Stress Enables Reinforcement-Elicited Serotonergic Consolidation of Fear Memory

Supplemental Information

SUPPLEMENTAL METHODS

Mice

Transgenic mice expressing Cre recombinase under the transcriptional control of the serotonin transporter promoter (SERT-Cre; generously provided by Xiaoxi Zhuang, The University of Chicago, Chicago, IL) [1] were backcrossed to C57BL/6 for at least seven generations prior to experimental use. Food and water were provided *ad libitum.* Mice (6-8 weeks old at the time of experimentation) were allowed to acclimate to colony conditions (68-72°F; 12-h light-dark cycle, 7 AM lights on) for 7-10 days prior to the start of experimental procedures. All mice were group-housed (4-5/cage). For experiments in which surgery was conducted, mice were singly housed post-surgery.

Virus

To construct adeno-associated viral (AAV) vectors, a flip-excision (FLEX) switch carrying two pairs of antiparallel loxP-type recombination sites (loxP and lox2722) was synthesized and transgenes encoding archaerhodopsin-3 fused with green fluorescent protein (Arch-GFP) or GFP alone (control) were inserted between the loxP and lox2722 sites in the reverse orientation. The final virus concentration was approximately 1.0 – 2.0 X 10^{11} infectious particles/mL. Aliquots of virus were stored at -80°C before stereotaxic injection.

Virus Delivery and Optical Fiber Implantation

Under isoflurane anesthesia (Webster Veterinary, Devens, MA), Cre-dependent AAV vectors carrying FLEX-Arch-GFP or control FLEX-GFP constructs were injected into the dorsal raphe nucleus (DRN; 4.4 mm posterior to bregma, 1.5 mm relative to the midline, and 2.5 mm ventral to the cortical surface, at a 20º angle to avoid puncturing the sinus) in SERT-Cre mice. Virus was delivered to the DRN using a 10-µl syringe and a thin 33-gauge metal needle with a

beveled tip (Hamilton Company, Reno, NV). The injection volume (1.0 µl) and flow rate (0.1 µl/min) were controlled with a microinjection pump (World Precision Instruments, Sarasota, FL). Following injection, the needle was left in place for an additional 10 min to allow diffusion of the virus.

For behavioral experiments, a multimode optical fiber (200 µm diameter core, NA 0.48; Thorlabs, Newton, NJ) coupled to a ceramic ferrule (225 µm diameter core; Kientec Systems Inc., Stuart, FL) was implanted over the same stereotactic coordinates as mentioned above. The optical fiber implant was secured to the skull with stainless steel screws and dental cement. SERT-Cre mice were allowed to recover for at least 3 weeks before behavioral and electrophysiological experimentation.

In Vivo **Recording**

Mice were anesthetized with a ketamine (100 mg/kg, i.p.) - xylazine (10 mg/kg, i.p.) cocktail. The scalp was shaved, and the mouse placed in a custom stereotactic unit, with ophthalmic ointment applied to the eyes. Three self-tapping screws (J.I. Morris Company, Southbridge, MA) were attached to the skull and a plastic head plate was subsequently affixed using dental acrylic, as previously described [2]. Once set, the mice were removed from the stereotactic unit and placed in a custom-built low profile holder. A dental drill was used to open up a rectangular (~1 mm x 2 mm) craniotomy over the previous injection site to allow insertion of a borosilicate glass pipette (Warner Instruments, Hamden, CT) attached to a 200 µm optical fiber.

Borosilicate glass pipettes were pulled using a filament micropipette puller (Flaming-Brown P97 model, Sutter Instruments, Novato, CA) and the intracellular pipette solution consisted of (in mM): 125 potassium gluconate, 0.1 $CaCl_2$, 0.6 $MgCl_2$, 1 EGTA, 10 HEPES, 4 Mg ATP, 0.4 Na GTP, 8 NaCl (pH 7.23, osmolarity 289 mOsm). Briefly, positive pressure (~200 mBar) was applied to the pipette and it was lowered to an approximate depth of -2.30 mm. The pressure was reduced to ~20 mBar and the pipette advanced in steps of 2-3 µm while constantly

monitoring the pipette resistance. Contact with a neuron was detected by a 30-60% increase in resistance, at which time the positive pressure in the pipette was released. Typically a seal for cell-attached recording stabilized after 3-4 min. An Axon Multiclamp 700B Microelectrode Amplifier (Molecular Devices Inc., Sunnyvale, CA) was used for signal amplification and an Axon Digitdata 1440A (Molecular Devices Inc.) for signal digitization. Signals were sampled at 30-50 KHz and Bessel filtered at 15 KHz. All data analyses were carried out in Clampfit (Molecular Devices Inc.) and Excel.

For photoinhibition, a green laser diode (λ = 532 nm; Shanghai Laser & Optics Century, Shanghai, China) was coupled to an optical fiber which was used to drive Arch in the DRN [3]. The irradiance at the fiber tip was measured to be ~200 mW/mm^2 prior to insertion. We identified putative serotonergic neurons based on their broad waveform shape and duration [4]; and we also evaluated firing frequency (typically 1.5-3 Hz), although this was not our primary criterion due to recent reports on the heterogeneity in the firing characteristics of serotonergic neurons [5-7].

Cannula Implantation and Microinfusion

In C57BL/6 mice, stainless steel guide cannulae (26 gauge; Plastics One, Roanoke, VA) were targeted to the basolateral amygdala (BLA; 1.4 mm posterior to bregma, ±3.1 mm relative to the midline, and 3.8 mm ventral to the cortical surface). The cannulae were secured with stainless steel screws and dental cement. SB242084, a selective serotonin 2C receptor (5-HT2CR) antagonist, was delivered to the BLA immediately following fear conditioning. Drug administration was controlled by a programmable microinjection pump (Harvard Apparatus, Holliston, MA) that delivered SB242084 (0.4 μg/0.4 μl) to the injection site over a one-minute period. Microinfusion volumes for these structures were similar to those used in other published reports [8,9]. The injector was left in place for an additional minute to allow diffusion from the needle tip before the injector was removed.

Fear Conditioning Apparatus

Conditioning occurred in clear plastic chambers (10 L x 8 W x 7 H inch) that were placed in a sound-attenuating cabinet. The cabinet had a tone generator and a 15 W clear light bulb mounted to the ceiling. The conditioning chambers rested on a removable floor of stainless-steel rods (ENV-3013WR; Med Associates, St. Albans, VT). Each rod was wired to a shock generator and scrambler (ENV-414S; Med Associates) for the delivery of footshock. The mounted tone generator delivered an 85 db, 2.2 kHz tone. Presentation of stimuli was delivered via a TTL pulse generator (National Instruments, Austin, TX) and controlled with Python 2.6 software.

Fear Conditioning

Prior to conditioning, each mouse was taken from its colony room and transported to a holding room for 1 h. Fear conditioning and testing took place in a room separate from that where immobilization occurred. The fear conditioning protocol consisted of 4 tone conditional stimulus presentations (CS; each 30 sec in duration) and 4 footshock unconditional stimulus presentations (US; 0.5 mA, each 2 sec in duration). The first CS presentation always occurred 2 min after placement of the subject in the conditioning chamber, and a 2 min interval separated all CSs and concluded the session. Importantly, the session duration, the number of CS presentations, and the number of US presentations were the same for all subjects. To achieve 50% CS-US pairing, two of the USs were paired with CSs (the 2 sec footshock coincided with the last 2 seconds of the 30 sec tone), while the remaining two US presentations occurred during the inter-CS intervals either 42, 52, or 72 seconds prior to the next CS presentation. To achieve 0% CS-US pairing, all four US presentations were presented during the inter-CS intervals, either 42, 52, or 72 seconds prior to the next CS presentation.

To measure auditory fear memory strength, mice were returned the following day to an altered context. In the novel test environment the original conditioning chamber was altered by removal of the shock grid and placement of a Plexiglas plate between two diagonally opposite

corners, forming a triangular chamber. The brightly lit conditioning chamber was replaced with a 25 W red light bulb. Further, the house light for the room was turned off. During the initial 3 min (pre-tone) the subject's freezing to the novel environment was scored. This was followed by presentation of the conditional tone for 3 min. Freezing was defined as the absence of all movement except that required for respiration [10]. For some experiments, behavior during the tone test was recorded by a digital video camera mounted directly above the chamber and freezing levels were scored by a male observer blind to the experimental groups using a time-sampling procedure every 10 seconds throughout the memory test. In some experiments, an infrared camera recorded behavior during conditioning and the tone test, and activity levels were determined with software using a proprietary formula that calculates a value for the average change of grayscale pixel values in the video (acquired at 30 Hz; VideoFreeze, Med Associates). In this case, the time spent freezing was calculated by the software after the experimenter determined a "threshold" value for freezing. Percent freezing was computed for each tone presentation and during 1 min bins before the presentation of the first tone; this yields an index of fear memory strength amenable to parametric statistics [10]. For assessing shock reactivity, the average raw value of the pixel change was used as a measure of motor activity (arbitrary units) during each 2 sec shock.

Photoinhibition

For Arch-mediated photoinhibition, a 532 nm green laser diode (Shanghai Laser & Optics Century Co.) was coupled to a 200-μm multimode silica-core optical fiber through an FC/PC adapter. A fiber-optic rotary joint (Doric Lenses, Quebec, Canada) was used to release torsion in the connector fiber caused by the animal's rotation. Photostimuli consisted of green light pulses of 30 sec duration and power levels that yielded a fiber tip irradiance approximately 225 mW/mm^2 as determined by an optical power meter (Newport, Irvine, CA).

ELISA

Thirty minutes after fear conditioning, mice were overdosed with isoflurane and the brain was rapidly dissected and placed into chilled 0.1 M phosphate-buffered saline (PBS; pH 7.4) for one minute. After placement in a chilled matrix, 1 mm thick coronal sections were taken. Bilateral punches (2 mm diameter) containing the BLA were removed from each mouse and placed in a low-binding Eppendorf tube, flash frozen, and stored at -80ºC.

Tissue was thawed on ice and homogenized using a motorized pestle (VWR, Radnor, PA) for 20 sec in lysis buffer (1:15; 15 µl of 1X PBS, pH 7.3, with 2% HALT, 0.15% NP-40, 0.1% ascorbic acid per 1 µg of tissue). Each sample remained on ice for 5 min before spinning at 17,200 *g* for 20 min at 4ºC; the supernatant was placed in a new tube. Serotonin was detected in individual samples in duplicate with a commercially available serotonin ELISA kit (ADI-900-175, Enzo Life Sciences, Farmingdale, NY) according to the manufacturer directions. Serotonin levels were normalized to the protein concentration for each homogenized sample.

Biotinylation of Surface Proteins

Ten minutes after fear conditioning, the BLA was microdissected and the tissue was processed for biotinylation of surface proteins using a protocol developed for hippocampal slices [11] and BLA punches [12]. Mice were overdosed with isoflurane and the brain was rapidly dissected and placed into chilled 0.1 M PBS (pH 7.4) for one minute. After placement in a chilled matrix, 1 mm thick coronal sections were taken. Bilateral punches (2 mm diameter) containing the BLA were removed from each mouse and coarsely minced into pieces of approximately 0.5 mm^3. Each tissue mince was placed into 500 µl of ice-cold Tris-buffered saline (TBS; pH 7.2) containing 5% HALT protease and phosphatase inhibitor cocktail and placed on ice. Pairs of samples were processed for surface biotinylation using a commercial kit (Pierce Cell Surface Protein Isolation Kit, PI89881; Pierce Biotechnology, Rockford, IL). All samples remained on ice throughout the procedure, except during incubations which were performed on an orbital shaker at 4ºC. All

centrifugation was conducted at 500 *g* and 4ºC and supernatants were removed and discarded after each spin. Samples were spun for 1 min and washed an additional two times with chilled TBS. Ice-cold biotin (1 mg/mL) was added to each tube (500 µl) and incubated (1 h). The reaction was quenched by incubating with quenching solution (100 µl; 20 min). After spinning, 500 µl of TBS containing 5% HALT was added to each tube. Tubes were inverted twice to mix, and samples were incubated for 20 min. After spinning, two additional washes and spins were performed. After the final spin, the supernatants were removed and the pelleted samples were stored at -80ºC until further processing.

To lyse the cells, samples were thawed on ice. All tubes remained on ice throughout the procedure. Chilled homogenization buffer [RIPA lysis and extraction buffer (Thermo Scientific) with 2.5% HALT] was added to each tube (labeled Set #0; 250 µl). After mixing with a pipette, samples were spun (10 min; 1000 *g*; 4ºC). The supernatant was removed to a new tube (labeled Set #1) and placed on ice. Chilled homogenization buffer was added (250 µl) to the pellet in tube Set #0 and a pipette was used to mix each sample. Each sample was mixed with motorized pestle (VWR, Radnor, PA) (30 sec) and placed on a vortex (5 sec; full power), followed by pipetting (1000 µl tip; 10 times). Each tube was then sonicated in chilled water in a cup sonicator (5 sec; 50% power), followed by incubation (30 min). Samples were sonicated a second time. A portion of this solution (50 µl) was saved (Set #2; stored at -20ºC) and used as the whole cell fraction for western blot. The remaining 200 µl was spun (5 min; 10,000 *g*; 4ºC) and the supernatant was combined with the supernatant in Set #1. The tubes containing the pellet were labeled Set #3 and stored at -80ºC.

To create the suspension for binding the biotinylated proteins, NeutrAvidin agarose was swirled to obtain an even suspension. The agarose was added to new tubes (labeled Set #4; 150 µl) and spun (1 min; 1000 *g*; room temperature). The supernatant was discarded and RIPA buffer (no HALT; 200 µl) was added. Inversion was used to mix the slurry, and the suspension

was spun (1 min; 1000 *g*; room temperature). The wash and spin procedure was repeated two additional times.

To bind the proteins to the suspension, the clarified supernatant from the tubes labeled Set #1 was added to the agarose. Tubes were sealed with Parafilm and incubated on a slowly rotating end-over-end shaker (18 h; 4ºC).

The next day, tubes were spun (2 min; 1000 *g*; 4ºC). The supernatants were transferred to a new set of tubes (Set #5; unbound fraction; stored at -80ºC).The agarose was washed (200 µl of RIPA with 2.5% HALT) and spun (1 min; 1000 *g*; room temperature) three times, with the supernatant discarded each time. SDS-PAGE sample buffer (containing 50 mM DTT) was added to each pellet (80 µl), and the tubes were vortexed (10 sec). Tubes were sealed with Parafilm and incubated on a slowly rotating end-over-end shaker (1 h; room temperature). The tubes were spun (2 min; 1000 *g*; room temperature). Supernatant (5 µl) was removed to another tube on ice (Set #6) for determination of protein concentration. The remaining supernatant was split across two set of tubes (Sets #7,8; stored at -20ºC) and used as the surface fraction for western blot. Immediately following sample elution, the protein concentration of each sample was determined.

Protein Assay

Protein concentrations of tissue homogenates were determined in duplicate using a commercial kit (Thermo Fisher Scientific, Inc., Waltham, MA). Manufacturer's instructions for the microplate assay procedure were followed except that a sufficient volume for either two wells of standard (20 µl) or each sample of unknown protein concentration (20 µl of either a 1:10 or 1:5 dilution in sterile water) was combined with two wells of protein assay reagent (300 µl) in a single Eppendorf tube before 160 µl was pipetted into each well of the microplate. For biotinylated tissue samples, ionic detergent compatibility reagent (Thermo Fisher Scientific, Inc.) was added

to the protein assay reagent (5% w/v) before combining this reagent with the standards and samples.

Western Blot

Protein samples (8 µg for 5-HT2CR and 30 µg for adenosine deaminase acting on RNA 1 (ADAR1)) were heated to 95°C for 10 min, and loaded into a standard polyacrylamide gel (NuPAGE Bis-Tris 4–12%; Life Technologies, Grand Island, NY). Protein was transferred to a nitrocellulose membrane electrophoretically using the iBlot dry-blotting system (175 V for 75 min; Life Technologies). Nonspecific binding was reduced with Odyssey blocking buffer for 1 h at room temperature (RT). Primary antibodies (in Odyssey blocking buffer containing 0.2% Tween-20 overnight at 4°C) were: rabbit anti-5-HT2CR (1:5,000; LifeSpan BioSciences, Seattle, WA) and rabbit anti-ADAR1 (1:1,000; Cell Applications, San Diego, CA). The loading control for samples was mouse anti-β-actin (1:200,000; Sigma). Blots were washed 4 x 5 min with PBS with 0.1% Tween-20, and probed with IRDye 800CW goat anti-rabbit and goat anti-mouse IgG secondary antibodies (1:10,000; LI-COR Biosciences, Lincoln, NE) for 1 h at RT. Each band was detected and quantified by the Odyssey Infrared Imaging System (LI-COR Biosciences). For each sample, the protein level was normalized to the loading control β-actin.

High-Performance Liquid Chromatography

5-Hydroxyindoleacetic acid (5-HIAA) content was measured by high-performance liquid chromatography (HPLC) with electrochemical detection. The system consisted of an ESA 5600A Coularray detector with an ESA 5014B analytical cell and an ESA 5020 guard cell. The column (Phenomenex, Torrance, CA) was maintained at 40°C, and the mobile phase was a 20 mM phosphate buffer (PB; pH 2.5). The analytical cell potentials were kept at +220 mV and the guard cell at +250 mV. Samples (25 µL) were injected with an ESA 542 autosampler that kept the samples at 6°C. External standards (Sigma) were run each day to quantify 5-HIAA.

Immunohistochemistry

Following experimentation, mice were anesthetized with isofluorane and perfused through the left cardiac ventricle with ice-cold physiological saline followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Brains were removed and post-fixed overnight, then transferred to 30% sucrose in PBS and stored at 4ºC until sectioning. DRN serial sections (30 µm) were obtained in a -20ºC cryostat and placed in 0.01 M PBS until processing.

Sections were washed three times in PBS containing 0.5% Triton X-100 (PBS-T) and then blocked overnight at 4ºC in PBS-T with 2.5% bovine serum albumin. Then, sections were incubated for 48 h at 4ºC with a mixture of primary antibodies: chicken anti-GFP (1:500; Millipore) and mouse anti-tryptophan hydroxylase (TPH; 1:500, Sigma). Sections were then washed with PBS-T and incubated (2 h) at RT with secondary antibodies conjugated to different dyes: goat anti-chicken Alexa Fluor 488 and goat anti-mouse Alexa Fluor 594 (1:500; Invitrogen). After several washes in PBS the sections were mounted onto SuperFrost Plus slides (Fisher Scientific) and coverslipped with VectaShield mounting medium with DAPI (Vector Laboratories, Burlingame, CA) and sealed with nail polish for microscopy.

The TPH antibody used does not distinguish between the TPH1 and TPH2 isoforms, however prior studies have demonstrated that TPH1 immunoreactivity is not detectable in rodent dorsal raphe serotonin neurons [13]. Thus, the immunofluorescence quantified most likely represents immunoreactivity to TPH2.

Tissue was examined on a confocal laser scanning microscope (Carl Zeiss, Jena, Germany) and images of DRN sections were taken by acquiring image stacks as provided by the microscope software for validation of virus injection sites. For quantification of labeling efficiency and colocalization of GFP-expressing and TPH-immunoreactive (ir) neurons, brain sections from GFP-transduced SERT-Cre mice were collected spanning the rostral-caudal axis of the DRN from approximately bregma -4.30 to -4.90 mm. The number of TPH-ir neurons coexpressing GFP, the number of GFP-ir neurons coexpressing TPH, and the total numbers of

TPH- and GFP-ir neurons were counted. For each subject, two brain sections at each rostral-caudal level of the DRN were quantified and averaged. GFP immunofluorescence was not observed in the median raphe nucleus, a serotonergic structure ventral to the DRN.

Statistics

All statistical comparisons were computed using StatView for Windows (Version 5.0.1; SAS Institute, Cary, NC). Data were analyzed by either Student's *t*-test or repeated-measures ANOVA followed by *post hoc* comparisons (Fisher's protected least significant difference). All data is expressed ± standard error of the mean. All group data were considered statistically significant if $P < 0.05$. All results are comprised of two or more independent replications for each experiment.

SUPPLEMENTAL RESULTS

Stress-Enhanced Fear Cannot Be Attributed to Enhanced Acquisition, Pain Processing, or Retrieval

We explored the possibility that repeated stress enhances fear memory by facilitating fear acquisition, potentiating shock reactivity during conditioning, or enhancing retrieval and/or performance during the long-term memory test. In groups of stressed and unstressed mice, stress had no impact on freezing levels during fear acquisition (Stress: $F_{(1,54)}$ = 1.53, Stress X Time interaction: $F_{(4,216)}$ = 1.40, *Ps* = n.s., **Figure S1A**). Thus, repeated stress did not enhance fear memory acquisition. The memory-enhancing effect of stress cannot be attributed to stress-related enhancement of pain processing during the aversive footshocks: repeated stress did not alter the motor response to the footshock (Stress: $F_{(1,54)}$ = 1.89; Stress X Trial interaction: $F_{(3,162)}$ = 0.993, *Ps* = n.s., *n* = 27-29/group, **Figure S1B**), consistent with previous studies [14]. Furthermore, prior immobilization stress did not alter general motor activity (total distance and velocity) during the pre-tone period prior to the auditory fear test (*Ps* = n.s., unpaired *t*-test, **Figure S1C,D**). The memory-enhancing effect of stress also cannot be attributed to changes in long-term fear memory retrieval or performance because exposure to repeated immobilization stress after fear conditioning had no effect on later fear retrieval (Stress: $F_{(1,18)}$ = 0.169, Stress X Tone interaction: $F_{(1,18)}$ = 3.42, *Ps* = n.s., *n* = 10/group, **Figure S2**), consistent with previous studies [14].

The observation that repeated stress initiated 24 h following fear conditioning has no impact on long-term fear memory suggests that the critical window in which stress influences consolidation occurs shortly after fear conditioning. This aligns with prior studies that have found selective enhancement of fear memory following pre-training but not post-training (given 24 h+ after training) stressor exposure [14,15], and also with an extensive literature showing that

immediate post-training manipulations of neuromodulators within the amygdala can affect the consolidation of aversive memories [16,17].

We also examined whether the immobilization stress had to be repeated to produce enhancement of learned auditory fear. We found that single session of immobilization stress did not produce fear enhancement (Stress: $F_{(1,17)}$ = 0.364, Stress X Tone: $F_{(1,17)}$ = 0.566, *Ps* = n.s., *n* = 9-10/group, **Figure S3**), consistent with other reports [18].

Selective Targeting of Arch to DRN Serotonergic Neurons

To determine whether serotonergic activity in the DRN is important for stress-enhanced fear, we used an AAV vector expressing the light-driven silencing opsin Arch. Arch was encoded in a Cre-inducible expression cassette (AAV-FLEX-Arch-GFP; **Figure S5A**). Arch or the control vector (AAV-FLEX-GFP) was delivered to the DRN of SERT-Cre transgenic mice.

Histological characterization of DRN tissue sections transduced with AAV-FLEX-Arch-GFP confirmed that Arch expression was specific to serotonergic neurons [demonstrated by GFP and TPH (the rate-limiting enzyme for serotonin synthesis) coexpression; **Figure S5B,C**]. GFP-positive neurons showed robust colabeling with TPH throughout the rostral to caudal regions of the DRN (mean: 98.66 ± 0.80%; **Figure S5C**, left panel). The virus transduced both the dorsal and ventral subdivisions of the DRN, representing the main subregions where BLA-projecting serotonin neurons are located [19]. GFP immunofluorescence was not observed in the median raphe nucleus, a serotonergic structure ventral to the DRN. A high percentage of TPH-positive cells were also colabeled with GFP (mean: 69.97 ± 1.81%; **Figure S5C**, right panel).

Temporally Precise Optical Silencing of Serotonergic Neuronal Activity

To assess the *in vivo* function of Arch, we performed loose cell-attached recordings from transduced DRN in SERT-Cre mice. Under ketamine-xylazine anesthesia, we recorded from the DRN in a head-fixed preparation, illuminating neural tissue with a 200 μm optical fiber coupled

to a 532 nm laser (fiber tip irradiance ~200 mW/mm^2, center-to-center distance between electrode tip and optical fiber tip ~800 μm) 3-4 weeks after viral injection. We identified putative serotonergic neurons based on their broad waveform shape and duration (**Figure S6A**) [4]; we also evaluated firing frequency (typically 1.5-3.0 Hz), although this was not our primary criterion due to recent reports on the heterogeneity in the firing characteristics of serotonergic neurons [5-7].

Clear silencing of the activity of putative DRN serotonergic neurons was observed in response to 30 sec of green light delivery to the DRN in animals injected with AAV-FLEX-Arch-GFP (**Figure S6B**, upper panel). Arch-mediated silencing of DRN activity was time-locked to the illumination period (87.09 ± 2.10% photoinhibition of firing rate relative to baseline, n = 11 recordings, 3 mice; **Figure S6B**, middle panel); the firing rate was restored to levels indistinguishable from baseline following light delivery (P = 0.519, paired t-test; **Figure S6B**, middle panel). In contrast, light delivery to the DRN in nontransduced mice had no significant impact on spiking activity (firing rate: 2.00 ± 0.32 Hz baseline versus 2.09 ± 0.27 Hz during light; P = 0.504, paired t-test; n = 14 recordings, 3 mice, **Figure S6B**, lower panel) in putative DRN serotonergic neurons, indicating that nonspecific effects of light on neurons, such as heat, were not responsible for the change in DRN neural activity in mice transduced with Arch.

SUPPLEMENTAL DISCUSSION

The fact that serotonergic consolidation of fear memory is present in mice with stress-enhanced receptivity to serotonin, together with our observation that DRN photoinhibition during only half of the aversive stimulus presentations was sufficient to fully reverse stress-related enhancement of fear, suggests that there is a cumulative, temporally integrated “threshold” of serotonin in DRN targets which must be exceeded for fear memory consolidation to be enhanced. This threshold is likely achieved both by the amount of aversive reinforcement, shown here to be the trigger for the serotonin release into the BLA, and also by stress-related changes in postsynaptic receptivity to serotonin mediated by 5-HT2CR.

Our data illuminate several aspects of the relationship between DRN serotonin and stress-related modulation of fear memory. First, these findings show that aversive reinforcers are the necessary triggers for serotonergic enhancement of fear memory, and suggest that serotonin resulting from either spontaneous or tone-elicited DRN firing does not contribute to this change in fear memory. Second, despite the existence of both excitatory and inhibitory responses to shock in different populations of DRN neurons [20], it must be specifically an increase in DRN firing that is important for stress-related enhancement of fear memory. If shock-elicited decreases in DRN firing were critical for stress-related enhancement of fear memory, optical silencing of DRN activity during shock would have further potentiated stress-enhanced fear memories, rather than eliminating this effect of stress. Third, silencing the DRN during footshock does not simply remove or reduce the aversive quality of the shock; were this the case, silencing during the paired tone-shock presentations would have reduced or eliminated conditional freezing, an effect which was not observed for unstressed animals (**Figure 4D**).

There are multiple mechanisms by which stress could potentially modulate serotonergic signaling in the BLA. For example, prolonged stress can enhance the release of corticotropin-releasing factor (CRF) [21], and activation of CRF receptor 1 can sensitize signaling through 5-

HT2Rs [22]. In addition, we have recently shown that repeated, but not single, experiences with stress elevate ghrelin, and this hormone acts on the ghrelin receptor 1a (GHS-R1a) to produce stress-enhanced fear [18]. Ghrelin has recently been shown to enhance mRNA of the 5-HT2CR and serotonin turnover in the amygdala, suggesting that stress-related increases in ghrelin could enhance serotonergic signaling [23]. Expression of the unedited 5-HT2CR has also recently been shown to depress signaling through GHS-R1a [24,25]; thus, the stress-related shift towards the edited 5-HT2CR that we report here could enhance 5-HT2CR signaling while concurrently promoting ghrelin-dependent enhancement of fear memory. Such a mechanism could help explain why repeated (**Figure 1**), but not acute (**Figure S3),** stress exposure is sufficient to elevate fear memory. Thus, while edited forms of the 5-HT2CR are often described as having less constitutive activity [26] and less agonist potency and affinity [27], the altered interactions of the edited 5-HT2CR with other molecules and receptors, rather than changes in signaling from serotonin *per se*, may be the critical factor in determining the cellular mechanisms of fear memory consolidation in the amygdala. The large number of potential isoforms of 5-HT2CR poses a formidable challenge for linking specific isoforms and their downstream signaling cascades to stress-enhanced fear and other forms of dysregulated brain function.

It is also important to note that our data indicate that the parameters of associative learning models are impacted by stress. For example, in the Rescorla-Wagner model, one of the most influential models of associative learning, the asymptote of learning (λ) is derived from the physical parameters of the reinforcer used (duration and intensity, for example). Our data show that a physically identical aversive reinforcer is processed differently in the stressed and unstressed brain, but not because of changes in pain perception (**Figure S1B**). Rather, serotonin is able to impact consolidation processes in the stressed brain but not the unstressed brain, in part, because of stress-enhanced receptivity to serotonin in the amygdala. Thus, λ does not simply reflect the attributes of the reinforcer, or even the animal's perception of the

reinforcer (which could be altered if pain processing was affected, for example). Instead, the true value of λ reflects a greater abstraction of the reinforcer, which includes the biochemical impact of reinforcement both during and following learning. Associative learning theories must become more sophisticated and recognize that the asymptote of learning can be determined, in part, by processing that occurs after training.

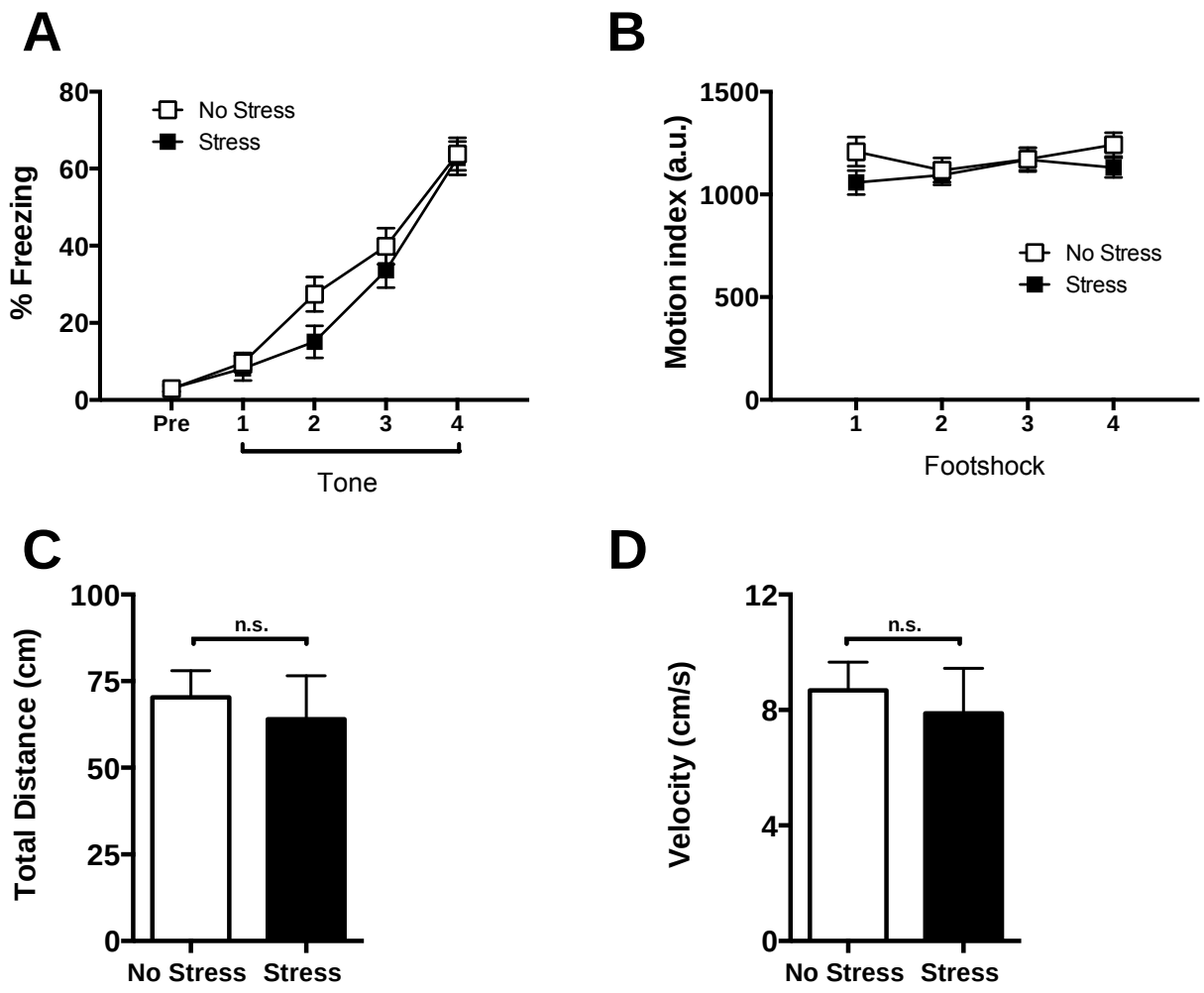

Figure S1. Prior stress does not alter freezing or pain sensitivity during conditioning or general motor activity prior to fear retrieval. (**A**) During the acquisition phase, the level of freezing behavior and (**B**) the motor response evoked by the conditioning footshocks did not differ between Stress and No Stress groups (*n* = 27-29/group). (**C**) During the pre-tone period of the auditory fear test, the total distance and (**D**) velocity of motor activity did not differ between Stress and No Stress groups. Data are means ± SEM.

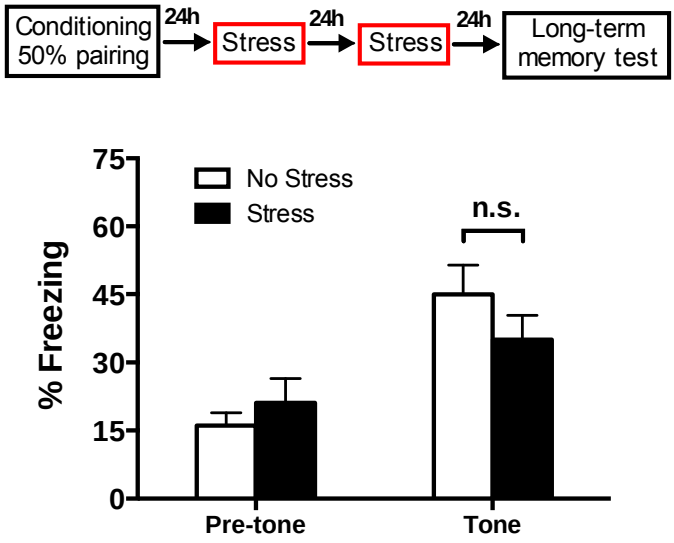

Figure S2. Repeated stress must precede conditioning to impact fear memory. Stress given *after* fear conditioning did not alter retrieval of the long-term auditory fear memory. Data are means ± SEM. Fisher's PLSD comparisons during auditory fear test: n.s. = not significant for Stress versus No Stress.

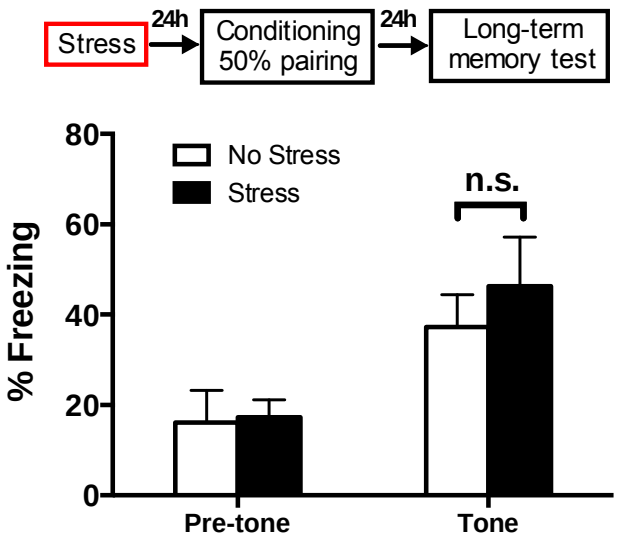

Figure S3. Acute stress does not alter long-term fear memory. A single session of immobilization stress prior to fear conditioning did not augment long-term fear memory (*n* = 9-10/group). Data are means ± SEM. Fisher's PLSD group comparisons during tone fear test: n.s. = not significant for Stress versus No Stress.

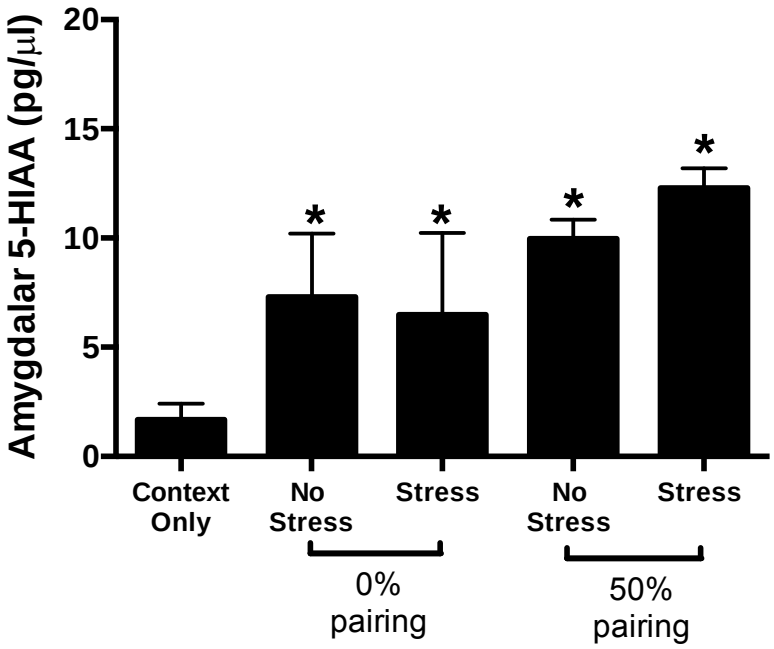

Figure S4. Stress does not affect conditioning-related increases in amygdalar 5-hydroxyindoleacetic acid (5-HIAA). Fear conditioning produced a significant elevation in BLA 5-HIAA, but this was not altered by previous stress exposure. Data are means ± SEM. Fisher's PLSD comparisons to the Context Only group: * $P < 0.05$.

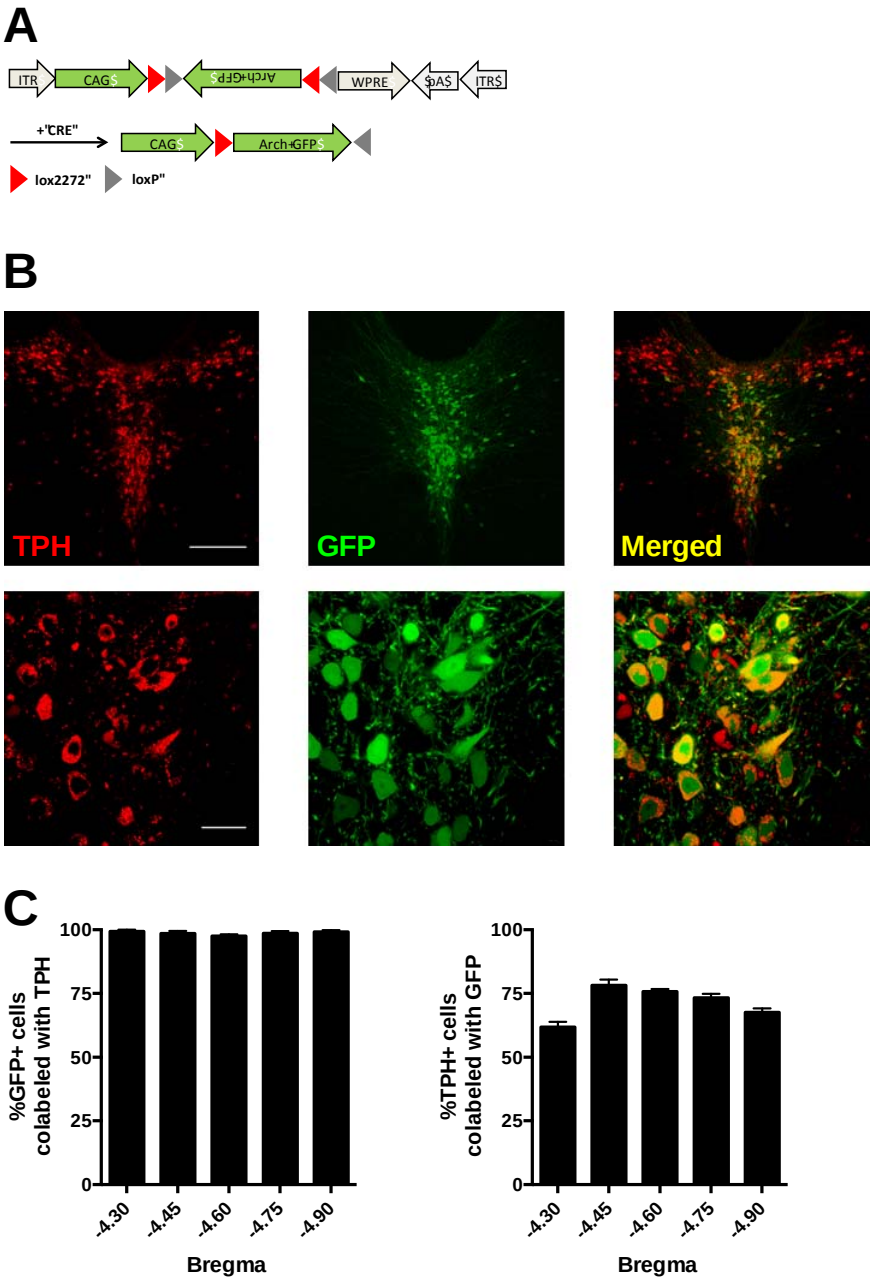

Figure S5. Specific expression of Arch in dorsal raphe serotonergic neurons. (**A**) Schematic of the construct for AAV-FLEX-Arch-GFP. In the presence of Cre recombinase, reversed Arch-GFP is inverted to the sense direction and expressed under the control of the cytomegalovirus early enhancer/chicken beta actin (CAG) promoter. (**B**) Representative confocal fluorescence images depicting colocalized expression of tryptophan hydroxylase (TPH; left column, red) and GFP (center column, green) from a SERT-Cre mouse with a transduced dorsal raphe nucleus (DRN). Top row, TPH and Arch expression in the DRN (scale bar, 200 μm); bottom row, individual neurons (scale bar, 25 μm). (**C**) Percentage of DRN GFP-immunoreactive cells that co-express TPH (left, n = 4) and percentage of TPH-immunoreactive cells that co-express GFP (right, n = 4). Tissue sections (30 μm) were taken across the rostral-caudal axis of the DRN. Data are means ± SEM. ITR, inverted terminal repeat; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element; pA, poly(A).

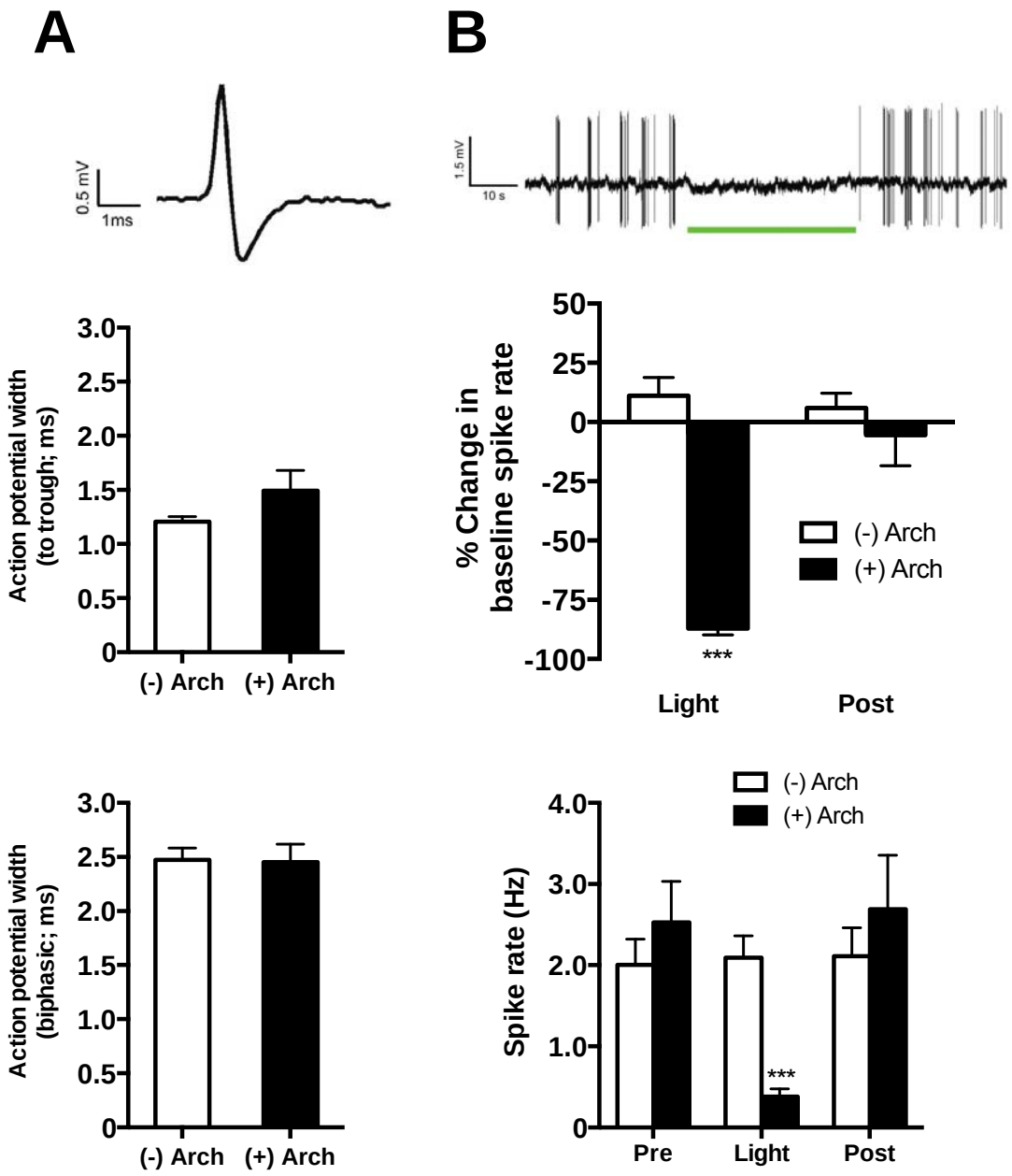

Figure S6. Arch-mediated optical silencing of serotonergic neurons in the dorsal raphe nucleus. (**A**) Representative waveform of an *in vivo* loose-cell attached recording from the dorsal raphe nucleus (DRN) of a SERT-Cre mouse. There were no differences in the mean waveform duration of action potentials (middle and lower) recorded from SERT-Cre mice (*n* = 15 recordings, 3 mice) with (+) or without (-) Arch expression in the DRN. (**B**) Top, Individual trace showing DRN single unit response to 30 sec of Arch-mediated silencing. Green bar indicates duration of light delivery. Middle, mean percent change in spike frequency during Arch silencing (Light) and during the 30 sec immediately after light offset (Post) versus baseline (*n* = 11 recordings, 3 mice). Lower, average spike frequency before, during, and after 30 sec of Arch silencing (*n* = 11 recordings, 3 mice). Data are means ± SEM. Fisher's PLSD comparisons: *** $P < 0.001$.

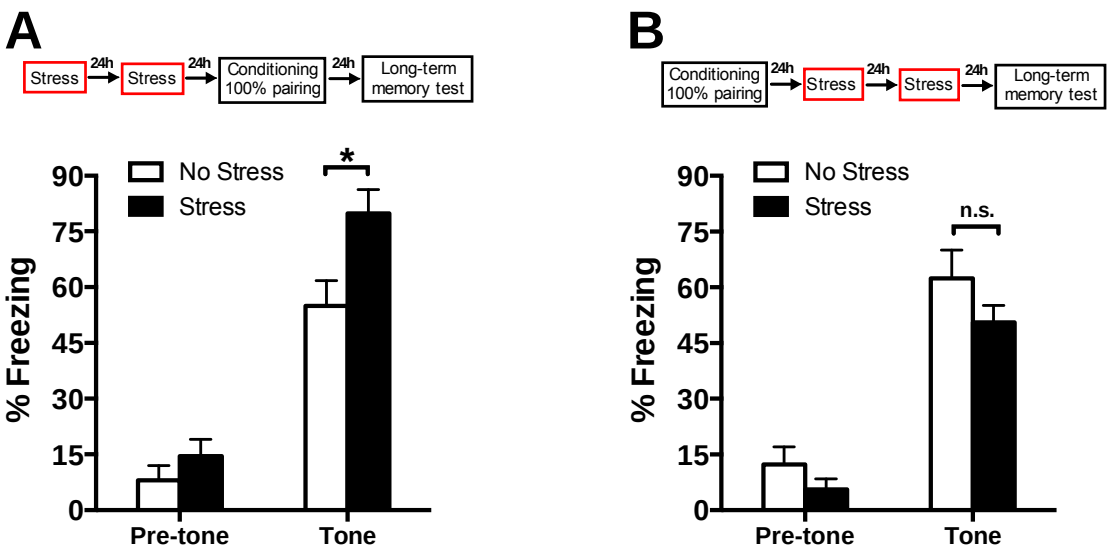

Figure S7. Prior stress enhances fear to unambiguous cues. (**A**) Prior stress enhances tone-elicited freezing in a conditioning paradigm with a tone-footshock contingency of 100%, (**B**) but no facilitation was observed when repeated stress followed conditioning (*n* = 8-10/group). Data are means ± SEM. Fisher's PLSD group comparisons during tone fear test: * $P < 0.05$ and n.s. = not significant for Stress versus No Stress.

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