cagemate could be due to social buffering[6] of the
167 distressed mouse's emissions, or to the intensity of direct, physical social contact with the
168 distressed cagemate.

169 Our findings provide evidence that socially-induced reactivation of memory engrams can
170 strengthen the behavioral expression of the memory. Consistent with this notion, the reactivation
171 of fear engrams is thought to be the mechanism by which multiple exposures to the contexts[47] or
172 cues[44] present during conditioning can strengthen memories in the days after learning. We
173 demonstrate that a single social encounter can reactivate a subset of the DG fear engram (**Fig. 3**)
174 and produce similar enhancement of memory expression (**Fig. 1**). Strikingly, the degree of cellular
175 reactivation during the social encounter was similar to previously reported results in animals re-
176 exposed to the original fear context[48]. Given the malleability of engrams[49, 50], the natural
177 reactivation of a fear memory ensemble during a stressful social experience might be expected to
178 add additional negative valence from the current experience to the representation of the former,
179 contributing to the observed enhancement of fear (**Fig. 1**) beyond the effects of reactivation in a
180 neutral context by associated cues or context.

181 Concurrent tracking of fear engrams in the DG and BLA during varied experiences
182 revealed distinct patterns of reactivation. During restraint, there is non-significant reactivation in
183 the DG, and this small amount of reactivation is potentially driven by the correlated significant
184 level of reactivation in the BLA (**Fig. 3e, 3h**). However, DG fear engram reactivation during social
185 experience with a stressed conspecific is discorrelated with BLA engram reactivation (**Table S3**),
186 suggesting that social inputs to the DG induce an engram excitation process distinct from non-

social stress. The increased level of DG activity during Juvenile Intruder compared to 1-way mirror (**Fig. 3d**) in the animal's homecage highlights the DG's role in encoding experiences beyond physical contexts per se[51]. We posit that the higher level of activity in the BLA and DG during exposure to a juvenile intruder than in the 1-way mirror paradigm reflects the richness of direct social interaction during the juvenile intruder's presence that is not permitted in the 1-way mirror setup.

Our data also suggest that the DG is concurrently reactivating a pre-existing fear engram and actively processing social experiences, as evidenced by the significantly different levels of cFos-positive cells (**Fig. 3d, left**) between types of social stress. Optogenetic tagging during a social experience revealed that the total number of cells was the same regardless of prior fear conditioning (**Fig. 4e**), and that stimulation drove fearful behavior only in fear conditioned animals (**Fig. 4j and 4k**), thus reflecting the prior-experience-dependent nature of this light induced freezing. The coactivation of ensembles leads to co-allocation and the linking of memories, as shown in both mouse[52] and human[53] studies. We therefore posit that the reactivated subset of the fear ensemble might therefore have become co-allocated to form part of the ensemble encoding the social encounter, perhaps explaining why activating DG cells tagged during the social experience drove fear only in animals that had previously been fear conditioned, although the total number of cells active during the social experience did not differ (**Fig. 4e-g, lower**). In the context of the social experience, however, this functional capacity of the active ensemble was quiescent; mice did not freeze during the social encounter itself (example **Movie S1** and **Movie S2**).

We propose that the capacity of social experiences to reactivate memories contributes to the enriching effects of a social environment on cognition[1,54]. From an evolutionary perspective, a stressed conspecific or their ambient auditory-olfactory emissions might signal a dangerous

210 situation in which it is adaptive to hone one's own memories to guide decision making. Together,
211 our findings illuminate how the mnemonic contents of an organism's brain may be modulated
212 throughout the course of social interactions to bias future behavioral responses.
213
214 **References and Notes:**

215 1. Heimer-McGinn, V. R., Wise, T. B., Hemmer, B. M., Dayaw, J. N. T. & Templer, V. L.
216 Social housing enhances acquisition of task set independently of environmental enrichment:
217 A longitudinal study in the Barnes maze. *Learn. Behav.* (2020) doi:10.3758/s13420-020-
218 00418-5.
219 2. Doulames, V., Lee, S. & Shea, T. B. Environmental enrichment and social interaction
220 improve cognitive function and decrease reactive oxidative species in normal adult mice. *Int.*
221 *J. Neurosci.* **124**, 369–376 (2014).
222 3. Dause, T. J. & Kirby, E. D. Aging gracefully: social engagement joins exercise and
223 enrichment as a key lifestyle factor in resistance to age-related cognitive decline. *Neural*
224 *Regeneration Res.* **14**, 39–42 (2019).
225 4. Kuhlmann, S., Piel, M. & Wolf, O. T. Impaired memory retrieval after psychosocial stress in
226 healthy young men. *J. Neurosci.* **25**, 2977–2982 (2005).
227 5. Hoppitt, W. & Laland, K. N. Chapter 3 Social Processes Influencing Learning in Animals: A
228 Review of the Evidence. *Advances in the Study of Behavior* 105–165 (2008)
229 doi:10.1016/s0065-3454(08)00003-x.
230 6. Leblanc, H. & Ramirez, S. Linking Social Cognition to Learning and Memory. *J. Neurosci.*
231 **40**, 8782–8798 (2020).
232 7. Williamson, J. A. & Austin Williamson, J. Social support, mood, and relationship satisfaction
233 at the trait and social levels. doi:10.17077/etd.vkde3xr7.

234 8. Reavis, R. D., Donohue, L. J. & Upchurch, M. C. Friendship, Negative Peer Experiences, and
235 Daily Positive and Negative Mood. *Social Development* vol. 24 833–851 (2015).
236 9. Neumann, R. & Strack, F. 'Mood contagion': The automatic transfer of mood between
237 persons. *Journal of Personality and Social Psychology* vol. 79 211–223 (2000).
238 10. Caplin, A., Leahy, J. & Matějka, F. Social Learning and Selective Attention. (2015)
239 doi:10.3386/w21001.
240 11. Peleh, T., Eltokhi, A. & Pitzer, C. Longitudinal analysis of ultrasonic vocalizations in mice
241 from infancy to adolescence: Insights into the vocal repertoire of three wild-type strains in
242 two different social contexts. *PLoS One* **14**, e0220238 (2019).
243 12. Castellucci, G. A., Calbick, D. & McCormick, D. The temporal organization of mouse
244 ultrasonic vocalizations. *PLoS One* **13**, e0199929 (2018).
245 13. Wöhr, M., Seffer, D. & Schwarting, R. K. W. Studying Socio-Affective Communication in
246 Rats through Playback of Ultrasonic Vocalizations. *Current Protocols in Neuroscience*
247 8.35.1–8.35.17 (2016) doi:10.1002/cpns.7.
248 14. Simola, N. & Brudzynski, S. M. Repertoire and Biological Function of Ultrasonic
249 Vocalizations in Adolescent and Adult Rats. *Handbook of Ultrasonic Vocalization - A*
250 *Window into the Emotional Brain* 177–186 (2018) doi:10.1016/b978-0-12-809600-0.00017-2.
251 15. Biasi, E., Silvotti, L. & Tirindelli, R. Pheromone detection in rodents. *Neuroreport* vol. 12
252 A81–A84 (2001).
253 16. Bredy, T. W. & Barad, M. Social modulation of associative fear learning by pheromone
254 communication. *Learn. Mem.* **16**, 12–18 (2009).
255 17. Ebbesen, C. L., Bobrov, E., Rao, R. P. & Brecht, M. Highly structured, partner-sex- and
256 subject-sex-dependent cortical responses during social facial touch. *Nat. Commun.* **10**, 4634

257 (2019).

258 18. Hertenstein, M. J. *The Communicative Functions of Touch in Humans, Nonhuman Primates,*
259 *and Rats: A Review and Synthesis of the Empirical Research*.

260 19. Williamson, C. M., Lee, W. & Curley, J. P. Temporal dynamics of social hierarchy formation
261 and maintenance in male mice. *Animal Behaviour* vol. 115 259–272 (2016).

262 20. Fan, Z. *et al.* Using the tube test to measure social hierarchy in mice. *Nat. Protoc.* **14**, 819–
263 831 (2019).

264 21. Barker, T. H., George, R. P., Howarth, G. S. & Whittaker, A. L. Assessment of housing
265 density, space allocation and social hierarchy of laboratory rats on behavioural measures of
266 welfare. *PLoS One* **12**, e0185135 (2017).

267 22. Jones, C. E. & Monfils, M.-H. Dominance status predicts social fear transmission in
268 laboratory rats. *Anim. Cogn.* **19**, 1051–1069 (2016).

269 23. Liu, N. *et al.* Single housing-induced effects on cognitive impairment and depression-like
270 behavior in male and female mice involve neuroplasticity-related signaling. *Eur. J. Neurosci.*
271 **52**, 2694–2704 (2020).

272 24. Josselyn, S. A. & Tonegawa, S. Memory engrams: Recalling the past and imagining the
273 future. *Science* **367**, (2020).

274 25. Davis, P. & Reijmers, L. G. The dynamic nature of fear engrams in the basolateral amygdala.
275 *Brain Res. Bull.* **141**, 44–49 (2018).

276 26. Janak, P. H. & Tye, K. M. From circuits to behaviour in the amygdala. *Nature* vol. 517 284–
277 292 (2015).

278 27. Duvarci, S. & Pare, D. Amygdala microcircuits controlling learned fear. *Neuron* **82**, 966–980
279 (2014).

280 28. Sun, X. *et al.* Functionally Distinct Neuronal Ensembles within the Memory Engram. *Cell*
281 **181**, 410–423.e17 (2020).
282 29. Montagrin, A., Saiote, C. & Schiller, D. The social hippocampus. *Hippocampus* vol. 28 672–
283 679 (2018).
284 30. Gothard, K. M. Multidimensional processing in the amygdala. *Nat. Rev. Neurosci.* **21**, 565–
285 575 (2020).
286 31. Felix-Ortiz, A. C. & Tye, K. M. Amygdala inputs to the ventral hippocampus bidirectionally
287 modulate social behavior. *J. Neurosci.* **34**, 586–595 (2014).
288 32. Defensor, E. B., Corley, M. J., Blanchard, R. J. & Caroline Blanchard, D. Facial expressions
289 of mice in aggressive and fearful contexts. *Physiology & Behavior* vol. 107 680–685 (2012).
290 33. Clark, J. W., Drummond, S. P. A., Hoyer, D. & Jacobson, L. H. Sex differences in mouse
291 models of fear inhibition: Fear extinction, safety learning, and fear–safety discrimination.
292 *British Journal of Pharmacology* vol. 176 4149–4158 (2019).
293 34. Forcato, C. *et al.* Reconsolidation of declarative memory in humans. *Learning & Memory* vol.
294 14 295–303 (2007).
295 35. Chan, J. C. K. & LaPaglia, J. A. Impairing existing declarative memory in humans by
296 disrupting reconsolidation. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 9309–9313 (2013).
297 36. Gisquet-Verrier, P. & Riccio, D. C. Memory integration: An alternative to the
298 consolidation/reconsolidation hypothesis. *Progress in Neurobiology* vol. 171 15–31 (2018).
299 37. Bluthe´, R.-M. & Dantzer, R. Social recognition does not involve vasopressinergic
300 neurotransmission in female rats. *Brain Research* vol. 535 301–304 (1990).
301 38. Thor, D. H. Testosterone and persistence of social investigation in laboratory rats. *Journal of*
302 *Comparative and Physiological Psychology* vol. 94 970–976 (1980).

303 39. Mobbs, D. *et al.* Viewpoints: Approaches to defining and investigating fear. *Nat. Neurosci.*
304 **22**, 1205–1216 (2019).
305 40. Tanaka, K. Z. *et al.* Cortical Representations Are Reinstated by the Hippocampus during
306 Memory Retrieval. *Neuron* vol. 84 347–354 (2014).
307 41. Mahler, S. V. & Aston-Jones, G. CNO Evil? Considerations for the Use of DREADDs in
308 Behavioral Neuroscience. *Neuropsychopharmacology: official publication of the American*
309 *College of Neuropsychopharmacology* vol. 43 934–936 (2018).
310 42. Liu, X. *et al.* Optogenetic stimulation of a hippocampal engram activates fear memory recall.
311 *Nature* **484**, 381–385 (2012).
312 43. Kalueff, A. V. *et al.* Neurobiology of rodent self-grooming and its value for translational
313 neuroscience. *Nat. Rev. Neurosci.* **17**, 45–59 (2016).
314 44. Kim, J., Kwon, J.-T., Kim, H.-S., Josselyn, S. A. & Han, J.-H. Memory recall and
315 modifications by activating neurons with elevated CREB. *Nat. Neurosci.* **17**, 65–72 (2014).
316 45. Sara, S. J. Strengthening the shaky trace through retrieval. *Nat. Rev. Neurosci.* **1**, 212–213
317 (2000).
318 46. Pickens, C. L., Golden, S. A., Adams-Deutsch, T., Nair, S. G. & Shaham, Y. Long-lasting
319 incubation of conditioned fear in rats. *Biol. Psychiatry* **65**, 881–886 (2009).
320 47. Inda, M. C., Muravieva, E. V. & Alberini, C. M. Memory retrieval and the passage of time:
321 from reconsolidation and strengthening to extinction. *J. Neurosci.* **31**, 1635–1643 (2011).
322 48. Chen, B. K. *et al.* Artificially Enhancing and Suppressing Hippocampus-Mediated Memories.
323 *Curr. Biol.* **29**, 1885–1894.e4 (2019).
324 49. Ramirez, S. *et al.* Creating a false memory in the hippocampus. *Science* **341**, 387–391 (2013).
325 50. Redondo, R. L. *et al.* Bidirectional switch of the valence associated with a hippocampal

326 contextual memory engram. *Nature* **513**, 426–430 (2014).

327 51. Stefanini, F. *et al.* A Distributed Neural Code in the Dentate Gyrus and in CA1. *Neuron* **107**,
328 703–716.e4 (2020).

329 52. Cai, D. J. *et al.* A shared neural ensemble links distinct contextual memories encoded close in
330 time. *Nature* **534**, 115–118 (2016).

331 53. Yetton, B. D., Cai, D. J., Spoormaker, V. I., Silva, A. J. & Mednick, S. C. Human Memories
332 Can Be Linked by Temporal Proximity. *Front. Hum. Neurosci.* **13**, 315 (2019).

333 54. Shafto, M. A. *et al.* Cognitive Diversity in a Healthy Aging Cohort: Cross-Domain Cognition
334 in the Cam-CAN Project. *J. Aging Health* 898264319878095 (2019).

335 55. Defensor, E. B., Corley, M. J., Blanchard, R. J. & Blanchard, D. C. Facial expressions of
336 mice in aggressive and fearful contexts. *Physiol. Behav.* **107**, 680–685 (2012).

337 56. Jendryka, M. *et al.* Pharmacokinetic and pharmacodynamic actions of clozapine-N-oxide,
338 clozapine, and compound 21 in DREADD-based chemogenetics in mice. *Sci. Rep.* **9**, 4522
339 (2019).

340 57. Roy, D. S., Park, Y. G., Ogawa, S. K., Cho, J. H. & Choi, H. Brain-wide mapping of
341 contextual fear memory engram ensembles supports the dispersed engram complex
342 hypothesis. *bioRxiv* (2019).

343 58. Pennington, Z. T. *et al.* ezTrack: An open-source video analysis pipeline for the investigation
344 of animal behavior. *Sci. Rep.* **9**, 19979 (2019).

345 59. Ollion, J., Cochennec, J., Loll, F., Escudé, C. & Boudier, T. TANGO: a generic tool for high-
346 throughput 3D image analysis for studying nuclear organization. *Bioinformatics* **29**, 1840–
347 1841 (2013).

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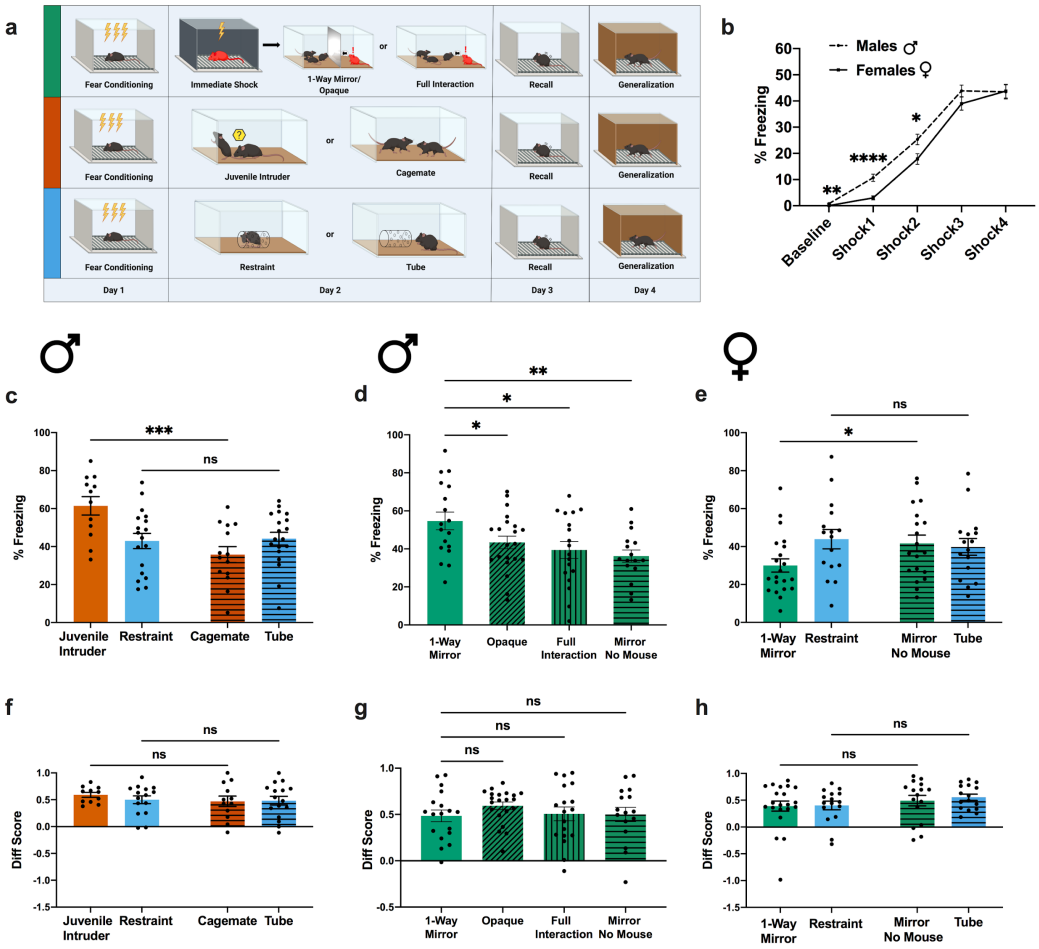

Figure 1. Social stimuli bidirectionally modulate fear memory recall for males and females. **a)** Schematic representation of behavioral schedule. **b)** Freezing levels during fear acquisition for males and females (2way ANOVA with Holm-Sidak's multiple comparison test, n=69 males, 63 females). **c,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

381 mean +/- SEM. n.s. $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, detailed statistics

382 shown in ST2.

383

384

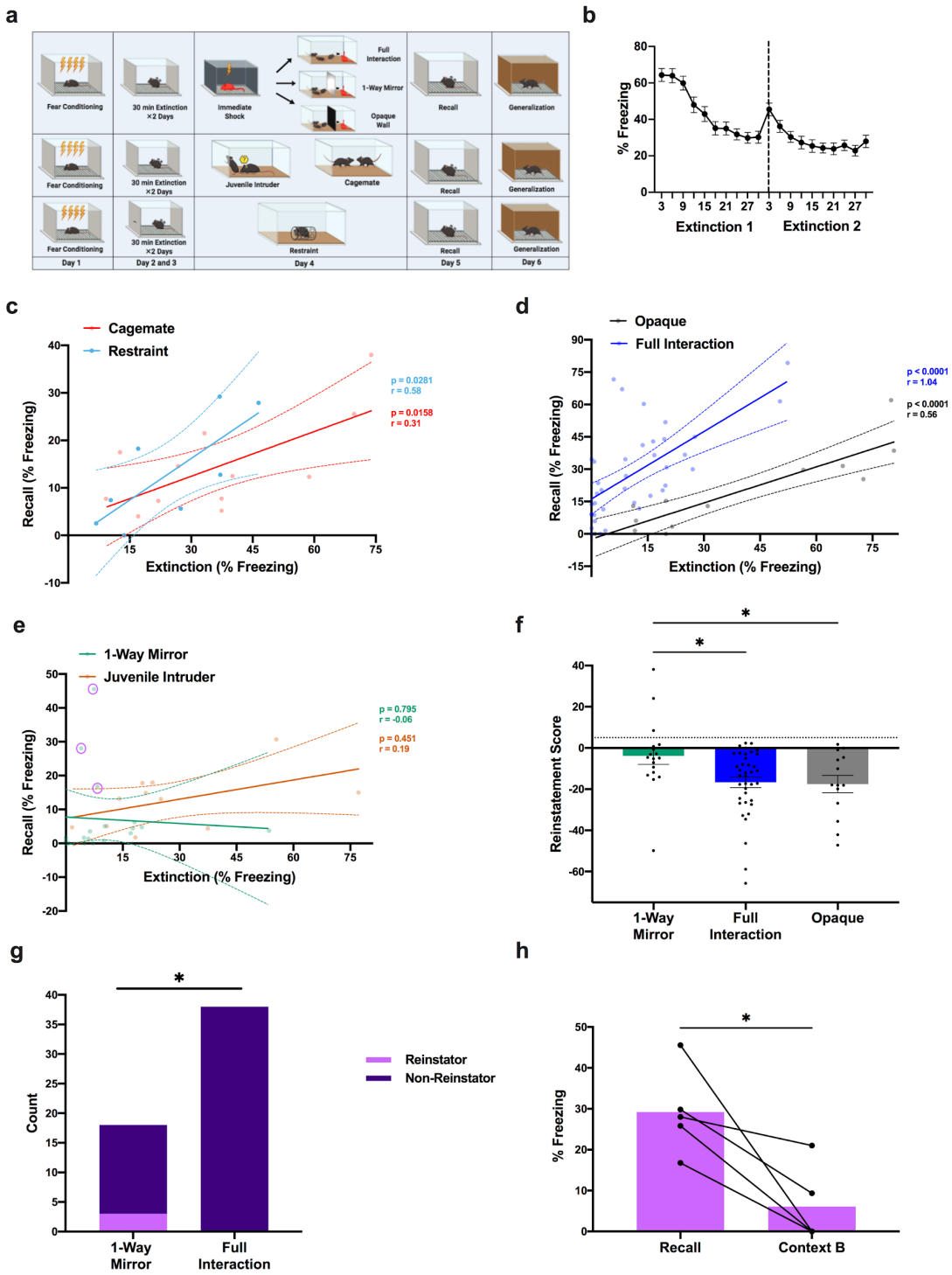

Fig. 2. A stressed mouse with unidirectional visual access to cagemates causes them to reinstate fear. a) Behavioral timeline for the 6 groups shown. **b)** Freezing throughout extinction, dashed line indicates first 3 minutes of last session. **c-e)** The relationship between freezing during

389 the first 3 minutes of the last extinction session and during the first 3 minutes of recall. Solid lines
390 show linear regressions, dotted lines at 95% confidence bands (Pearson correlation tests). **f)**
391 Comparison of the increase in freezing from the first 3 minutes of the last extinction session to the
392 first 3 minutes of recall for the three types of exposure to a recently shocked cagemate; i.e.
393 reinstatement score. (1 Way ANOVA, n=18 1-Way Mirror, n=38 Full Interaction; n=14 Opaque)
394 **g)** Proportion of reinstators (animals who freeze >5% more during recall than extinction) in the 1-
395 Way Mirror and Full Interaction group (Fisher's Exact test, n=18 1-Way Mirror, n=38 Full
396 Interaction). **h)** Freezing during recall test compared to generalization test for reinstators
397 (Student's paired t test, n=5). In e-f, data shown as mean +/- SEM. n.s. $p>0.05$, *$p<0.05$, **$p<0.01$,
398 detailed statistics shown in Supplementary Text and Table S1).
399

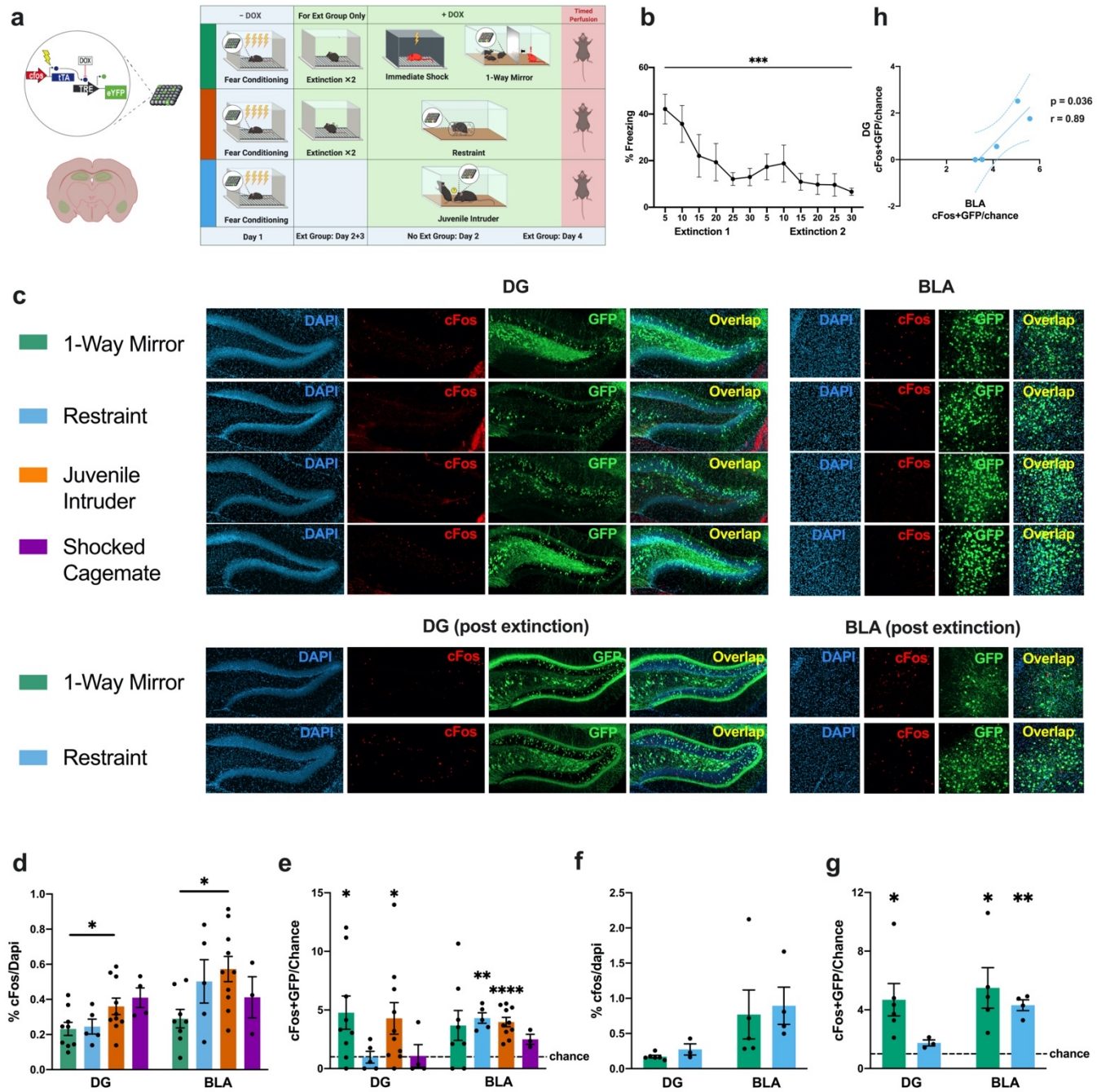

Figure 3. Social experience reactivates subsets of pre-existing fear engrams. a) Schematic representation of the doxycycline mediated viral tagging construct (left) and experimental design (right). **b)** Freezing throughout extinction training (RM 1Way ANOVA, Holm-Sidak's multiple comparisons, n=9). **c)** Confocal visualization of DG and BLA histology showing cFos-tTa+Tre-

405 EYFP labelled cells (active during FC, green) and cFos+ cells (active during the different types of
406 stress exposure, red). Left - without fear extinction, Right - following fear extinction. **d, f)**
407 Proportion of cFos+ cells per DAPI in the DG and BLA (1way ANOVA with Holm-Sidak's
408 multiple comparisons test), d - without extinction, f - with extinction. **e, g)** Amount of overlap
409 between the two labeled populations normalized over chance (1-sample t tests compared to
410 chance=1), e - without extinction, g - with extinction. **h)** Correlation between overlap in the DG
411 and BLA for the restraint stress group (Pearson's R, n=6). Other than in h, data shown as mean +/-
412 SEM. n.s. $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, detailed statistics shown in
413 ST3.

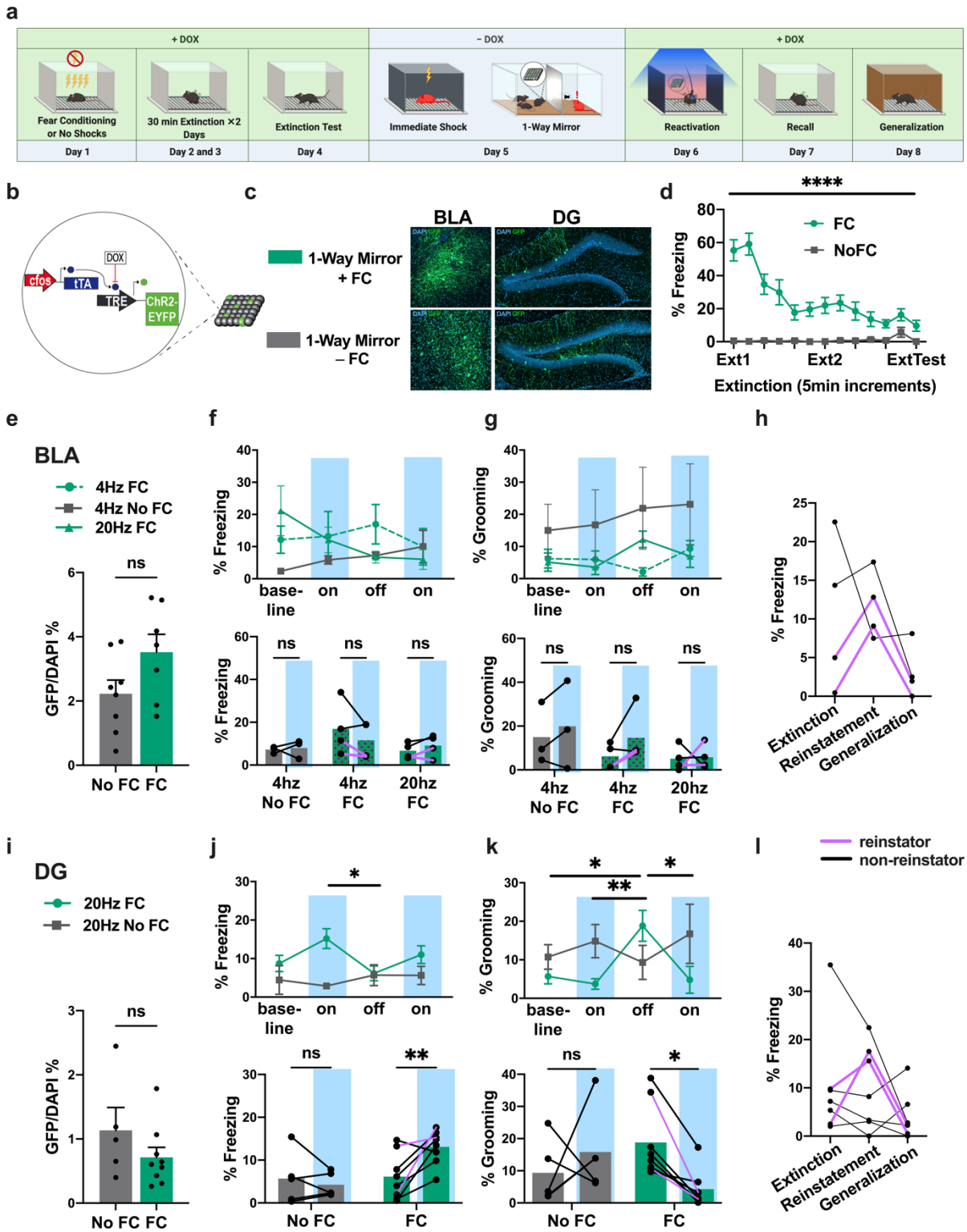

Figure 4. Optogenetic stimulation of hippocampus cells active during a social experience drives freezing only in previously fear-conditioned animals. a) Behavioral schedule. **b)**

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.

Supplementary Materials for

Social Reactivation of Fear Engrams Enhances Recall and Reinstatement

Abby Basya Finkelstein, Héloïse Leblanc, Rebecca Cole, Troy Gallerani, Anahita Vieira, Yosif Zaki, Steve Ramirez

Correspondence to: afinkelstein@fas.harvard.edu and dvsteve@bu.edu

Supplemental materials include:

- Materials and Methods
- Supplementary Text
- Figs. S1 to S5
- Tables S1 to S3
- Captions for Movies S1 to S2
- Captions for Data S1 to S5

Other Supplementary Materials for this manuscript include the following:

- Movies S1 to S2

467 **Materials and Methods**

468 Surgery

469 For all surgeries, mice were initially anesthetized under 3.5% isoflurane inhalation and then
470 maintained during surgery at 1.0-2.0% isoflurane inhalation through stereotaxic nosecone delivery.
471 Ophthalmic ointment was applied to both eyes to provide adequate lubrication and prevent corneal
472 damage. The hair above the surgical site was removed with scissors and subsequently cleaned with
473 alternating applications of betadine solution and 70% ethanol. 2.0% Lidocaine HCl was injected
474 subcutaneously as local analgesia prior to 10-15mm midsagittal incision of the skin. For
475 optogenetic implant surgeries, two bone anchor screws were secured into the cranium, one anterior
476 and one posterior to the target injection and fiber placement sites. All animals then received
477 bilateral craniotomies with a 0.6 mm drill-bit for dorsal dentate gyrus (dDG) and basolateral
478 amygdala (BLA) injections. For all dDG and BLA surgeries, a 10µL air-tight Hamilton syringe
479 with attached 33-gage beveled needle was lowered to the coordinates for DG of -2.2mm
480 anteroposterior (AP), ±1.3mm mediolateral (ML), -2.0mm dorsoventral (DV), and for BLA of -
481 1.35mm (AP), ±3.25mm mediolateral (ML), and -5.0mm (DV). All coordinates are given relative
482 to bregma (mm). For overlap surgeries, a volume of 300 nL of AAV9-cFos-tTa + TRE-EYFP viral
483 cocktail was bilaterally injected at 200 nL/min using a micro-infusion pump for each coordinate
484 (2x300 nL for dDG and 2x300 nL for BLA) (UMP3; World Precision Instruments). After injection
485 was complete, the needle was left in place 2 minutes prior to incremental retraction of the needle
486 from the brain. For dDG chemogenetic surgeries, a 300nL viral cocktail of AAV9-cFos-tTa +
487 AAV9-TRE-hM4Di was bilaterally injected into the dDG at the coordinates listed above. For dDG
488 **or** BLA optogenetic surgeries, a 300nL viral cocktail of AAV9-cFos-tTa + AAV9-TRE-ChR2-
489 EYFP was bilaterally injected into the dDG **or** BLA (separate surgeries and animals for each brain

490 region; see overlap surgeries for coordinates and procedure for injection). Following viral
491 injection, bilateral optical fibers (200µm core diameter; Doric Lenses) were placed 0.4mm above
492 the injection sites (dDG: -1.6mm DV; BLA: -4.6mm DV). The implants were secured to the skull
493 with a layer of adhesive cement (C&M Metabond) followed by multiple layers of dental cement
494 (Stoelting). Mice were injected with a 0.1 mg/kg intraperitoneal dose of buprenorphine (volume
495 dependent on weight of animal) and placed in a recovery cage atop a heating pad until recovered
496 from anesthesia. Viral targeting was confirmed by histological study and only animals with proper
497 viral expression were utilized for data analysis.
498
499 Immunohistochemistry
500 Mice were overdosed with 3% isoflurane and perfused transcardially with cold (4°C)
501 phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. Brains were
502 extracted and kept in PFA at 4°C for 24-48 hours and then transferred to PBS solution. Brains
503 were sectioned into 50µm thick coronal sections with a vibratome and collected in cold PBS.
504 Sections were blocked for 1-2 hours at room temperature in PBS combined with 0.2% triton
505 (PBST) and 5% normal goat serum (NGS) on a shaker. Sections were incubated in primary
506 antibody (1:1000 rabbit anti-c-Fos [SySy]; 1:5000 chicken anti-GFP [Invitrogen]) made in PBST-
507 NGS at 4°C for 48 hours. Sections then underwent three washes in PBST for 10 minutes each,
508 followed by a 2 hour incubation period at room temperature with secondary antibody (1:200 Alexa
509 555 anti-rabbit [Invitrogen]; 1:200 Alexa 488 anti-chicken [Invitrogen]) made in PBST-NGS.
510 Sections then underwent three more wash cycles in PBST. Sections were mounted onto
511 microscope slides (VWR International, LLC). Vectashield HardSet Mounting Medium with DAPI
512 (Vector Laboratories, Inc) was applied and slides were coverslipped and allowed to dry overnight.

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

515 Behavior

516 *Fear Conditioning* - Fear conditioning (FC) for all experiments took place in a 18.5 × 18 × 21.5
517 cm chamber with aluminum side walls and plexiglass front and rear walls (Context A). Each cage
518 animal was placed in a separate chamber (4) and received shocks in parallel during the session.
519 The session consisted of 4 shocks over a time span of 500s (shocks at 198s, 278s, 358s, 438s).
520 Depending on the experiment, the length and strength of the shock varied. For the experiments
521 without extinction training (recall 24hrs after social session), the four shocks were 1 second each at
522 1mA. For the experiments with extinction, in order to obtain homogenous and reliable fear
523 responses at a more remote time point, more intense fear conditioning was utilized, with shocks at
524 1.5mA for 2 seconds. After the session, animals were placed back into their home cage and in an
525 isolated holding area until all animals in a cohort had been fear conditioned.
526

527 *Extinction Training* - Animals were placed into Context A (see above) for a duration of 30 minutes
528 on the day following fear conditioning. A second extinction training session occurred on the next
529 day (30 minutes). All animals in a cage of four underwent extinction training simultaneously.
530 After the session, animals were placed back into their home cage and in an isolated holding area
531 until all animals in a cohort had undergone extinction training.
532

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

536 As in fear conditioning and extinction training, all animals in a cage of four underwent testing
537 simultaneously and were placed back into their home cage and in an isolated holding area until all
538 animals in a cohort were tested.
539
540 *Recall Test* - For the recall test, animals were placed into Context A for a duration of 5 minutes. As
541 in fear conditioning and extinction training, all animals in a cage of four underwent testing
542 simultaneously and were placed back into their home cage and in an isolated holding area until all
543 animals in a cohort were tested.
544
545 *Generalization* - To assess generalization, 24 hours after the recall session animals were placed
546 into Context B (see above for description) for a duration of 5 minutes. All animals in a cage of four
547 underwent testing simultaneously and were placed back into their home cage and in an isolated
548 holding area until all animals in a cohort were tested.
549
550 *Juvenile Intruder/Cagemate Control* - Animals were removed from the home cage and placed into
551 a separate, clean cage with access to food and water. The lid of the cage and the feeder were
552 removed from the home cage, which served as the interaction chamber. An experimental mouse
553 was placed back into the home cage and allowed 1 minute to acclimate. An unfamiliar younger
554 (p43-49) intruder mouse was placed into the home cage for ten minutes of interaction with the
555 experimental resident mouse; younger mice were used to avoid confounding effects of mutual
556 aggression[31,55]. A clear acrylic top was placed over the home cage during interaction. This was
557 repeated for each experimental cage animal. A different intruder mouse was used in each session.
558 After each session, both mice were removed from the home cage and placed into a separate

559 holding cage for their respective group. For cagemate control experiments, a cagemate of the
560 resident mouse was used for interaction in lieu of the unfamiliar intruder. The social session was
561 conducted with a procedure otherwise identical to that used for RIT.
562
563 *Immediate Shock* - Immediate shock was administered in a 18.5 × 18 × 21.5 cm chamber with
564 vertical black and white striped (3 cm width) sides, a plastic container holding gauze soaked in
565 almond extract under the chamber floor, and red light as room illumination (Context B). The
566 animal received a single 1.5mA shock if after extinction or 1mA shock if without extinction at the
567 2 second time point over a 60s session.
568
569 *Full Interaction* - The lid of the cage and the feeder were removed for social interaction and a clear
570 acrylic top was placed over the home cage. The recently shocked cagemate was then placed into
571 the cage and all animals were left in the testing room for one hour of social interaction. After one
572 hour, the home cage was returned to its normal condition.
573
574 *1-Way Mirror/Mirror No Mouse Control/Opaque Control* - Mirror inserts were created out of
575 laminated cardboard and one way mirror material (OuBay). Wooden dowels were placed on the
576 bottom to support the structure. Dimensions were based to fit tightly into the cage (7" x 11" x 5")
577 to separate the container into two sections (a smaller area for the recently shocked cagemate and
578 larger area for the rest of the cagemates). The lid of the cage and the feeder were removed for
579 social interaction and a clear acrylic top was placed over the home cage. In order to enhance the
580 effect of the mirror by creating a large light difference, black covers were created to cover the
581 section of the cage with the recently shocked cagemate. Refer to **Figure S1** for a visual

582 representation of the set-up. After Immediate Shock, the recently shocked cagemate was
583 immediately placed into the section opposite of and separate from the other cage mates. Animals
584 were left in the testing room for one hour of social interaction except in the chemogenetic
585 inhibition experiment, in which they were weighed and injected 15 minutes prior to Immediate
586 Shock and then left to experience the 1-Way Mirror manipulation for 15 minutes to ensure efficacy
587 of CNO in brain tissue throughout the entire experience[56]. After one hour, or 15 minutes for the
588 chemogenetic experiment, the recently shocked cagemate was removed and immediately
589 euthanized by overdose with sodium pentobarbital to ensure any subsequent effects were due to
590 the manipulation rather than any social interaction afterward, and the home cage was returned to
591 its normal condition. For the Mirror No Mouse Control, all procedural steps were identical to the
592 one-way mirror experiments, except that there was no shocked cagemate placed on the other side
593 of the mirror. For opaque control experiments, the one way mirror insert was altered through the
594 addition of a black insert on the side of the shocked cagemate so that the wall was bidirectionally
595 blocking visual input from the other side. Social interaction was conducted with a procedure
596 otherwise identical to that used for 1-way mirror experiments.
597
598 *Restraint/Tube Control*
599 One at a time, animals were removed from the home cage and enclosed in a plastic restraint tube
600 containing holes to permit air flow. The tube was then placed in the center of a clean cage for a
601 duration of two minutes. At the end of the two minutes, the animal was released from the tube into
602 a separate clean cage. For the tube control test, all animals remained in the home cage and a clean
603 restraint tube was placed into the home cage for a duration of two minutes.
604

605 *Dominance Tube Test*- 30mm (width) x 30cm (length) Plexiglass tubes were wide enough for only
606 one mouse to pass through at a time. Protocol for the dominance test was adapted from[20]. In short,
607 cages of mice were trained for two days to walk through the small acrylic tube in one, forward
608 direction. Following training, each mouse was paired against one another to test dominance. Each
609 mouse in the pair was placed on either side of the tube so that they met in opposite directions
610 inside the tube. Dominance was defined as one of the mice pushing the other out or forcing them
611 to retreat back. After all mice were tested against one another in one day, they were ranked in a 1
612 to 4 fashion. This was repeated each day until the hierarchy was kept stable at least 4 days in a
613 row. Once the hierarchy determined, the most dominant or least dominant mouse was chosen to
614 become the recently shocked cagemate in the 1-Way Mirror paradigm to determine if hierarchy
615 could predict reinstatement of the fear memory.
616
617 *Open Field Test*- An open 24in x 24in arena with black plastic walls was used for the test, with a
618 red-taped area of 12.5in x 12.5 in the middle delineating "center" from "outside". The lights in the
619 room were adjusted to minimize unwanted shadows. A camera was placed above the open field
620 area in order to record the session. The mouse was taken out of its cage and immediately placed in
621 one corner of the arena. The mouse was then left to explore the space for 10 minutes. Once 10
622 minutes had passed, the mouse was placed in a separate cage until all of the cage mates had also
623 gone through the behavioral test. They were then all placed back in their homecage.
624
625 <u>Optogenetic Stimulation</u>
626 The optogenetic stimulation session occurred 24 hours after the tagging experience (1-Way
627 Mirror). Prior to the start of the session, optogenetic patch cords were tested to ensure a minimum

628 10mA laser output. Mice were given stimulation in a separate room than Context A or B. For
629 stimulation, the mice were attached to the optogenetic cords in the palm of ABF's hand and placed
630 into a striped acrylic chamber with either white or dimmed white + red light and either almond or
631 orange scent (Context C). The session lasted 8 minutes and consisted of 4 alternating 2 minute
632 periods of laser stimulation (off/on/off/on; dDG: 450 nm, 20 Hz, 10 ms pulse width[42]; BLA: 450
633 nm, 4 Hz and 20 Hz, 10 ms pulse width[57]). The BLA group received 4 Hz and 20 Hz stimulation
634 counterbalanced in different contexts (orange vs almond scent, white vs red light) separated by
635 about 1.5hrs.

636 Behavior Scoring

637 *FreezeFrame* - Videos of behavioral sessions were obtained using cameras secured to the chamber
638 either above or to the side of the subject. For extinction, extinction test, and recall sessions,
639 FreezeFrame/View (Coulbourn Instruments; Whitehall, PA) was used to score freezing behavior,
640 defined as 1.25 seconds of animal immobility.
641
642 *ezTrack* - Videos of behavioral sessions were obtained using cameras secured to the chamber
643 either above or to the side of the subject. Videos of the open field test were obtained using a
644 webcam secured above for an aerial view. ezTrack[58] was used to analyze all open field videos. An
645 ROI of the center was defined manually according to the lines present on the floor of the open field
646 arena. The software tracked the movement of the mouse and calculated the amount of frames in
647 which the mouse moved less than specified parameters (both pixel change and distance traveled).
648 The software then tracked and calculated the amount of frames the mouse spent in the center,
649 which was converted to the amount of time spent in the center according to video parameters.
650

651 *Manual scoring* - For optogenetic sessions, due to cord movement and lighting conditions
652 interfering with automated scoring, all freezing and grooming quantification was done manually.
653 This was then converted to a percentage of time that the mouse spent freezing within the bins of
654 stimulation in the session. Each video was scored for grooming by HL, and for freezing by 2
655 separate observers (ABF and HL or ABF and RC) whose scores were averaged to mitigate
656 variation in bout length perception. Grooming was defined as any syntactic-chain cephalocaudal
657 grooming, scratching, licking, or other observable forms of non-chain self-grooming performed by
658 the animal[43]. Freezing was defined as any observable complete cessation of movement, other than
659 breathing, by the animal.

660 Imaging and Cell Counting

661 All coronal brain slices were imaged through a Zeiss LSM 800 epifluorescence microscope
662 with a 20x/0.8NA objective using the Zen2.3 software. Images of the basolateral amygdala were
663 captured in a 2x2 tile (640x640 microns) Z-stack. Images of the dentate gyrus were captured in a
664 4x2 tile (1280x640 microns) Z-stack. DAPI and GFP were imaged as separate channels for target
665 verification and ensemble size quantification. For overlap counts, DAPI, cFos, and GFP were
666 imaged (DAPI and cFos simultaneously and GFP as a separate channel). 3-4 different slices were
667 imaged for each animal for averaging.
668

669 *FIJI Software* - Images were processed for greater clarity before quantification. The Subtract
670 Background tool was used to enhance contrast of cells to background, and the Despeckle was used
671 to minimize noise that may interfere with quantification. ROIs were selected using Freehand
672 Selection so that only cells within the brain region would be analyzed. The DAPI channel was
673 segmented using a custom pipeline involving 3D Object Counter. The cFos and GFP channels

674 were initially segmented using a custom pipeline utilizing the 3D Iterative Thresholding tool of 3D
675 ImageSuite [59]. Once cells segmented, the z-stacks were Z-Projected into a single slice image and
676 saved.
677
678 *Cell Profiler* - Segmented images of DAPI, cFos, and GFP were loaded into CellProfiler and run
679 through a pipeline that identified cells with more stringent parameters of size and shape. For
680 overlap quantification, the final step in the pipeline counted all identified objects between the cFos
681 and GFP images that had an overlap of greater than 80%.
682
683 *Cell Quantification and Analysis* - Once cells were counted, the relative amounts of $cFos^+$ only,
684 GFP^+ only, and $cFos^+GFP^+$ (overlap) cells in each slice was normalized over the total amount of
685 DAPI cells present ($cFos^+$/DAPI, GFP^+/DAPI, Overlap/DAPI). Chance of an overlap was defined
686 as ($cFos^+$/DAPI)x (GFP^+/DAPI). Overlap over Chance was then calculated by dividing
687 Overlap/DAPI by the Chance Overlap calculated in the previous step.
688

689 **Supplementary Text**

690 Statistical Analysis Details

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

692 2 Way ANOVA with Sidak's multiple comparison tests of fear acquisition for males vs
693 females, n=69 males, n=63 females; Time x Sex $F(4, 520)=2.910$, $p=0.0212$; Time $F(2.717,$
694 $353.3)=323.8$, $p<0.0001$; 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.

741

742 Reactivation of DG and BLA fear ensembles during various experiences

743 *Extinction of fear* - Repeated Measures 1 Way ANOVA with Holm-Sidak's multiple comparisons

744 test, n=9. Comparing the first five minutes to the last, t=5.688, p=0.0005.

745

746 *Number of cFos+ cells during different experiences* -

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

748 **DG**: n=10 Juvenile Intruder, 9 1-Way Mirror, 4 Shocked Cagemate, 5 Restraint; F=2.825,

749 p=0.0820. Comparing Juvenile Intruder vs 1-Way Mirror, t=2.249, p=0.0339; **BLA**: n=10 Juvenile

750 Intruder, 8 1-Way Mirror, 5 Restraint, 3 Immediate Shock, F=3.961, p=0.0356. Comparing

751 Juvenile Intruder vs 1-Way Mirror, t=2.782, p=0.0118.

752

753 Extinction - **DG:** Welch's t test (due to different variances, F=8.788, p=0.0462), n=6 1-Way

754 Mirror, 3 Restraint, t=1.261, p=0.3232. **BLA**: two-tailed, unpaired t test, n=4 Restraint, 5 1-Way

755 Mirror, t=0.2716, p=0.7938.

756

757 *Reactivation of fear ensemble during different experiences* -

758 One sample t test comparing to chance overlap of 1:

759

760 No Extinction - **DG**: n=10 Juvenile Intruder, t=2.436, p=0.0376; n=9 1-Way Mirror, t=2.656,

761 p=0.0290; n=5 Restraint, t=0.06673, p=0.9500; n=4 Immediate Shock, t=0.08473, p=0.9378.

762 **BLA:** n=10 Juvenile Intruder, t=7.411, p<0.0001; n=8 1-Way Mirror, t=2.101, p=0.0737; n=5

763 Restraint, t=7.462, p=0.0017; n=3 Immediate Shock, t=3.492, p=0.0731.

764

765 Extinction: **DG**: n=6 1-Way Mirror, t=3.340, p=0.0206; n=3 Restraint, t=3.575, p=0.0701. **BLA:**

766 n=4 1-Way Mirror, t=3.257, p=0.0312; n=4 Restraint, t=9.031, p=0.0029.

767

768 Optogenetic stimulation of cells active during social experience

769 *Extinction training* - Mixed-effects model, n=11 FC, n=7 No FC, time x FC F(12, 186)=8.201,

770 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

771 comparisons, first five minutes of extinction vs extinction test, t=5.054, p<0.0001.

772

773 *Ensemble size* -

774 **DG:** two tailed, unpaired t test, n=9 Fear Conditioned, 5 Non Fear Conditioned, t=1.264,

775 p=0.2300.

776 **BLA**: two tailed, unpaired t test, n=7 Fear Conditioned, 8 Non Fear Conditioned, t=1.873,

777 p=0.0838.

778

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

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

781 **Freezing:**

782 For individual sessions -light x FC F(3, 33)=2.093, p=0.1201; light F(3,33)=0.7303; FC

783 F(1,11)=8.695, p=0.0132; Subject F(11, 33)=1.254, p=0.2931; Holm-Sidak's multiple

784 comparisons test, FC first light-on vs light-off t=3.021, p=0.0145.

785

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 $F(8, 8)=4.779$, $p=0.0121$.

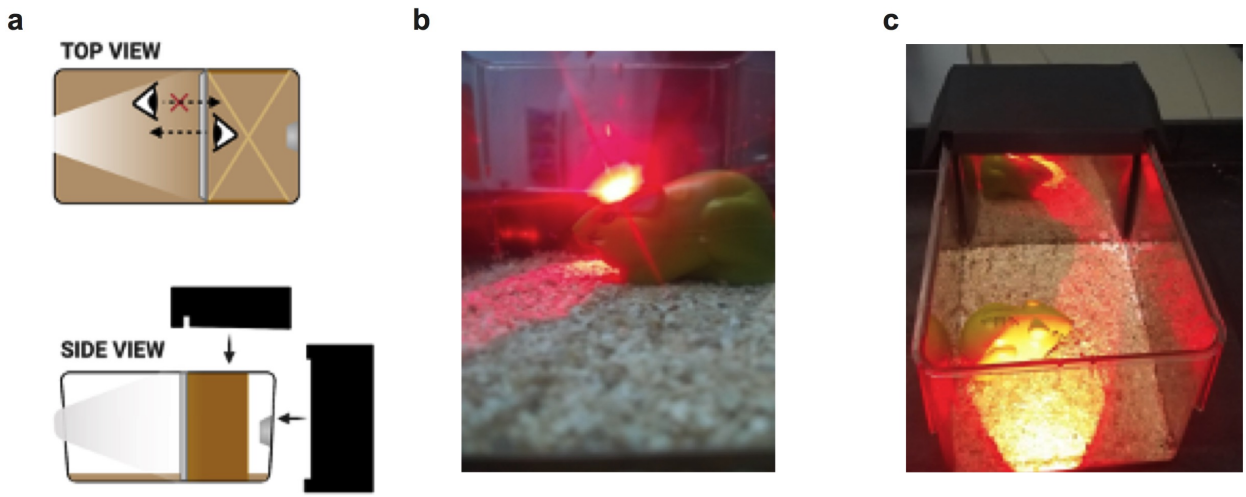

Fig. S1. 1-Way Mirror Prototype. **a)** Unidirectional visual access allows the recently shocked mouse in the darkened compartment to see the cagemates on the other side of the insert, in the bright compartment. **b)** The cagemates do not see the shocked cagemate in the dark compartment but rather see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

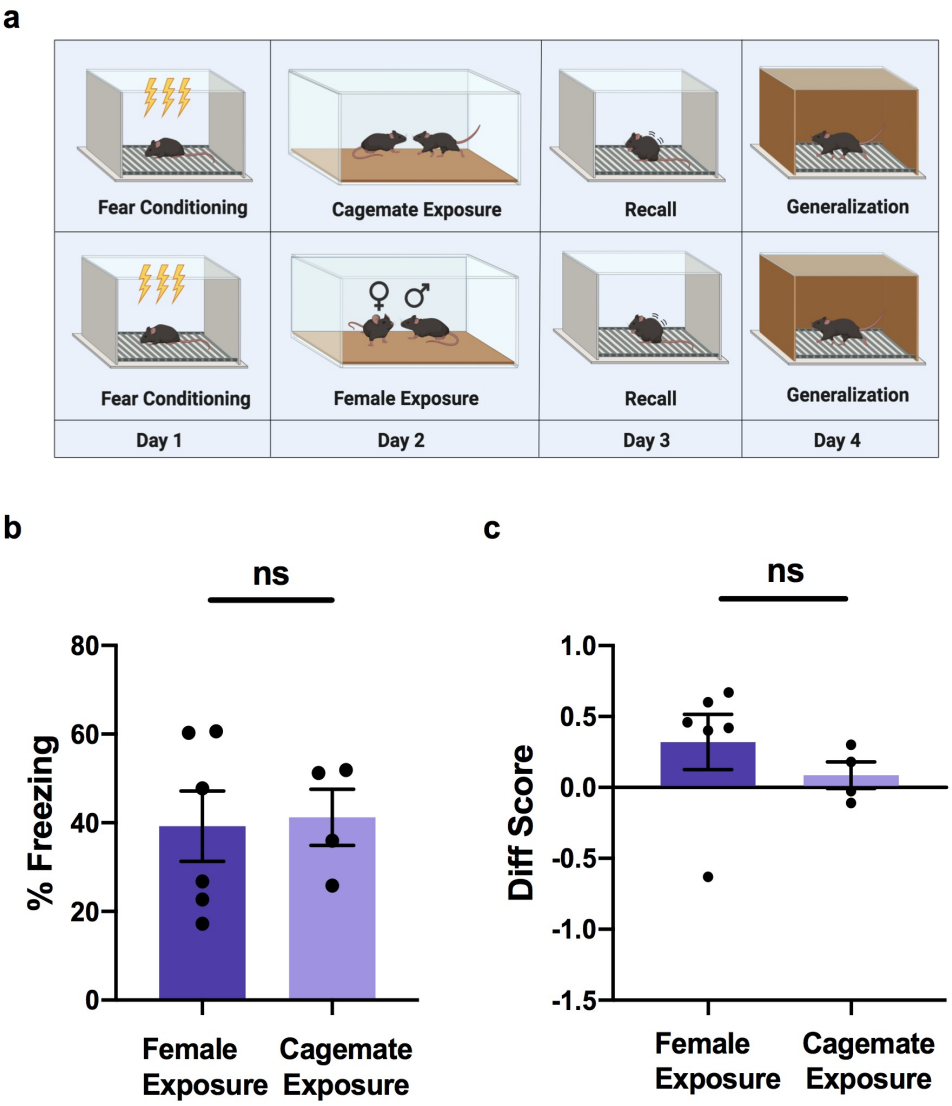

Fig. S2. Exposure to females does not impact males' fear memory recall compared to cagemate controls. a) Schematic behavioral paradigm. **b)** Freezing during recall (two tailed, unpaired t test, n=6 female exposure, 4 cagemate controls, t=0.1799, p=0.8617). **c)** Generalization represented by difference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

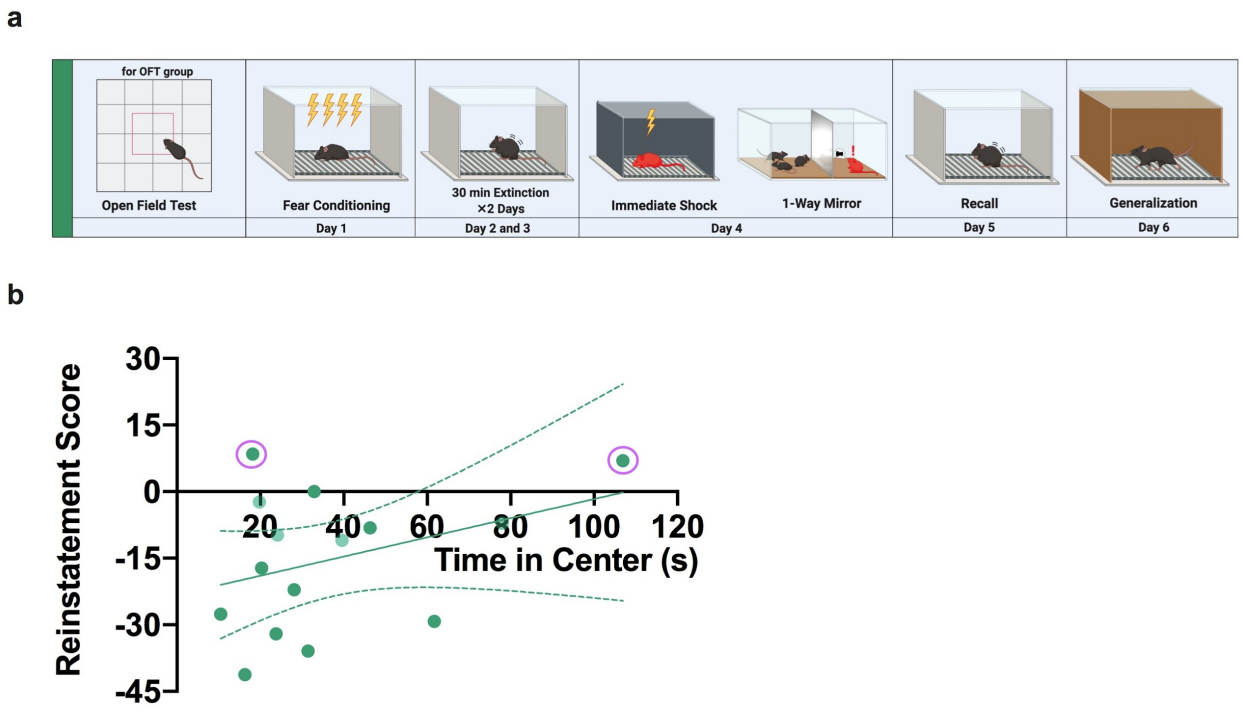

Fig. S3. Open Field Test performance does not predict socially-induced reinstatement of fear. Time in the center of the Open Field plotted on the x-axis, freezing during the first 3 minutes of last extinction session subtracted from freezing during the first 3 minutes of recall plotted on the y-axis. 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.

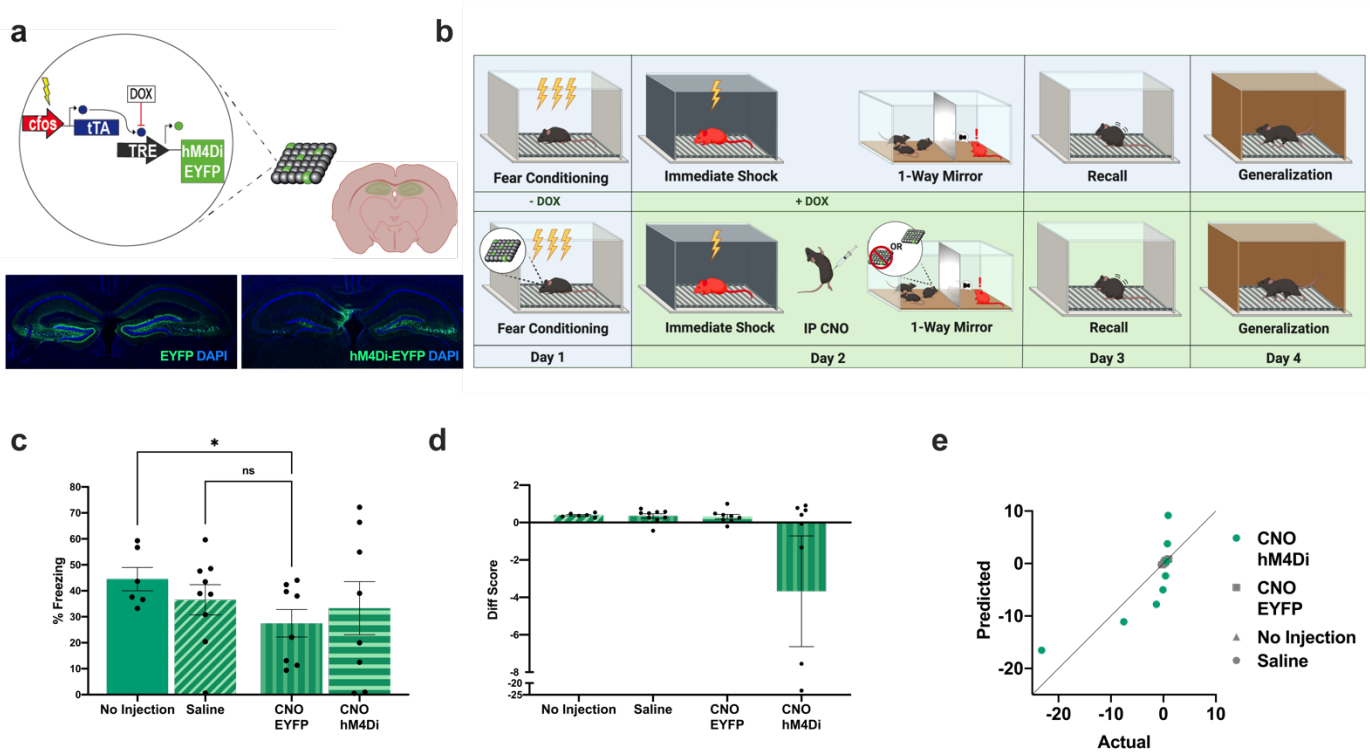

Fig. S4. CNO and injection-related stress blocks social enhancement of memory, thereby occluding the impact of chemogenetic inhibition. a) Schematic of viral construct (upper) and representative histology (lower). **b)** Behavioral timeline. **c)** Freezing during recall, n=8 CNO EYFP, 8 hM4Di, 6 No 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.

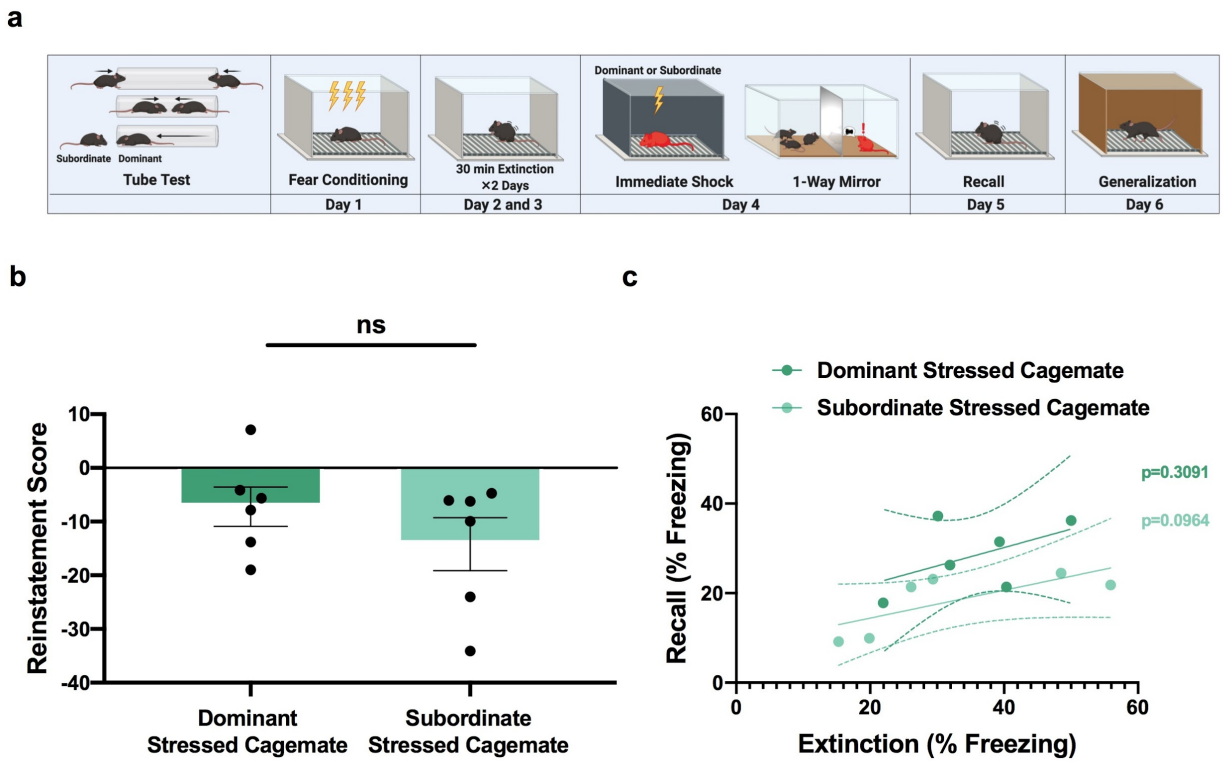

Fig. S5. Hierarchical role of the stressed animal does not impact cagemates' reinstatement. a) Behavioral timeline. **b)** Reinstatement scores, calculated as (%freezing first three minutes of recall) - (%freezing first three minutes of second extinction session). n=6 subordinate stressed cagemate, n=6 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.