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ARTICLE

Stress-induced generalization of negative memories is mediated by an extended hippocampal circuit

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Memories of negative experiences exert important control of behavior in the face of actual or anticipated threat. Sometimes, however, this control extends to non-threatening situations, a phenomenon known as overgeneralization of negative memories. Overgeneralization is a reliable cognitive phenotype of major depressive disorder, generalized anxiety disorder, and post-traumatic stress disorder. We therefore sought to develop an animal model to study stress-induced generalization of negative memories (SIG) and determine its dependence on the episodic-like memory circuit. We found that male and female mice, which were trained to differentiate a threatening from neutral context, exhibited robust SIG in response to subsequent social stress. Using chemogenetic circuit manipulations during memory retrieval, we demonstrated that both excitatory afferents to the dorsal hippocampus (DH) from the ventral tegmental area (VTA), and excitatory efferents from the DH to the retrosplenial cortex (RSC) contribute to SIG. Based on the known roles of these projections, we suggest that (1) by targeting subcortical VTA circuits that provide valence signals to the DH, stress prioritizes the retrieval of negative over neutral memories, and (2) by forwarding such information to the RSC, stress engages cortical mechanisms that support the retrieval of general relative to specific memory features. Altogether, these results suggest that various components of the extended hippocampal circuit can serve as treatment targets for memory overgeneralization.

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INTRODUCTION

Generalized anxiety disorder (GAD), major depressive disorder (MDD), and post-traumatic stress disorder (PTSD) are often accompanied by debilitating cognitive symptoms, which impede recovery from these disorders, often persist after antidepressant treatment of other symptoms, and increase the likelihood of relapse [1–7]. Additionally, they precede and predict associated affective symptoms [1, 8, 9]. A recurrent cognitive symptom across MDD, GAD, and PTSD is overgeneralization of negative memories [10–20], which involves excessive control of behavior by negative memories, even in neutral situations. Stress can promote generalization of negative memories [21, 22]; however, the neurobiological and circuit mechanisms underlying this effect are not well-understood. Notably, current knowledge of memory generalization focuses largely on processes related to the encoding [23–27] of new memories in a manner that promotes their generalization, whereas much less is known about memory retrieval processes that act upon consolidated memories [28, 29].

The dorsal hippocampus (DH) is crucial for mediating valence-memory integration, memory formation, memory retrieval, and memory generalization [30–38]. These functions partly rely on vGlut2-positive afferents from the ventral tegmental area (VTA) that provide a valence signal to the DH [33] and DH vGlut1-positive efferents to the retrosplenial cortex (RSC), which contribute to the long-lasting representations of episodic-like memories [39–45]. We therefore hypothesized that these components of the extended hippocampal circuit might contribute to stress-induced generalization (SIG).

Chronic social stressors, such as social defeat stress and social instability stress, induce a spectrum of anxiety-like and depressive-like behaviors in rodents [46–54]. Here, we demonstrate that exposure to subacute versions of these social stressors induces SIG in both male and female mice that is not associated with stress-induced anxiety-like and depressive-like behavioral phenotypes. Using chemogenetic manipulations, we show that SIG occurs at memory retrieval and is mediated by circuits involved in processing negative valence (VTA to DH) and long-term memory representation (DH to RSC).

METHODS

Mice

We performed these experiments using male and female C57BL/6J mice, vGlut1-Cre, and vGlut2-Cre mice. Wild-type C57BL/6J mice were purchased from Harlan (Indianapolis, IN) and wild-type CD-1 aggressor male mice were purchased from Charles Rivers (Wilmington, MA). All other mouse lines were obtained from Jackson Laboratory (Bar Harbor, ME). The vGlut1-Cre knockin mice, also known as Slc17a7-IRES2-Cre-D and Vglut1-IRES2-Cre-D, express Cre-recombinase in glutamatergic neuron cell bodies containing vesicular glutamate transporter 1 (vGlut1). The vGlut2-Cre knockin mice, also known as Slc17a6tm2(cre)Lowl and Vglut2-IRES-Cre, express Cre-recombinase in glutamatergic neuron cell bodies containing vesicular glutamate transporter 2 (vGlut2). Heterozygous mice were

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backcrossed with wild-type C57BL/6J for six generations in our facility to achieve offspring with a genetic identity closer to the C57BL/6J strain. The colony was subsequently expanded by homozygous breeding and confirmed by genotyping using primers and protocols posted on the Jackson Laboratory website.

All mice were 8 weeks old at the beginning of the experiments. The mice were maintained under standard housing conditions (12/12 h light–dark cycle with lights on at 7 a.m., temperature 20–22 °C, humidity 30–60%) in our satellite behavioral facility. Randomization was performed by assigning similar numbers of littermates to the different treatment conditions. Behavioral experiments were performed by an experimenter blind to drug treatments and experimental groups. All animal procedures used in this study were approved by the Northwestern University IACUC and complied with federal regulations set forth by the National Institutes of Health.

Contextual fear conditioning and generalization

Contextual fear conditioning was performed in an automated system (TSE Systems, Chesterfield, MO) [45]. Context A (Cxt A) was scented with ethanol, had a metal grid floor, was rectangular in shape, and had dim room lighting. Context B (Cxt B) was scented with acetic acid, had a smooth plastic floor, was oval in shape, and had brighter room lighting. Cxts A and B were cleaned with 70% ethanol and 1% acetic acid, respectively, between mice. Mice were pre-exposed to Cxt A and Cxt B for 3 min each. Exposures to these contexts were spaced 3 h apart, with half of the mice in each experimental group being exposed to Cxt A first and the other half being exposed to Cxt B first. 24 h later, mice underwent 3 days of contextual fear conditioning. Each day consisted of 3 min of exposure to the conditioning context, followed by a footshock (2 s, 0.7 mA, constant current). Twenty-four hours after the last day of contextual fear conditioning, mice were tested for memory generalization by placing them into Cxt B for 3 min, as adapted from Xu et al. [55]. Freezing was scored every 10 s during context exposures and expressed as a percentage of the total number of observations during which the mice were motionless. Mice were also tested for memory retrieval by returning them to Cxt A for 3 min. The Generalization Index was adapted from discrimination indices that are commonly used to assess context discrimination [24, 55] and was calculated as follows:

$$0.5 - [(\text{Cxt A freezing} - \text{Cxt B freezing})/(\text{Cxt A freezing} + \text{Cxt B freezing})]$$

A higher generalization index indicated increased generalization.

Mice were exposed to social defeat stress or social instability stress (see below for details) 24 h after pre-stress memory tests. Twenty-four hours after the completion of social defeat stress or social instability stress, mice underwent post-stress memory tests.

Social defeat stress and social instability stress

Social defeat stress (SDS) [51] was conducted in male mice by placing an "intruder" (the experimental mouse) into the home cage of an aggressive "resident" (CD-1 male mouse) for 5 min. Intruder mice were returned to their own home cage after 5 min. Experimental mice underwent social defeat once per day for 4 consecutive days and were exposed to a different aggressive resident mouse each day.

Social Instability Stress (SIS) [52, 53] was conducted in female mice by alternating mice between 72 h of housing with three other unfamiliar females and 24 h of isolation. During each round of 72-h housing, the mice were exposed to three new cage-mates. This continued for a total of 11 days.

Elevated plus maze test, open-field test, sucrose preference test, and light–dark emergence test

During the sucrose preference test (SPT), mice were allowed to freely drink from two 50 mL graduated bottles. One bottle contained 1% sucrose (vol/vol) in distilled water, and the other bottle contained distilled water alone. Mice underwent 48 h of habituation to these bottles, during which bottle locations were alternated after 24 h. After habituation, sucrose and water consumption were measured every 24 h for a total of 96 h. The mass of liquid consumed was measured to a resolution of ±0.01 g. Water and sucrose bottle locations were alternated after every measurement to eliminate position bias.

During the open-field test (OFT), mice were placed in the center of a 50 × 50 cm platform for 5 min. The time spent in the center region (25 × 25 cm), velocity, and total distance traveled were measured using automatic video tracking software (VideoMot, TSE Systems). The platform was cleaned with 0.5% ammonium between mice.

During elevated plus maze (EPM) testing, mice were placed in the center of a plus shaped platform with two open arms and two closed arms for 5 min. The dimensions of each arm were 30 × 5 cm. The time spent in the open and closed arms, velocity, and total distance traveled were measured using automatic video tracking software (VideoMot). The platform was cleaned with 0.5% ammonium between mice.

During light–dark emergence (LDE), mice were placed into a dark box (10 ×10 x 10 cm) in the center of a 50 ×50 cm platform and the latency for the mice to emerge from the dark box was measured. The platform was cleaned with 0.5% ammonium between mice.

Stereotaxic surgeries and infusions of viral vectors and drugs

Mice were anesthetized with 1.2% tribromoethanol (vol/vol; Avertin) for viral vector intracranial infusion and cannula implantation. AAV8-hSyn-DIO-GFP (Addgene 50457, Addgene, Watertown, MA), AAV8-hSyn-DIO-mCherry (Addgene 50459), or AAV8-hSyn-DIO-hM4D(Gi)-mCherry (Addgene 50475) were bilaterally infused into the VTA (3.4 mm posterior, ±0.5 mm lateral, 4.3 mm ventral to bregma) or DH (1.8 mm posterior, ±1 mm lateral, 2.25 mm ventral to bregma). Infusions were performed using an automatic pump controller (Micro4-WPI, Sarasota, FL) connected to a microsyringe (Hamilton, Reno, NV). Viral vectors were infused into the VTA at a volume of 0.35 µL per site or into the DH at 0.5 µL per site, at a titer $\geq 3 \times 10^{12}$ vg/mL, over 2 min. Syringes were left in place for 5 min to allow for virus diffusion. Mice were allowed 4 weeks for virus expression before behavioral experiments.

Bilateral 26-gauge guide cannulas (Plastics One, Roanoke, VA) were placed in the DH (1.8 mm posterior, ±1.0 mm lateral, 2.25 mm ventral to bregma) or RSC (1.8 mm posterior, ±0.4 mm lateral, 0.75 mm ventral to bregma). Mice were allowed 1 week of recovery before behavioral experiments. Clozapine-N-Oxide (CNO; Sigma, St. Louis, MO; 0.3 µg/mL in 0.7% DMSO and 99.3% aCSF (vol/vol)) or vehicle (Veh; 0.7% DMSO and 99.3% aCSF (vol/vol)) was infused through the cannulas (0.25 µL per side into DH or 0.20 µL per side into RSC over 30 s) 30 min prior to context retrieval or the generalization test. After the completion of behavioral testing, mice were intracardially perfused and cannula placements and virus spread were confirmed by immunohistochemical analysis of mCherry signals.

Immunohistochemistry and immunofluorescence

Mice were anesthetized with an i.p. injection of 240 mg/kg Avertin and transcardially perfused with ice-cold 4% paraformaldehyde in phosphate buffer (pH 7.4). Brains were removed and post-fixed for 24 h in the same fixative solution and then immersed for 24 h each in 20% and 30% sucrose in phosphate buffer. Brains was frozen and 50 µm sections were cut for use in free-floating immunohistochemistry with primary antibodies against mCherry (1:16000, chicken polyclonal, ab205402, abcam, Cambridge, MA) or tyrosine hydroxylase (1:2000, mouse monoclonal, Immunostar 22941, Immunostar, Hudson, WI) and biotinylated goat anti chicken IgG or biotinylated goat anti mouse IgG secondary antibodies (1:200, Vector Laboratories, Burlingame, CA). Signals were amplified using ABC-HRP (1:100, Vector Laboratories) and visualized with TSA tetramethylrhodamine (1:62.5, Perkin Elmer, Waltham, MA) using fluorescent microscopy.

Quantification and statistical analyses

Statistical analyses were performed using Graphpad (San Diego, CA). All statistical tests were two-sided. Data used for analyses included: (1) mice with virus expression in the DH or VTA of 50% or more of the mean number of DH or VTA-infected neurons across all experiments, (2) mice with detectable VTA-originating terminals in the DH or DH-originating terminals in the RSC, and (3) mice with correctly implanted cannulas in the DH or RSC. For the behavioral studies, two-way RM ANOVA using either (1) context and treatment as factors or (2) context and stress as factors was used to determine differences in conditioning or altered context freezing between groups, as well as the effect of stress on conditioning context and altered context freezing. Significant *F*-values were followed by post-hoc multiple comparisons using Tukey's or Sidak's test. To calculate the generalization index, the same mice were exposed to both Cxt A and Cxt B. Differences in the generalization index were also determined for treatment (CNO or Veh) or stress (Pre-Stress vs Post-Stress) using two-tailed Mann–Whitney test or Wilcoxon matched-pairs signed rank test. Differences in behavioral measures during EPM, OFT, LDE, or SPT paradigms were determined for stress (No Stress vs Stress) using two-tailed Students T test. Statistical differences were considered significant for all *p*-values <

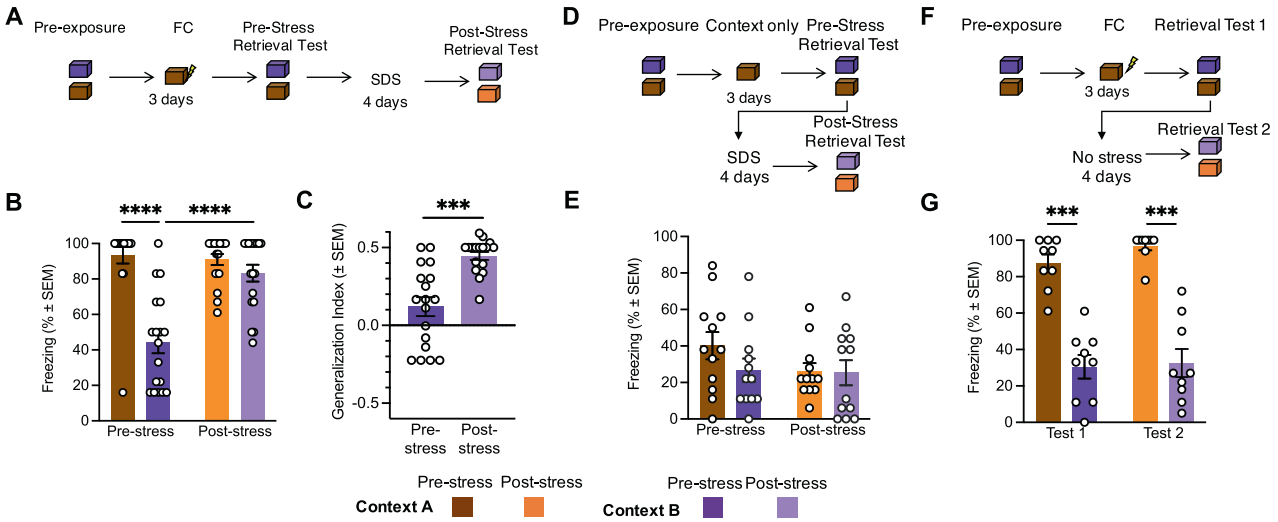

Fig. 1 Stress induces generalization of negative memories in male mice. A Behavioral schematic for SIG performed in male mice and shown in **B**, **C**. FC, fear conditioning. Arrows indicate the passage of 24 h. **B** Freezing in Context A and Context B in male mice before and after SDS. Two-way RM ANOVA. Factor: Stress, $F(1,17) = 15.15$, $p = 0.0012$. Factor: Context, $F(1,17) = 44.66$, $p < 0.0001$. Factor: Stress x Context, $F(1,17) = 34.83$, $p < 0.0001$. Post-hoc Tukey ****$p < 0.0001$. $n = 18$ mice per group. **C** Generalization index (see methods for formula) in male mice before and after SDS. Wilcoxon matched-pairs signed rank test, ***$p = 0.0001$. $n = 18$ mice per group. **D** Behavioral schematic for **E**. Note that mice were exposed to context without footshock. **E** Freezing in Context A and Context B after SDS in the absence of FC. Two-way RM ANOVA. Factor: Stress, $F(1,11) = 1.702$, $p = 0.2187$. Factor: Context, $F(1,11) = 1.032$, $p = 0.3315$. Factor: Stress x Context, $F(1,11) = 0.8979$, $p = 0.3637$. $n =$ 12 mice per group. **F** Behavioral schematic for **G**. Note that mice were exposed to FC but not SDS. **G** Freezing in Context A and Context B after FC in the absence of SDS. Two-way RM ANOVA. Factor: Timepoint, $F(1,8) = 1.702$, $p = 0.2283$. Factor: Context, $F(1,8) = 88.30$, $p < 0.0001$. Factor: Timepoint x Context, $F(1,8) = 0.3995$, $p = 0.5450$. Post-hoc Tukey ***$p < 0.001$. $n = 9$ mice per group.

0.05. All data are presented as mean ± SEM with overlaid values for individual mice. Details of statistical analyses are found in figure legends.

RESULTS

Stress induces generalization in male and female mice

In the absence of stress (pre-stress retrieval, Fig. 1A), male mice exhibited significantly more freezing in Cxt A compared to in Cxt B (Fig. 1B), indicating weak generalization. After SDS, however, mice no longer showed significant differences in freezing between Cxt A and Cxt B due to significantly higher freezing in Cxt B (Fig. 1B). Accordingly, the generalization index was significantly higher post-stress compared to pre-stress (Fig. 1C). To control for the possibility that this effect was due to an overall increase in fear due to stress, rather than interaction between stress and memory systems, we repeated the same experiment without delivery of footshocks in the conditioning context (Fig. 1D). Under this condition, we found no increase in freezing in Cxt A or Cxt B after SDS, suggesting that SDS alone does not increase freezing (Fig. 1E). Additionally, to control for potential generalization of memories over time, we repeated the same experiment with a 4-day waiting period instead of SDS (Fig. 1F) and also found no increase in freezing in Cxt B (Fig. 1G). Finally, we found that pre-stress retrieval tests were not necessary for the SIG phenotype to develop (Fig. S1), and the order of Cxt A or Cxt B testing during memory retrieval did not affect SIG (Fig. S2). Together, these results suggest that SIG arises from the effects of stress on memory systems.

Next, we sought to study SIG in female mice. SIS was performed on female mice in lieu of SDS, as male CD-1 mice used as aggressors in SDS do not reliably show aggression towards female mice. Similar to male mice, we found that after SIS (Fig. 2A), female mice exhibited significantly higher freezing in Cxt B and a significantly higher generalization index, as compared to before SIS (Fig. 2B, C). Accordingly, mice no longer had significant differences in freezing between Cxt A and Cxt B after SIS (Fig. 2B). To control for the possibilities that the effect was due to an overall increase in fear or due to generalization of negative memories over time, we repeated the same experiment without delivery of footshocks in Cxt A (Fig. 2D) or with a 11-day waiting period instead of SIS (Fig. 2F). We found no increase in freezing in Cxt A or B after stress under either of these conditions (Fig. 2E, G). As in our previous study, the order of Cxt A or Cxt B testing did not affect SIG (Fig. S2). Altogether, these results show that social stress can be a strong trigger of SIG in both male and female mice.

Stress-induced generalization is not associated with anxiety-like or depressive-like behaviors in male and female mice

SDS and SIS have been shown to induce depression-like and anxiety-like behaviors when applied over 10 days and 28 days, respectively [46–54]. We therefore examined whether these behaviors could be related to SIG, which was induced by subacute versions of these stressors in this study. In both male and female mice, we found no significant differences between stressed and non-stressed mice (Figs. S3 and S4), in their latency to emerge in the LDE paradigm or sucrose preference in the SPT. We also found no significant differences in time spent in the open arms in the EPM or time spent in the center region in the OFT, despite the trends towards significance in these two tests in female mice (Fig. S4). Altogether, these results demonstrate that SIG is not associated with anxiety-like and depression-like behaviors that are induced by chronic SDS and SIS.

vGlut2-expressing VTA projections to DH contribute to stress-induced generalization

We recently showed that vGlut2 + VTA afferents to the DH provide a negative valence signal that is sufficient to trigger fear reinstatement after extinction [33]. We therefore examined

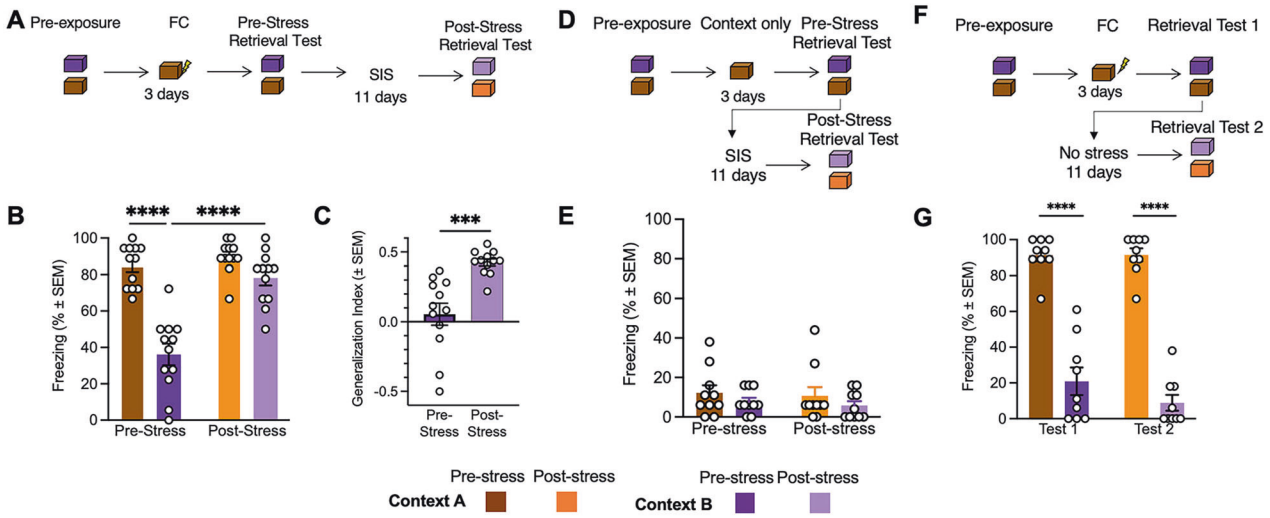

Fig. 2 Stress induces generalization of negative memories in female mice. A Behavioral schematic for SIG performed in female mice and shown in **B**, **C**. **B** Freezing in Context A and Context B in female mice before and after SIS. Two-way RM ANOVA. Factor: Stress, $F(1,11) = 34.81$, $p = 0.0001$. Factor: Context, $F(1,11) = 72.44$, $p < 0.0001$. Factor: Stress x Context, $F(1,11) = 28.66$, $p = 0.0002$. Post-hoc Tukey, $^{****}p < 0.0001$. $n = 12$ mice per group. **C** Generalization index (see methods for formula) in female mice. Wilcoxon matched-pairs signed rank test, $^{***}p = 0.0010$. $n = 12$ mice per group. **D** Behavioral schematic for **E**. Note that mice were exposed to context without footshock. **E** Freezing in Context A and Context B after SIS in the absence of FC. Two-way RM ANOVA. Factor: Stress, $F(1,9) = 0.2024$, $p = 0.6634$. Factor: Context, $F(1,9) = 1.802$, $p = 0.2124$. Factor: Stress x Context, $F(1,9) = 0.006574$, $p = 0.9372$. $n = 10$ mice per group. **F** Behavioral schematic for **G**. Note that mice were exposed to FC but not SIS. **G** Freezing in Context A and Context B after FC in the absence of SIS. Two-way RM ANOVA. Factor: Timepoint, $F(1,8) = 1.776$, $p = 0.2193$. Factor: Context, $F(1,8) = 285.1$, $p < 0.0001$. Factor: Timepoint x Context, $F(1,8) = 2.570$, $p = 0.1476$. Post-hoc Tukey $^{****}p < 0.001$. $n = 9$ mice per group.

whether these projections, by providing a negative valence signal during retrieval of Context B, might also contribute to SIG. In agreement with our previous work [33], GFP-positive terminals in DH were observed after injection of AAV8-hSyn-DIO-GFP into the VTA of vGlut2-Cre mice (Fig. 3A, B). To determine the role of these projections in SIG, we infused AAV8-hSyn-DIO-hM4Di-mCherry into the VTA of vGlut2-Cre male and female mice. Both male (Fig. 3E, F) and female mice (Fig. 3G, H) that received infusion of CNO into DH after stress and before memory retrieval showed significantly reduced generalization indices and Cxt B freezing compared to mice that received infusion of Veh (Fig. 3E–H). Additionally, CNO-infused mice showed significant differences between freezing in Cxt A and Cxt B, whereas Veh-infused mice did not (Fig. 3E, G for males and females, respectively). To rule out the possibility that this effect was due to off-target effects of CNO in the DH, we infused control virus (AAV8-hSyn-DIO-mCherry) into the DH of vGlut1 + mice, as vGlut1 is the predominant glutamatergic neuron subtype in the DH. In these mice, we found no differences in Cxt A or B freezing in mice that received CNO vs. Veh (Fig. S5), suggesting that SIG was not caused by CNO in the absence of hM4Di. To explore the role of these projections in generalization in the absence of stress, male and female mice underwent three days of fear conditioning but were not exposed to social stress afterwards. CNO or Veh was infused before memory retrieval tests. In this case, CNO-infused male and female mice showed no differences in freezing in Cxt A or Cxt B and showed no difference in generalization index compared to Veh-infused mice (Fig. S6). Altogether, these results suggest that VTA vGlut2 + projections to the DH contribute to SIG.

vGlut1-expressing DH projections to RSC contribute to stress-induced generalization

vGlut1 + and vGlut2 + DH projections to RSC are the predominant hippocampal efferents to the neocortex required for the formation (vGlut1 + and vGlut2 +) and retrieval (vGlut1 +) of context memories [39]. We next determined whether these projections also participate in SIG. First, we infused AAV8-hSyn-DIO-mCherry into DH and observed their projections in ventral RSC in vGlut1-Cre (Fig. 4A) and vGlut2-Cre mice (Fig. S7). In agreement with our previous work [39], we found robust mCherry + axon terminals in ventral RSC (Fig. 4B and Fig. S7). Next, we infused AAV8-hSyn-DIO-hM4Di-mCherry into the DH of vGlut1-Cre and vGlut2-Cre mice. After stress, we found that, in both male and female vGlut1-Cre mice (Fig. 4F–H), but not vGlut2-Cre male mice (Fig. S7), infusion of CNO into the RSC before memory retrieval tests led to a significant reduction in the generalization index and Cxt B freezing compared to the Veh-infused group. Additionally, in both male and female vGlut1-Cre mice (Fig. 4E, G), infusion of CNO before memory retrieval tests led to a significant difference in freezing between Cxt A and Cxt B after stress that did not occur in the Veh-infused group. Such effects were not seen in vGlut2-Cre mice (Fig. S7). These results suggest that SIG-relevant information was also conveyed from DH to RSC through vGlut1 + glutamatergic projections.

DISCUSSION

Here, we present a preclinical model for studying SIG by exposing mice to subacute social stress after training them to discriminate aversive from neutral contexts. While we cannot directly establish sex-specific effects due to differences in the social stressors used, it is noteworthy that despite this limitation, different social stressors similarly engaged the extended DH circuit in generating SIG. Furthermore, SIG was not associated with anxiety-like and depressive-like phenotypes that are typically reported after longer exposures to SDS or SIS [46–54]. This suggests that SIG is a separate phenotype and raises an interesting possibility that SIG may be an early cognitive change that ultimately contributes to increased negative affective states.

Memory generalization is often viewed as a problem of memory encoding [23–26], occurring either due to the inability to form sufficiently non-overlapping representations of contexts (e.g., Context A and B) [56], or due to the predominant encoding of general rather than specific features of individual contexts [24].

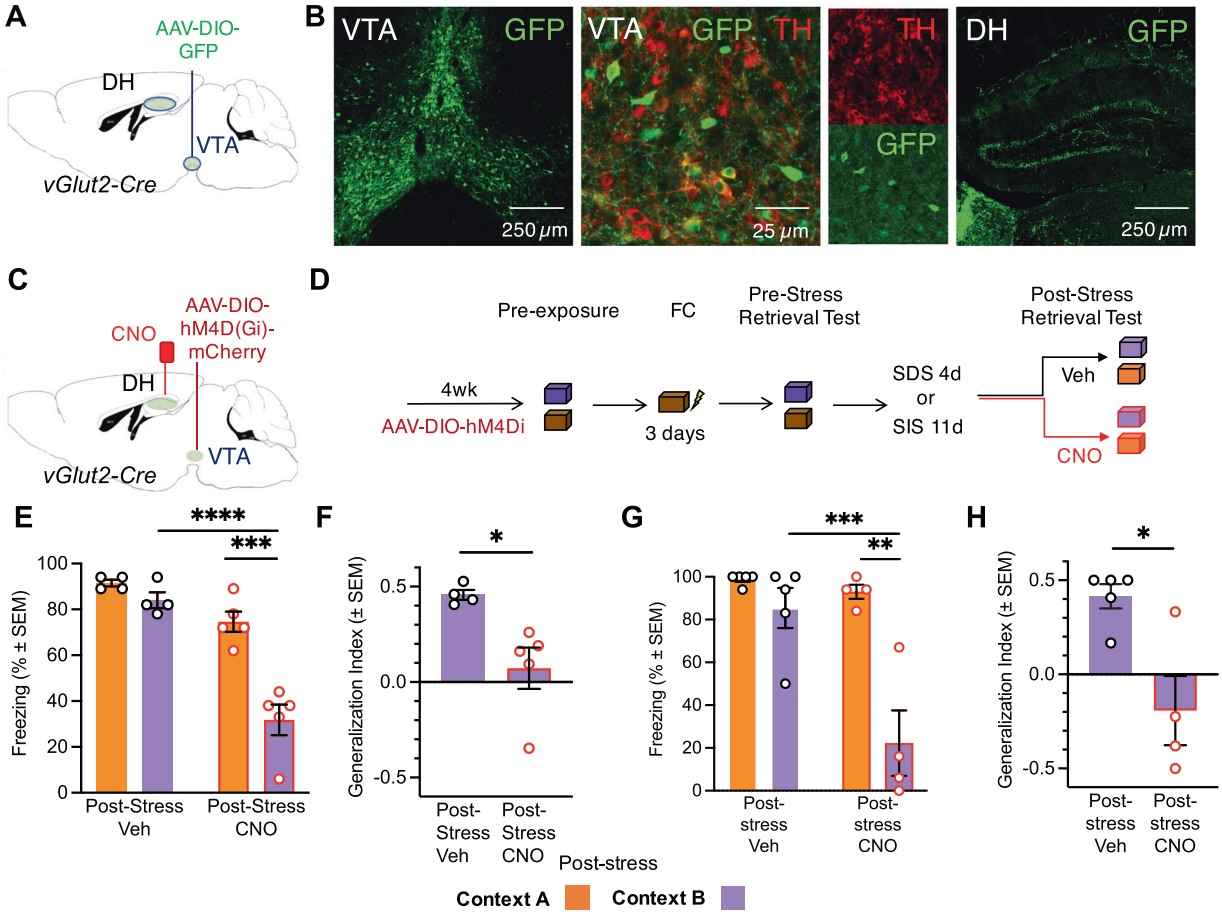

Fig. 3 Inhibition of vGlut2 + VTA projections to DH reduces stress-induced generalization in male and female mice. A Schematic of AAV-DIO-GFP injection in the VTA of vGlut2-Cre mice. **B** Cell bodies of GFP + neurons in VTA and TH immunohistochemistry (left 3 panels) and cell bodies of GFP + neurons in DH (rightmost panel). **C** Schematic of AAV-DIO-hM4D(Gi)-mCherry injection in vGlut2-Cre mice. **D** Behavioral schematic for inhibition of vGlut2 + VTA- >DH projections in **E**, **H**. **E** Freezing in Context A and Context B in SDS-exposed male mice injected with Veh or CNO into the DH before memory retrieval. Two-way RM ANOVA. Factor: Treatment, $F(1,7) = 44.26$, $p = 0.0003$. Factor: Context, $F(1,7) = 32.05$, $p = 0.0008$. Factor: Treatment x Context, $F(1,7) = 15.79$, $p = 0.0054$. Post-hoc Sidak, $^{***}p < 0.001$. $^{****}p < 0.0001$. $n = 4$ mice in Veh group, 5 mice in CNO group. **F** Generalization index in SDS-exposed male mice injected with Veh or CNO into the DH before memory retrieval. Mann–Whitney Test, Sum of Ranks: 30, 15 (Veh, CNO). $^{*}p = 0.0159$. $n = 4$ mice in Veh group, 5 mice in CNO group. **G** Freezing in Context A and Context B in SIS-exposed female mice injected with Veh or CNO into the DH before memory retrieval. Two-way RM ANOVA. Factor: Treatment, $F(1,7) = 14.83$, $p = 0.0063$. Factor: Context, $F(1,7) = 24.73$, $p = 0.0016$. Factor: Treatment x Context, $F(1,7) = 11.49$, $p = 0.0116$. Post-hoc Sidak, $^{**}p < 0.01$, $^{***}p < 0.001$. $n = 5$ mice in Veh group, 4 mice in CNO group. **H** Generalization index in SIS-exposed female mice injected with Veh or CNO into the DH before memory retrieval. Mann–Whitney Test, Sum of Ranks: 34, 11 (Veh, CNO). $^{*}p = 0.0317$. $n = 5$ mice in Veh group, 4 mice in CNO group.

However, such encoding mechanisms are not likely to contribute to SIG, given that several circuit manipulations that we applied at retrieval reversed generalization, thereby revealing that mice were able to encode the contexts in a manner that allowed them to be distinguished from each other. Recent work however, including empirical research and computational models, has emphasized that generalization is also controlled by memory retrieval mechanisms [28, 29]. Our findings show that these mechanisms are highly susceptible to social stress, which may act on the components of the extended hippocampal circuit related to both negative valence [33] and memory [39]. These findings are based on usage of chemogenetic circuit manipulations that we previously extensively validated for their ability to silence both neurons [57] and terminals [33, 39] using slice electrophysiology.

The densest DH glutamatergic projections from VTA terminate in the dentate gyrus and CA2/CA3. There are several mechanisms by which these afferents could contribute to generalization. Based on our recent work [33], we posit that VTA projections to DH provide a negative valence signal that results in reinterpretation of aspects of Context B at retrieval. At the cellular level, this can occur by reactivation of cell ensembles that encode general memory features [24] or by activation of CA3 recurrent loops that support memory generalization [58–60] by VTA afferents, or both.

DH glutamatergic projections to RSC primarily stem from the subiculum. Thus, it is likely that there are one or more intermediary intrahippocampal synapses between VTA glutamatergic projections to DH and DH glutamatergic projections to RSC. Indeed, integration and transmission of information through these microcircuits may then ultimately activate cortical memory representations, which tend to be less specific and more general than hippocampal representations [61, 62]. Therefore, SIG might also target systems consolidation mechanisms mediated by vGlut1-positive DH projections to RSC to bias retrieval toward general rather than specific context features. It should be noted, however, that the effects of our manipulations of vGlut1-positive DH projections to RSC were modest and did not completely diminish SIG, suggesting that targeting vGlut1-positive DH projections to RSC may not be necessary for the full effect of SIG. Consistent with our previous findings [39] in non-stressed mice, vGlut2 + DH- >RSC projections did not significantly

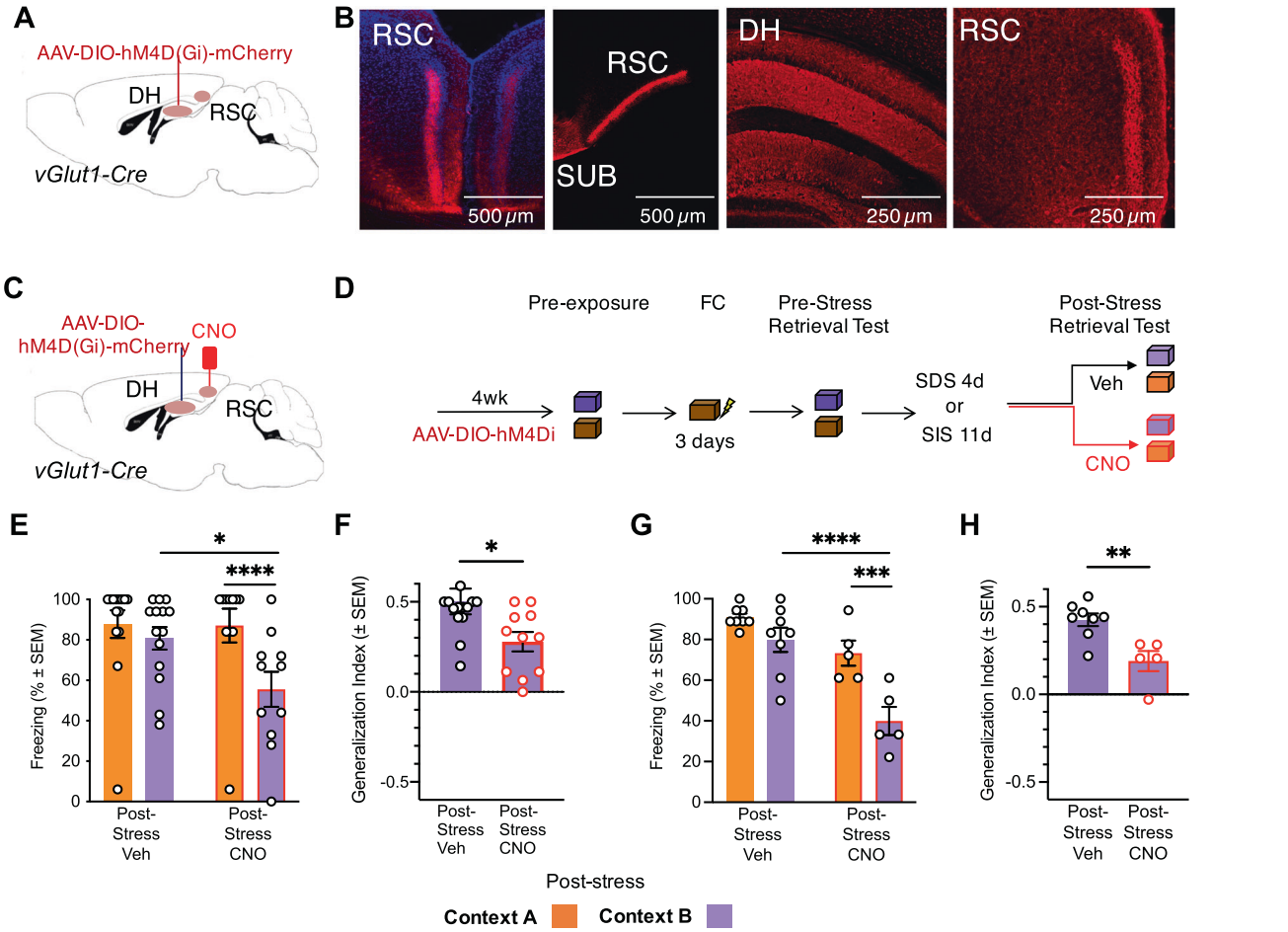

Fig. 4 Inhibition of vGlut1 + DH projections to RSC reduces stress-induced generalization in male and female mice. A Schematic of AAV-hSyn-DIO-mCherry injection in vGlut1-Cre mice. **B** mCherry + neurons in DH and their projections in RSC. **C** Schematic of AAV-DIO-hM4D(Gi)-mCherry injection in vGlut1-Cre mice. **D** Behavioral schematic for inhibition of vGlut1 + DH- >RSC projections during SIG paradigm in **E**–**H**. **E** Freezing in Context A and Context B in SDS-exposed male mice injected with Veh or CNO into the RSC before memory retrieval. Two-way RM ANOVA. Factor: Treatment, $F(1,23) = 1.894$, $p = 0.1820$. Factor: Context, $F(1,23) = 21.41$, $p = 0.0001$. Factor: Treatment x Context, $F(1,23) = 8.681$, $p = 0.0072$. Post-hoc Sidak, $^{*}p < 0.05$, $^{****}p < 0.001$. $n = 14$ mice in Veh group, 11 mice in CNO group. **F** Generalization index in SDS-exposed male mice injected with Veh or CNO into the DH before memory retrieval. Mann–Whitney Test, Sum of Ranks: 226, 99 (Veh, CNO). $^{*}p = 0.0137$. $n = 14$ mice in Veh group, 11 mice in CNO group. **G** Freezing in Context A and Context B in SIS-exposed female mice injected with Veh or CNO into the RSC before memory retrieval. Two-way RM ANOVA. Factor: Treatment, $F(1,11) = 19.24$, $p = 0.0011$. Factor: Context, $F(1,11) = 34.95$, $p = 0.0001$. Factor: Treatment x Context, $F(1,11) = 8.715$, $p = 0.0132$. Post-hoc Sidak, $^{***}p < 0.001$, $^{****}p < 0.001$. $n = 8$ mice in Veh group, 5 mice in CNO group. **H** Generalization index in SIS-exposed female mice injected with Veh or CNO into the RSC before memory retrieval. Mann–Whitney Test, Sum of Ranks: 74, 17 (Veh, CNO). $^{**}p = 0.0062$. $n = 8$ mice in Veh group, 5 mice in CNO group.

contribute to the retrieval of recent context memories either in stressed mice, supporting the specificity of our manipulations and the specificity of the effects of stress on individual projections within the extended hippocampal circuit.

The finding that glutamatergic VTA projections to DH and DH projections to RSC play a role in SIG but not negative memory retrieval is very interesting, especially given that our previous work showed that inhibition of vGlut1 + DH to RSC projections before memory retrieval in the conditioning context reduced conditioning context freezing in the absence of stress [39]. This suggests that either stress alters the neurobiology of this circuit so that it is no longer necessary for retrieval of the original memory, or that the stronger training protocol (3 context-shock associations over 3 days) used in this study might lead to a more robust contextual memory that is represented more redundantly in neuronal networks and may thus be less dependent on this circuit than a single context-shock association [63]. Additionally, this more robust fear memory may contribute to a ceiling effect in freezing levels, which may require more widespread manipulations (e.g.,: co-inhibition of several DH afferents or efferents) to observe a behavioral effect. Finally, it cannot be ruled out that mechanisms involved in retrieval of weaker aversive memories are repurposed towards sub-serving generalization during retrieval of strong memories.

Our results are translationally relevant for several reasons. First, we found that SIG is not associated with other anxiety-like and depressive-like behaviors, suggesting that SIG may be a separate cognitive change independent of affective symptoms, much like overgeneralization is a cognitive phenotype in MDD, GAD, and PTSD that can persist after treatment of affective symptoms. This also leads to the interesting possibility that SIG precedes other anxiety-like and depressive-like behaviors in a similar way as overgeneralization precedes and predicts affective symptoms of MDD, GAD, and PTSD [1, 8, 9]. Second, our finding that SIG occurs during memory retrieval may inform retrieval-based therapeutics for overgeneralization, which may be more clinically effective than

encoding-based therapeutics for overgeneralization. This is primarily due to the fact that patients have already encoded many negative memories by the time they present for treatment. Thus, SIG may be a promising model for researching therapeutic strategies for the prevention and the development of MDD, GAD, and PTSD. Finally, the inclusion of female mice in this study is crucial as MDD, GAD, and PTSD affect significantly more women than men in the United States as well as globally [64–67]. Altogether, these results provide a rodent model to study SIG, elucidate a neural circuit mediating SIG, and provide a framework for future therapeutics targeting overgeneralization.

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AUTHOR CONTRIBUTIONS

LYR and JR designed and LYR, MAAM, VSG and PG conducted the experiments. LYR and ALG bred and genotyped the transgenic mice. LYR analyzed the data. LYR and JR wrote the paper, and MAAM provided significant edits. All authors discussed and commented on the paper.

ADDITIONAL INFORMATION

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