Archival Report

Biological Psychiatry

Stress Enables Reinforcement-Elicited Serotonergic Consolidation of Fear Memory

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ABSTRACT

BACKGROUND: Prior exposure to stress is a risk factor for developing posttraumatic stress disorder (PTSD) in response to trauma, yet the mechanisms by which this occurs are unclear. Using a rodent model of stress-based susceptibility to PTSD, we investigated the role of serotonin in this phenomenon.

METHODS: Adult mice were exposed to repeated immobilization stress or handling, and the role of serotonin in subsequent fear learning was assessed using pharmacologic manipulation and western blot detection of serotonin receptors, measurements of serotonin, high-speed optogenetic silencing, and behavior.

RESULTS: Both dorsal raphe serotonergic activity during aversive reinforcement and amygdala serotonin 2C receptor (5-HT2CR) activity during memory consolidation were necessary for stress enhancement of fear memory, but neither process affected fear memory in unstressed mice. Additionally, prior stress increased amygdala sensitivity to serotonin by promoting surface expression of 5-HT2CR without affecting tissue levels of serotonin in the amygdala. We also showed that the serotonin that drives stress enhancement of associative cued fear memory can arise from paired or unpaired footshock, an effect not predicted by theoretical models of associative learning.

CONCLUSIONS: Stress bolsters the consequences of aversive reinforcement, not by simply enhancing the neurobiological signals used to encode fear in unstressed animals, but rather by engaging distinct mechanistic pathways. These results reveal that predictions from classical associative learning models do not always hold for stressed animals and suggest that 5-HT2CR blockade may represent a promising therapeutic target for psychiatric disorders characterized by excessive fear responses such as that observed in PTSD.

Keywords: Amygdala, Fear, 5-HT2C receptor, Optogenetics, PTSD, Serotonin

http://dx.doi.org/10.1016/j.biopsych.2015.06.025

Stress exposure is a risk factor for the development of posttraumatic stress disorder (PTSD) in humans (1,2). Humans with PTSD often have strong memories for the traumatic experiences that underlie their disorder (3) but also exhibit heightened fear conditioning in laboratory settings (4,5). In preclinical studies, the relationship between stress exposure and subsequent trauma-related memory can be studied by exposing rodents to stressors and examining the impact on Pavlovian fear conditioning. In this model, fear conditioning itself does not lead to PTSD; only stress-exposed animals display the excessively strong fear memories that are also observed in humans with PTSD. The exaggerated fear response typically observed in stress-exposed animals (6) is often attributed to either strengthened encoding (7) or consolidation processes (8).

Serotonin plays a critical role in the regulation of emotion, and dysregulation of serotonergic systems is associated with stress-related affective disorders (9), including PTSD. Multiple lines of evidence suggest that excess serotonin is linked to altered threat processing. For instance, individuals that carry the short variant of the gene encoding the serotonin transporter (*SLC6A4*), which is thought to impair synaptic serotonin uptake, display increased amygdala reactivity to briefly presented (phasic) aversive stimuli (10). In rodent studies, during aversive learning, serotonin is released into projection regions of the dorsal raphe nucleus (DRN) via phasic firing changes in response to discrete stimuli (11–13). The extracellular serotonin levels in downstream DRN targets, like the basolateral amygdala (BLA), can remain elevated for at least an hour after learning is completed (14,15). Although serotonin acts through several receptor subtypes in the BLA, the serotonin 2C receptor (5-HT2CR) is of interest because these receptors are heavily expressed in BLA neurons that regulate anxiety (16) and 5-HT2CR agonists promote anxiety in humans (17). Furthermore, viral-mediated overexpression of 5-HT2CR in amygdala produces anxiogenic effects (18), while pharmacologic blockade of amygdala 5-HT2CR prevents stress-induced anxiety-like behaviors (19).

Here, we examine behavior in a rodent paradigm in which repeated exposure to stress produces a vulnerability to heightened fear learning (6) and demonstrate that this vulnerability emerges from a serotonergic fear memory consolidation

ISSN: 0006-3223

process that is not present in unstressed mice. This consolidation process requires serotonergic activity in the DRN during aversive reinforcement and 5-HT2CR signaling in the BLA, a major target structure of the DRN (20–24), after aversive learning. Interestingly, we also show that serotonin activation by either signaled or unsignaled footshocks is sufficient to enhance associative fear memory in stressed animals, an effect not predicted by classic theoretical models of associative learning. We show that stress enhances cell surface expression of 5-HT2CRs in the amygdala without affecting total serotonin levels during fear conditioning. Thus, aversive reinforcement is processed differently in the brain of a stress-exposed animal, and this profoundly impacts memory for later aversive experiences. These findings reveal fundamental mechanisms underlying the operation of a critical neural system in affective processing and provide new principles both for associative learning theory and the prevention of stress-related psychiatric disorders.

METHODS AND MATERIALS

Subjects

Adult male C57BL/6 mice (Taconic, Germantown, New York) or transgenic mice (25) were used in all experiments. All procedures were approved by the Committee on Animal Care at the Massachusetts Institute of Technology and the Animal Care and Use Review Office at the U.S. Army Medical Research and Materiel Command.

Virus

Adeno-associated virus vectors were serotyped with adeno-associated virus 2/8 capsids and packaged by the Vector Core at The University of North Carolina at Chapel Hill. The final viral concentration was approximately 1.0 to 2.0 $\times$ 10^{11} infectious particles per milliliter.

Surgical Procedures

For some experiments, mice received cannulae implants, optical fiber implants, or virus infusions, as described in Supplement 1.

In Vivo Recording. Single-unit recordings were conducted in anesthetized SERT-Cre mice weeks after stereotactic delivery of virus to the DRN. Cell-attached recordings, which enabled well-isolated single-unit recordings, were obtained using a standard blind in vivo patching technique (26). See Supplement 1 for details.

Drugs. The selective 5-HT2CR antagonist 6-chloro-2,3-dihydro-5-methyl-N-[6-[(2-methyl-3-pyridinyl) oxy]-3-pyridinyl]-1H-indole-1-carboxyamide dihydrochloride (SB242084; Tocris Bioscience, Minneapolis, Minnesota) was dissolved in .9% sterile saline.

Immobilization Stress. Mice were transferred to an experimental room and placed in ventilated plastic Decapicone bags (Braintree Scientific, Braintree, Massachusetts) for 1 hour on each of 2 consecutive days. While fear conditioning is also a type of stress exposure, here we use the term stress to exclusively refer to immobilization stress.

See Supplement 1 for additional procedures and assays.

RESULTS

Repeated Stress Enhances the Consolidation of Fear Memories Established Under Degraded Contingency

Stress exposure can enhance learned fear memories (6,27,28), modeling the way in which a history of stress exposure can predispose humans to disorders of fear or anxiety (1,29). Here, we exposed mice to either 2 days of immobilization stress (stress; 1 hour/day) or handling (no stress), followed by auditory fear conditioning (Figure 1). Unlike previous studies that examined the relationship between stress and subsequent auditory fear memory (6,27), we used an auditory fear conditioning protocol in which two of four tone and footshock presentations were explicitly unpaired (50% pairing), thereby reducing the tone-footshock contingency. Such a paradigm may be more sensitive to the effects of stress than a conventional protocol where the pairing is 100% (30). Conditional fear to the tone was assessed in a novel environment either 2 hours (short-term memory) or 24 hours (long-term memory) after fear conditioning (Figure 1A).

Prior stress did not impact the amount of conditional freezing to the tone during fear acquisition (Figure S1A in Supplement 1) or the short-term memory test (stress: $F_{1,19}$ = .020; stress $\times$ tone interaction: $F_{1,19}$ = .384, ps = ns, n = 10–11/group; Figure 1A, left) but did enhance tone-elicited freezing in mice tested 24 hours later (stress: $F_{1,18}$ = 1.64, p = ns; stress $\times$ tone interaction: $F_{1,18}$ = 11.790, $p < .01$; Fisher's protected least significant difference [PLSD] comparing no stress = 37.22 $\pm$ 9.22% and stress = 62.78 $\pm$ 6.26%, $p < .05$, n = 10/group; Figure 1A, right). All groups exhibited comparable, low levels of freezing during the 3-minute baseline period of the auditory fear test (Fisher's PLSD comparing no stress with stress, ps $> .230$; Figure 1A, left and right), indicating no generalization between the conditioning and testing contexts. Stress did not enhance fear memory via changes in pain processing, general motor activity, or memory retrieval (Figures S1B–D and S2 in Supplement 1). Enhanced fear memory was also observed only after repeated stress (Figure S3 in Supplement 1). The findings that repeated stress enhances long-term but not short-term fear memory when given before fear conditioning suggests that immobilization stress enhances fear responses by strengthening fear memory consolidation.

Serotonergic Fear Memory Consolidation Is Selectively Enabled by Stress

Because our stress paradigm enhanced fear memory consolidation and serotonin is also implicated in the consolidation of memories (31–34), we determined whether stress-related enhancement of long-term fear memory consolidation is mediated by serotonin signaling in the BLA. Mice were implanted with bilateral cannulae in the BLA before stress or handling. Intra-BLA administration of the highly selective 5-HT2CR antagonist SB242084 (.4 μg/.4 μL) (24) immediately following fear conditioning completely blocked stress-induced

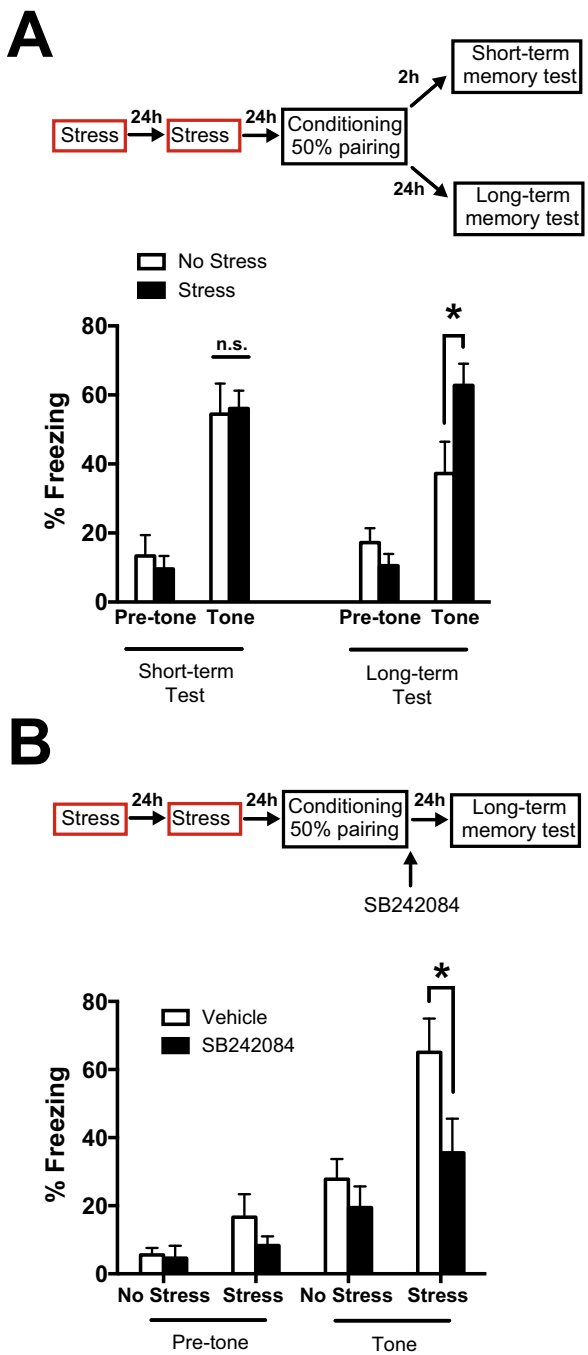

Figure 1. Stress recruits serotonergic fear memory consolidation. **(A)** Prior immobilization stress did not impact short-term (2 hours) fear memory (left) but increased long-term (24 hours) fear memory (right) to the tone. **(B)** Postconditioning infusion of the serotonin 2C receptor antagonist SB242084 into the lateral/basolateral amygdala (24) blocked the immobilization stress-induced enhancement of fear consolidation. Data are mean ± SEM. Fisher's protected least significant difference comparisons during auditory fear test: *$p < .05$. n.s., not significant for stress versus no stress.

enhancement of fear when mice were tested for long-term fear memory 24 hours later (stress: $F_{1,24} = 6.83$; stress × tone interaction: $F_{1,24} = 4.277$, $ps < .05$; Fisher's PLSD comparing stress-vehicle = 65.08 ± 9.90% and stress-SB242084 = 35.56 ± 10.01%, $p < .05$, $n = 6–10$/group; Figure 1B) but did not affect fear levels in the absence of prior stress (Fisher's PLSD comparing no stress-vehicle = 27.78 ± 5.91% and no stress-SB242084 = 19.44 ± 6.21%, p = ns). These findings reveal that serotonin-mediated consolidation of fear memory occurs through amygdalar 5-HT2CRs and is selectively enabled by a prior history of immobilization stress exposure.

Stress Enhances Amygdala Sensitivity to Serotonin

There are at least two possible mechanisms by which repeated stress may selectively engage serotonergic consolidation of fear memory through 5-HT2CR. One possibility is that stress enhances the release of serotonin from DRN afferents to the BLA during fear conditioning. As an alternative or concurrent change, it is possible that stress may increase the membrane expression of postsynaptic serotonin receptors in BLA neurons (35,36), leading to enhanced postsynaptic sensitivity to serotonin release by the DRN.

First, we determined whether prior stress impacts BLA serotonin levels during conditioning. In addition to the 50% pairing fear conditioning protocol (two tone-shock pairings with two unpaired tones and two unpaired footshocks), a 0% pairing protocol was used (four unpaired tones and four unpaired footshocks). This allowed us to determine whether BLA serotonin levels differ when negative reinforcement is uncoupled from the auditory cue. Two control groups were included: one remained in the home cage (home cage group) and the other was placed in the conditioning context without tones or footshocks (context only group). Mice were sacrificed 30 minutes following fear conditioning, a time point where extracellular serotonin in the amygdala is maximally elevated by the conditioning procedure (14,15).

The serotonin content of the BLA was increased by fear conditioning (conditioning: $F_{1,36} = 4.381$, $p < .05$, $n = 4–8$/group; Fisher's PLSD comparing all stress and no stress groups with control groups, $p < .05$; Figure 2) but not exposure to the novel context (Fisher's PLSD comparing context only and home cage groups, p = ns; Figure 2), consistent with other studies showing that fear conditioning and other stressors increase extracellular serotonin in the amygdala (14,37). Within the groups that received fear conditioning, there was no effect of pairing on serotonin levels (pairing: $F_{1,27} = .46$, p = ns; Figure 2), and, most critically, serotonin was similarly elevated in stress and no stress mice (pairing × stress: $F_{1,27} = .002$, p = ns; $n = 7–8$/group; Figure 2).

Because the BLA homogenates contain both extracellular and vesicular serotonin content in the BLA, it is possible that the lower levels of serotonin observed in the control groups reflect greater release of serotonin. To clarify this, we used high-pressure liquid chromatography to measure the primary serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) in a subset of the homogenates (Figure S4 in Supplement 1). We found that 5-HIAA levels were low in the context only control group and significantly increased by fear conditioning (conditioning: $F_{1,14} = 10.54$, $p < .01$; Fisher's PLSD comparing all stress and no stress groups with context only, $p < .05$) but similarly elevated in the stress and no stress groups

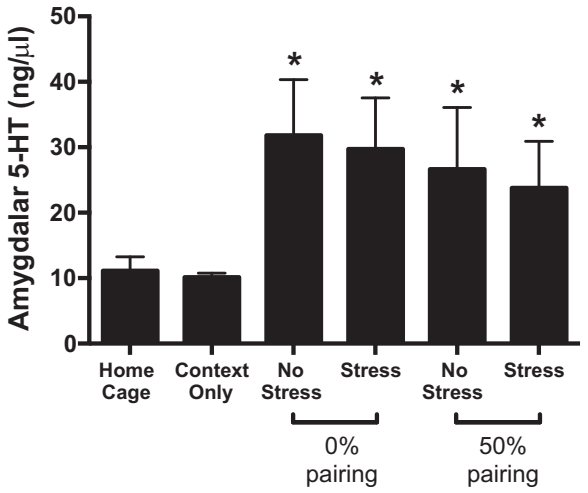

Figure 2. Stress does not affect conditioning-related increases in amygdalar serotonin. Fear conditioning produced a significant elevation in serotonin (5-HT) in the basolateral amygdala, but this was not altered by previous immobilization stress exposure. Data are mean ± SEM. Fisher's protected least significant difference comparisons with the home cage group: $^{*}p < .05$.

(pairing × stress: $F_{1,8} = .558$, p = ns, n = 3–4/group; Figure S4 in Supplement 1). This suggests that the changes we observed in serotonin may predominantly reflect extracellular release, even though our method of detection is not specific for extracellular serotonin. Conservatively, our results show that BLA serotonin content is elevated by fear conditioning, but this is not influenced by the prior immobilization stress history of the animals. The similar postconditioning levels of serotonin and its metabolite 5-HIAA when comparing subjects receiving the 0% pairing with the 50% pairing paradigm also suggests that footshock is the primary factor in determining conditioning-related increases in BLA serotonin.

We next examined the postsynaptic sensitivity of BLA neurons to serotonin following stress by measuring the surface expression of 5-HT2CR in the BLA. Mice received either 2 days of immobilization stress (stress groups) or handling (no stress groups), followed by auditory fear conditioning with 50% pairing. Mice were sacrificed 10 minutes after fear conditioning ended. 5-HT2CR density was assessed at this postconditioning time point because it corresponds roughly to both the time when serotonin is first significantly elevated by fear conditioning (14,15) and a time when cellular consolidation of fear memory is occurring (38).

We found that repeated stress produced a significant increase in surface membrane expression of the 5-HT2CR in the amygdala measured shortly following fear conditioning (stress: $F_{1,26} = 4.887$, $p < .05$, n = 12–16/group; Figure 3A), without affecting the total pool of 5-HT2CR (stress: $F_{1,10} = 1.504$, p = ns, n = 6/group; Figure 3B). This finding suggests that repeated stress alters trafficking of 5-HT2CR, as opposed to an upregulation of gene transcription or protein translation. Stress is known to trigger editing of the pre-messenger RNA for the 5-HT2CR (39) through adenosine deaminase acting on RNA 1 (40). Because edited forms of the 5-HT2CR are known to have less internalization from the membrane surface (41), we also examined expression of adenosine deaminase acting on RNA 1 protein. We found that repeated stress significantly enhances total levels of this protein in the BLA (stress: $F_{1,10} = 4.975$, $p < .05$, n = 6/group; Figure 3C). Such a finding is consistent with other reports showing that edited 5-HT2CR is more prevalent in the membrane of amygdala cells in mice that display increased anxiety and responsiveness to stress (42). Together, these data show that the amygdala exhibits an enhanced membrane presence of 5-HT2CR following repeated stress.

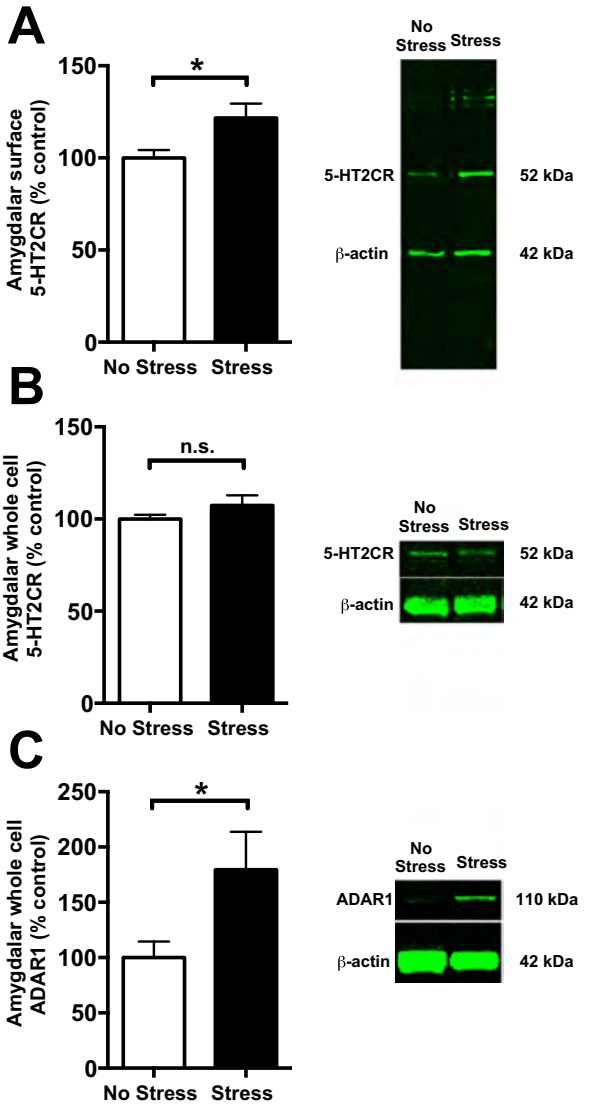

Figure 3. Stress enhances surface expression of serotonin 2C receptors (5-HT2CR) in basolateral amygdala (BLA). Immobilization stress enhanced membrane expression of the 5-HT2CR in the BLA **(A)** without affecting the total levels of 5-HT2CR **(B)**, suggesting a change in trafficking of the receptor. **(C)** Stress also produced a concurrent increase in the whole-cell levels of the messenger RNA editing enzyme adenosine deaminase acting on RNA 1 (ADAR1) in the BLA. Images on the right depict all bands detected in representative samples. Data are mean ± SEM. Fisher's protected least significant difference comparisons: $^{*}p < .05$. n.s., not significant for stress versus no stress.

Serotonergic DRN Activity During Aversive Reinforcement Is Required for Stress Facilitation of Fear Memory

Our data reveal that stress recruits a serotonergic consolidation mechanism for BLA-dependent fear memory, but the conditions during fear learning that lead to serotonin release into the BLA are unclear. Serotonergic DRN neurons exhibit heterogeneous, transient responses to a wide variety of discrete stimuli (11), including footshock (12). Most DRN neurons are unresponsive to acoustic stimuli (43), but excitation is observed in a very small population of cells (13). Thus, it is possible that stress could enhance fear memory by altering BLA responses to serotonin released by the auditory or shock stimuli or their contingent pairing during fear conditioning.

The DRN of SERT-Cre mice was transduced with the light-driven opsin Arch (Arch-green fluorescent protein [GFP] groups), which enables rapid and reliable large hyperpolarizing currents in neurons in response to pulses of green-yellow light (Figures S5 and S6 in Supplement 1) (44). Control groups received a virus expressing only GFP (GFP groups). The lightweight optical fiber system used for light delivery did not impair movement or exploration within the conditioning chamber (group: $F_{1,6}$ = .002 and .131; p = ns, n = 4/group; Figure 4A).

Typically, a conditional fear response is established by pairing 100% of neutral tones with aversive footshock. Stress does enhance long-term fear memory (group: $F_{1,15}$ = 6.581, $p < .05$; Fisher's PLSD comparing no stress = 54.94 ± 6.83% and stress = 79.86 ± 6.38%, $p < .05$, n = 8–9/group; Figure S7A in Supplement 1) without potentiating fear retrieval or performance (group: $F_{1,17}$ = 3.274, p = ns, n = 9–10/group; Figure S7B in Supplement 1) when such a paradigm is used. However, consistent with the prior experiments, we used 50% pairing to enable selective silencing of DRN serotonergic activity during the presentation of noncontingent or contingent cues and reinforcers, appropriate for parsing the relationship between serotonergic activity and temporally limited stimulus presentation during auditory fear conditioning. This also ensured that unstressed animals would exhibit moderate levels of conditional freezing in the long-term memory test, with ample room for potentiation by stress. Continuous light was applied for 30-second periods, a duration corresponding to the length of the tone used, across the three experimental conditions (Figure 4), equating the length of silencing across the different groups.

Photoinhibition during noncontingent footshocks (Figure 4B), noncontingent tones (Figure 4C), or contingent tones and footshocks (Figure 4D) produced different effects on fear memory. Notably, silencing serotonin activity during unpaired footshocks blocked stress-related enhancement of freezing in stress-Arch relative to stress-GFP mice (stress: $F_{1,18}$ = 1.39, p = ns; stress × tone × virus interaction: $F_{1,18}$ = 5.904; $p < .05$; Fisher's PLSD, $p < .05$, n = 5–6/group; Figure 4B). In contrast, despite an enhancement of fear memory by stress exposure (stress: $F_{1,20}$ = 9.08, $p < .01$; stress × tone interaction: $F_{1,20}$ = 12.285, $p < .001$, n = 6–8/group; Figure 4C), photoinhibition of serotonergic activity during unpaired tones did not result in a difference in freezing levels between stress-Arch and stress-GFP control mice (p = .551, Fisher's PLSD). As might be expected if the shock were the salient stimulus for the release of serotonin into its efferents, photoinhibition of DRN serotonin neurons during paired tones and footshocks prevented the stress-enhancement of fear (stress: $F_{1,16}$ = .498, p = ns; stress × tone × virus interaction: $F_{1,16}$ = 7.228, $p < .05$, n = 4–7/group; Figure 4D). Similar to the effect observed following silencing of DRN neurons during the footshock alone, conditional freezing to the tone in stress-Arch mice was reduced compared with stress-GFP ($p < .05$, Fisher's PLSD).

DISCUSSION

Perhaps the most surprising finding of our study was that serotonergic fear memory consolidation was only engaged in mice with a history of repeated stress exposure. This was demonstrated by the selective reduction of fear in stressed, but not unstressed, mice by postconditioning intra-BLA infusion of a 5-HT2CR antagonist (Figure 1B) and the lack of effect of DRN photoinhibition on long-term fear memory in unstressed animals under any conditions (Figure 4B–D). This cannot be attributed to floor levels of tone-induced freezing in the long-term fear memory test for the unstressed animals: posttone freezing levels were significantly higher than pretone freezing levels for most conditions (Figure 1, Figure 4B–D). Rather, repeated immobilization stress increases the expression of 5-HT2CR membrane receptors in the BLA measured in the postconditioning consolidation period, and this illuminates a mechanism by which 5-HT2CR-dependent fear memory consolidation is engaged following stress exposure. We measured 5-HT2CR density in the BLA after conditioning because this time point falls within the consolidation window that we identified as critical for stress-related enhancement of fear memory (Figure 1B). We do not know whether immobilization stress altered surface 5-HT2CR expression after stress exposure or whether it altered trafficking of these receptors after fear conditioning; this issue remains an important open question for future studies. Previous studies reported that lesions or pharmacologic inactivation of the DRN did not alter fear conditioning processes in unstressed animals but did block potentiation of fear produced by a prior stressor (45,46), consistent with our finding that serotonin signaling through the 5-HT2CR has a nonessential role in fear learning in animals lacking a history of stress exposure.

A second surprising finding from our study relates to the observation that DRN serotonin activity during unpaired footshocks regulates the associative memory strength of the tone-footshock pairings (Figure 4B). Typically, when unsignaled reinforcement is given between cue-reinforcer pairings, as in a degraded contingency paradigm (cue-reinforcer pairings held constant and extra reinforcers given) or a reduced temporal overlap paradigm as used here (total number of cue and reinforcer presentations held constant but number of pairings reduced), it reduces the overall level of associative learning for the cue-reinforcer pairing (47–49). Such a finding is accounted for in associative learning theory by positing that a context-reinforcer association competes with the cue-reinforcer association either at the time of encoding or the time of retrieval (50,51). Thus, one might predict that if stress enhances

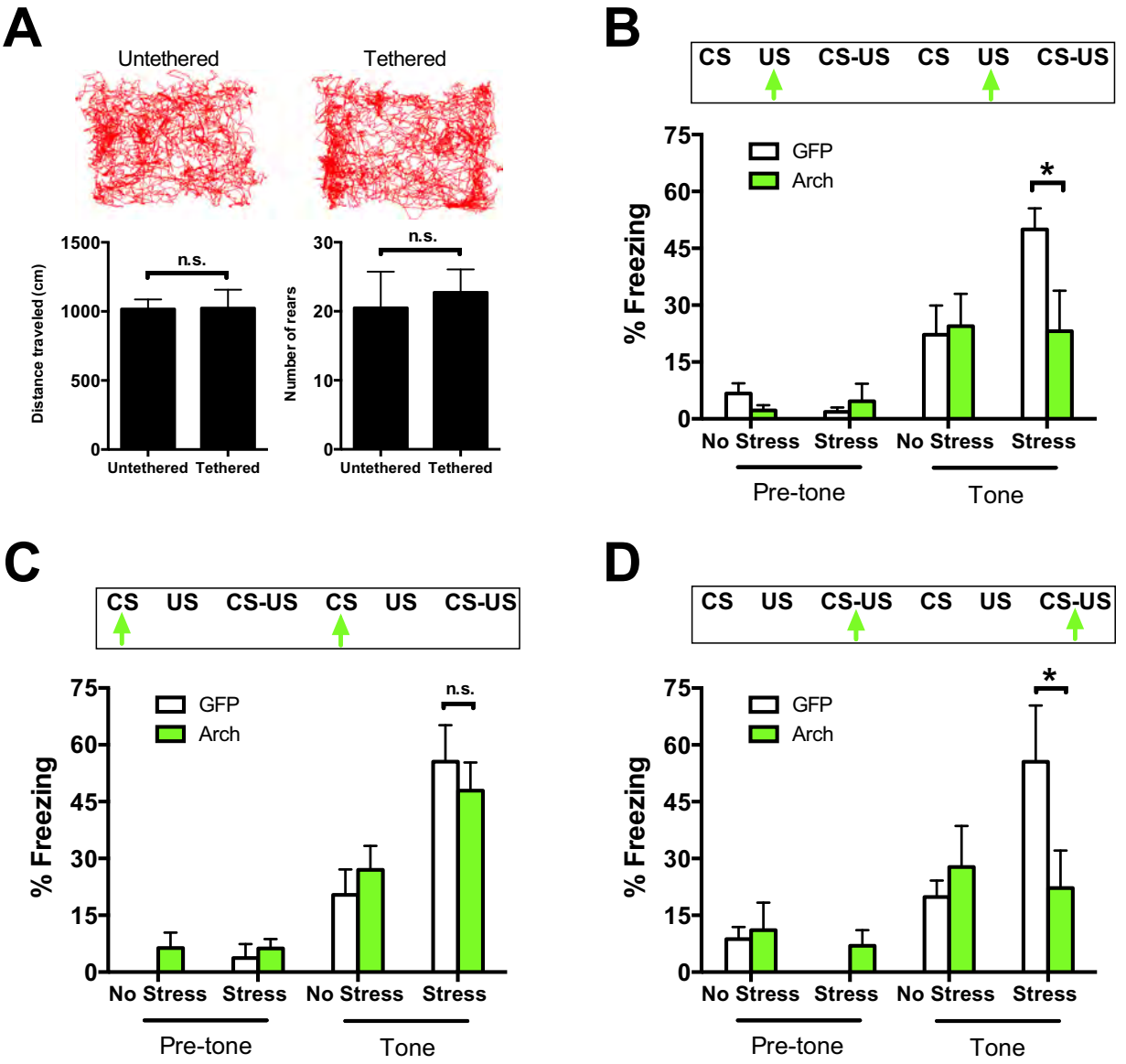

Figure 4. Dorsal raphe serotonergic activity is required for the stress enhancement of fear in a stimulus-dependent manner. **(A)** Upper, representative traces of mice tethered or untethered to the fiber optic patch cord freely exploring the fear conditioning apparatus. Lower, integration of fiber optic cable system with the fear conditioning apparatus does not interfere with voluntary motor behavior in the conditioning chamber. In three separate sets of mice, light was delivered to the dorsal raphe nucleus (DRN) for 30-second periods encompassing noncontingent footshocks, noncontingent tones, or contingent tones and footshocks. **(B)** Arch-mediated silencing of serotonin activity in the DRN during unpaired footshocks blocked stress-induced facilitation of fear. **(C)** Photoinhibition of DRN serotonin activity during unpaired tone presentation failed to affect stress enhancement of fear. **(D)** Photoinhibition of DRN serotonin activity during tone-shock pairings completely prevented stress-related enhancement of fear. Green arrows indicate green light delivery (30 seconds). Data are mean ± SEM. Group comparisons during auditory fear test: $^{*}p < .05$. CS, unpaired tone; CS-US, paired tone and footshock; GFP, green fluorescent protein; n.s., not significant for stress-Arch versus stress-GFP; US, unpaired footshock.

signaling of the neurotransmitter released by unpaired reinforcement, it should be augmenting the reduction of the associative learning for the cue-reinforcer pairing, and thus blockade of this signaling should actually enhance learning for the cue-reinforcer association. Here, we show that, contrary to this prediction, eliminating serotonergic activity during the unpaired footshocks reduces associative memory strength (Figure 4B), revealing that the serotonergic activity driven by unsignaled footshock enhances associative memory strength for the tone-footshock pairings but only in mice with a history of stress. The ability of the unsignaled footshocks to affect associative learning for the tone-footshock pairing occurs, in part, because the relevant biochemical signal for consolidation (serotonin, persisting for tens of minutes postconditioning; Figure 2) greatly outlasts the trigger for a necessary signal (aversive footshocks, persisting for seconds; Figure 4), an

effect that is typically not accounted for in classical associative learning models that explain learning through variation in the ability of the aversive reinforcer to support learning (52). Our results reveal a novel mechanism by which unsignaled aversive reinforcers modulate associative aversive learning and also reveal a specific set of circumstances in which the rules of learning theory are affected by state variables, such as stress. While many associative learning theories have been criticized for a failure to specifically account for the influence of state variables (53), there has been little consideration of this issue by neurobiologists who study stress and other state variables (Supplemental Discussion in Supplement 1). Given the importance of associative learning theory for motivating both behavioral and computational approaches to learning (54), we argue that thoughtful consideration of how experience influences learning theory is worthwhile.

Our observation that repeated stress administered after fear learning during a presumed consolidation window does not enhance fear memory (Figure S2 in Supplement 1) may seem to conflict with our claim that stress enhances fear memory by augmenting consolidation (Figure 1A,B). Additionally, the observation that optogenetic inhibition of the serotonergic dorsal raphe during conditioning is sufficient to prevent stress-related enhancement of fear (Figure 4) also may appear at odds with the claim that serotonin is important for consolidation. However, there are at least two viable resolutions for this apparent conflict. First, while aversive reinforcement triggers activity in serotonergic neurons (12) (Figure 4), it is clear that synaptic serotonin can remain elevated in projection regions such as the BLA for at least an hour following conditioning (14). Thus, serotonin may bind to its receptors during both fear learning and a brief (∼hours) posttraining consolidation window. Our finding that stress enhances long-term, but not short-term, fear memory (Figure 1A; Figure S1A in Supplement 1) via postconditioning activity at 5-HT2CRs in the BLA (Figure 1B) is consistent with this. Given this temporal constraint, repeated stress started 24 hours after fear learning does not alter fear memory strength for prior learning (Figure S2 in Supplement 1) because it cannot alter either the critical time period for serotonergic consolidation shortly following fear learning or the release of serotonin, most likely triggered by footshock during fear learning. Alternatively, our data are also consistent with a model in which serotonin release by aversive footshocks prepares the amygdala for a prolonged period of enhanced consolidation by acting at 5-HT2CRs shortly following fear learning. The prolonged elevation of extracellular serotonin observed after fear conditioning (14,15) may then act through 5-HT2CRs or other serotonin receptors to further stabilize fear memories.

In summary, during fear learning, serotonergic neurons make a critical contribution to the fear-enhancing effect of stress (Supplemental Discussion in Supplement 1), elicited by the presentation of aversive stimuli during fear conditioning. Furthermore, this effect is mediated by postsynaptic actions at 5-HT2CRs in the BLA, which enhance fear memory consolidation, though additional mechanisms may contribute (Supplemental Discussion in Supplement 1). These results show that while the triggers leading to serotonin release (i.e., presentation of aversive stimuli) are temporally delimited, the effects of serotonin on downstream targets like the BLA are persistent. This mechanism may explain why polymorphisms in human serotonergic genes are often associated with enhanced aversive processing, especially following a history of traumatic life events (10,55,56).

While our rodent model of PTSD is simple, it does capture critical features of the disorder. The strong fear memory of the fear conditioning experience in stressed animals mirrors the strong memories for traumatic events often observed in humans with PTSD (57). While PTSD involves additional symptoms, the intrusive nature of the traumatic memory may contribute to some other symptoms, such as hypervigilance or sleep disturbance (3,58). Also, the dose-response relationship between stress exposure and enhancement of fear in our model (Figure 1; Figure S3 in Supplement 1) parallels the relationship between stress exposure and vulnerability to PTSD in humans (59). Our demonstration that pharmacologic and optogenetic inhibition of a serotonergic subcircuit selectively reduces fear in stressed animals with pathologic (exaggerated) fear levels, without affecting fear responding in unstressed animals, overcomes a critical barrier to the successful treatment of stress-induced anxiety disorders such as PTSD. The benchmark for the successful treatment of PTSD should not be the elimination of fear but simply its reduction to normal, adaptive levels. Our results suggest that administration of a 5-HT2CR antagonist, such as agomelatine, already Food and Drug Administration-approved for human use, might prevent or treat PTSD by reducing the consolidation or reconsolidation of traumatic memories. One case report has shown that agomelatine produced full remittance of PTSD in one patient (60); clearly, additional studies are warranted.

ACKNOWLEDGMENTS AND DISCLOSURES

ESB acknowledges funding by Jerry and Marge Burnett; Department of Defense Congressionally Directed Medical Research Programs Posttraumatic Stress Disorder Program; Human Frontiers Science Program; Massachusetts Institute of Technology (MIT) Intelligence Initiative; MIT McGovern Institute and McGovern Institute Neurotechnology Program; MIT Media Lab; MIT Mind-Machine Project; National Alliance for Research on Schizophrenia and Depression; National Institutes of Health Director's New Innovator Award (1DP2OD002002); National Institutes of Health Grants 1R01DA029639, 1R43NS070453, and 1RC1MH088182; and the Alfred P. Sloan Foundation. KAG acknowledges funding by National Institute of Mental Health R01 MH084966 and the U.S. Army Research Laboratory and the U.S. Army Research Officer under Grant 58076-LS-DRP.

We thank Matthew B. Pomrenze and Philip H. Siebler for technical assistance and Elia S. Harmatz and Drs. Susana Correia and Liang Liang for comments on the manuscript. We also thank Dr. Steven F. Maier for providing equipment and laboratory space to perform western blot and high-performance liquid chromatography analysis of samples.

The authors report no biomedical financial interests or potential conflicts of interest.

ARTICLE INFORMATION

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Received Dec 23, 2014; revised Jun 2, 2015; accepted Jun 17, 2015.

Supplementary material cited in this article is available online at http://dx.doi.org/10.1016/j.biopsych.2015.06.025.

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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.

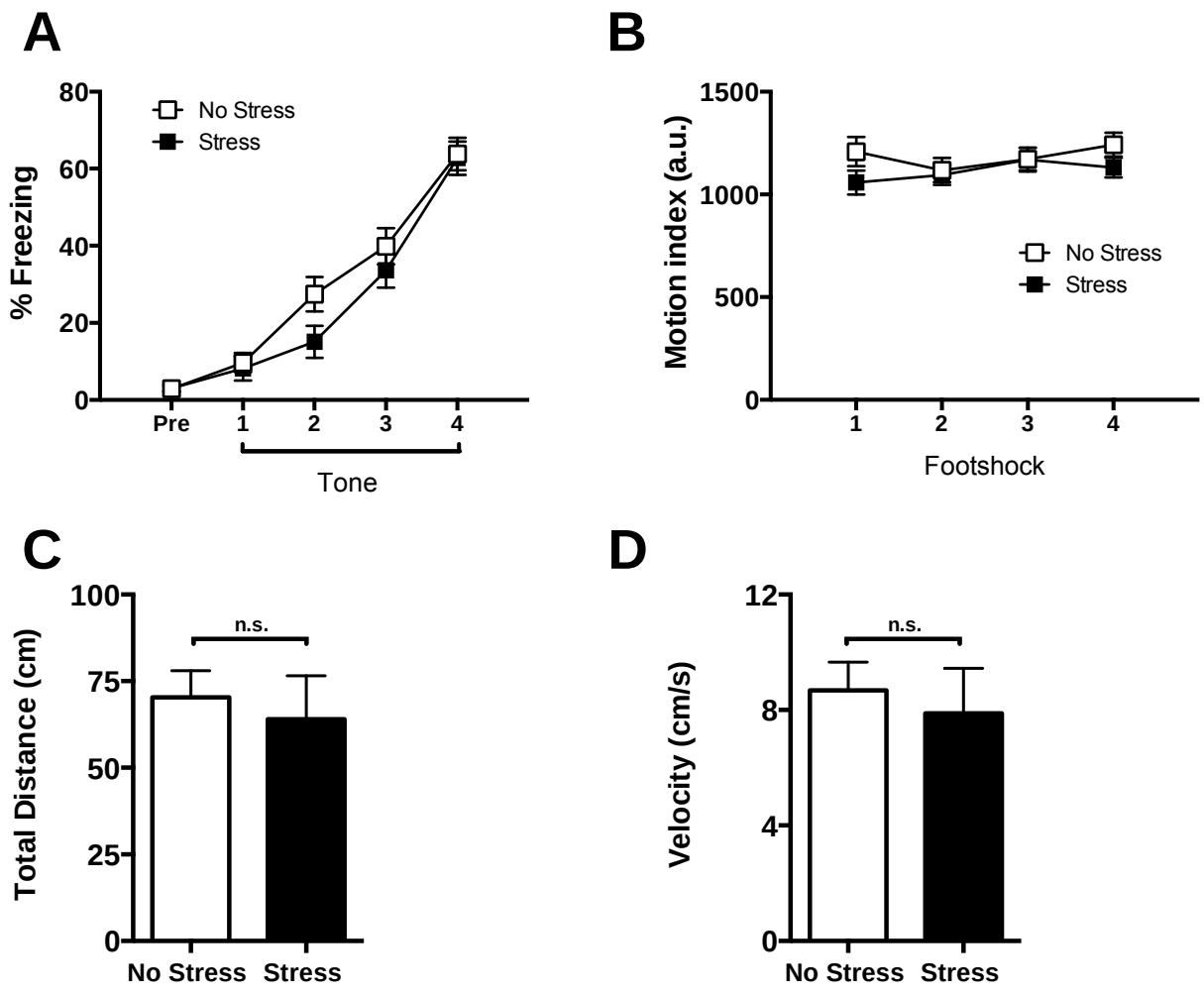

Figure S1. Prior stress does not alter freezing or pain sensitivity during conditioning or general motor activity prior to fear retrieval. (**A**) During the acquisition phase, the level of freezing behavior and (**B**) the motor response evoked by the conditioning footshocks did not differ between Stress 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.

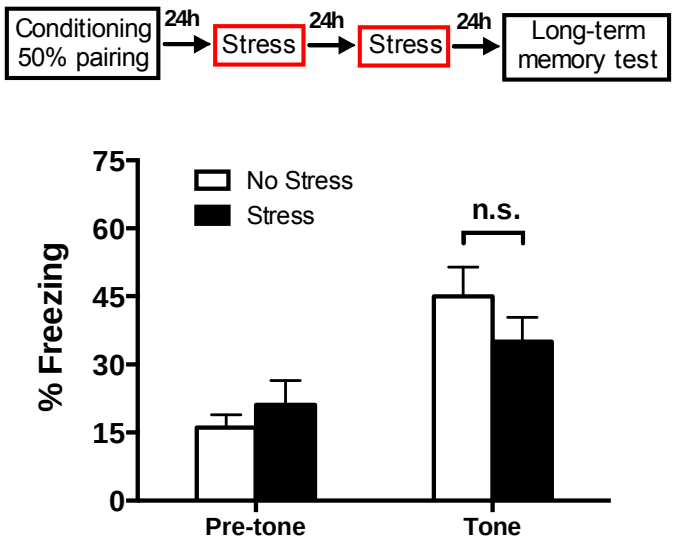

Figure S2. Repeated stress must precede conditioning to impact fear memory. Stress given *after* fear conditioning did not alter retrieval of the long-term auditory fear memory. Data are means ± SEM. Fisher's PLSD comparisons during auditory fear test: n.s. = not significant for Stress versus No Stress.

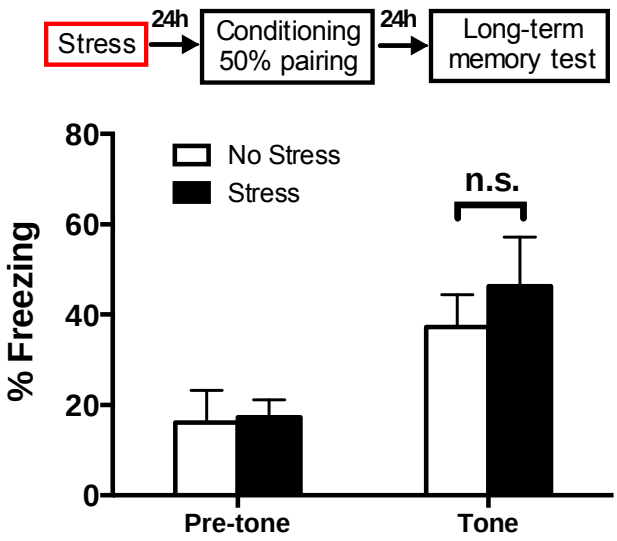

Figure S3. Acute stress does not alter long-term fear memory. A single session of immobilization stress prior to fear conditioning did not augment long-term fear memory (*n* = 9-10/group). Data are means ± SEM. Fisher's PLSD group comparisons during tone fear test: n.s. = not significant for Stress versus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

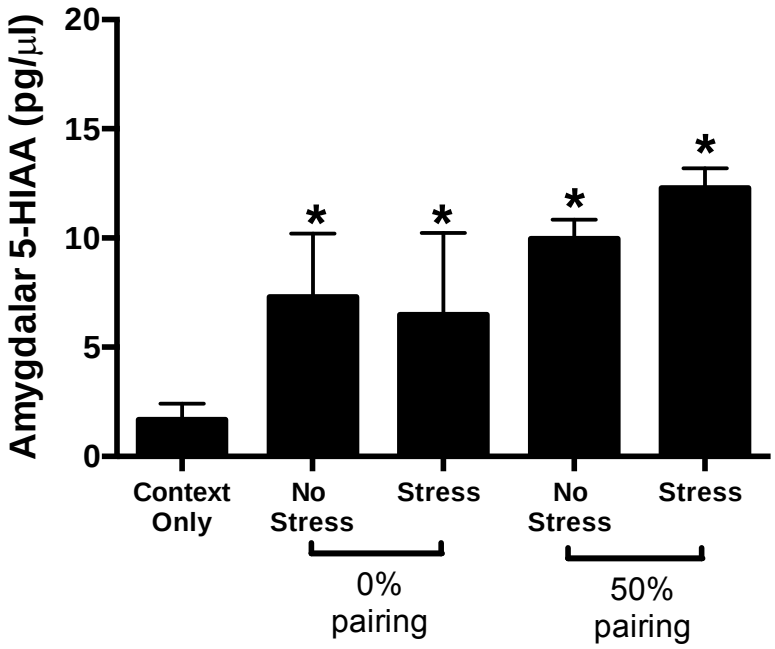

Figure S4. Stress does not affect conditioning-related increases in amygdalar 5-hydroxyindoleacetic acid (5-HIAA). Fear conditioning produced a significant elevation in BLA 5-HIAA, but this was not altered by previous stress exposure. Data are means ± SEM. Fisher's PLSD comparisons to the Context Only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

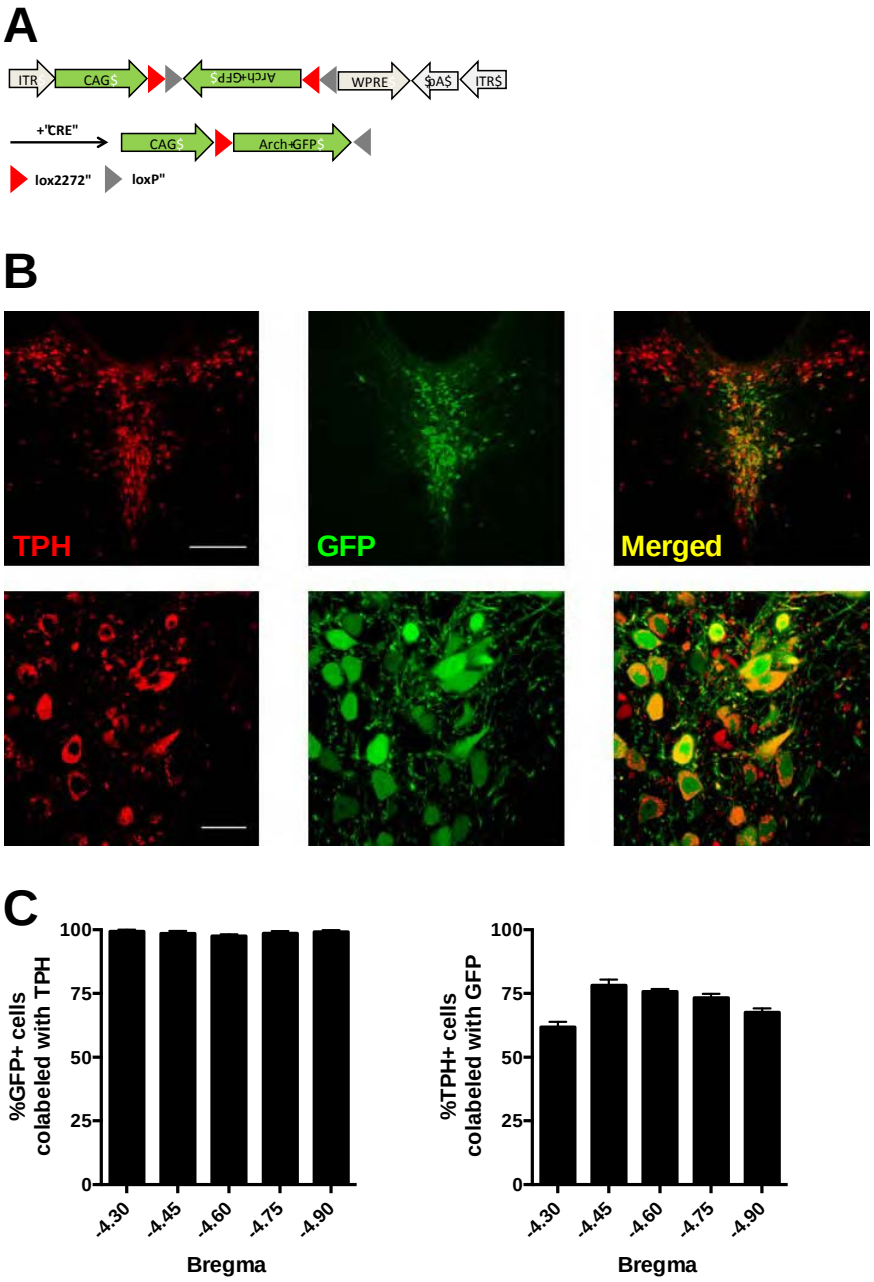

Figure S5. Specific expression of Arch in dorsal raphe serotonergic neurons. (**A**) Schematic of the construct for AAV-FLEX-Arch-GFP. In the presence of Cre recombinase, reversed Arch-GFP is inverted to the sense direction and expressed under the control of the cytomegalovirus early enhancer/chicken 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).

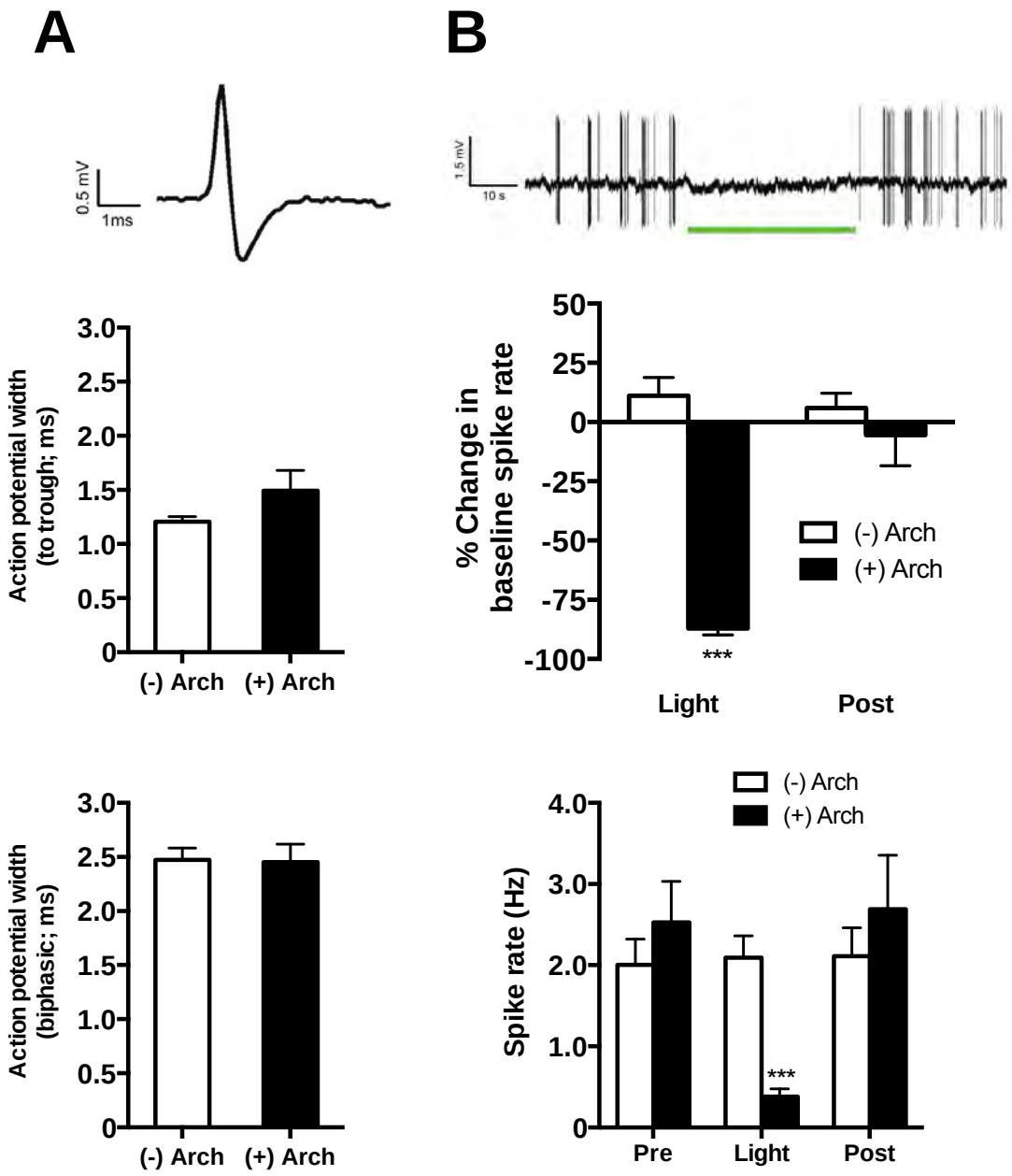

Figure S6. Arch-mediated optical silencing of serotonergic neurons in the dorsal raphe nucleus. (**A**) Representative waveform of an *in vivo* loose-cell attached recording from the dorsal raphe nucleus (DRN) of a SERT-Cre mouse. There were no differences in the mean waveform duration of action potentials (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: *** $P < 0.001$.

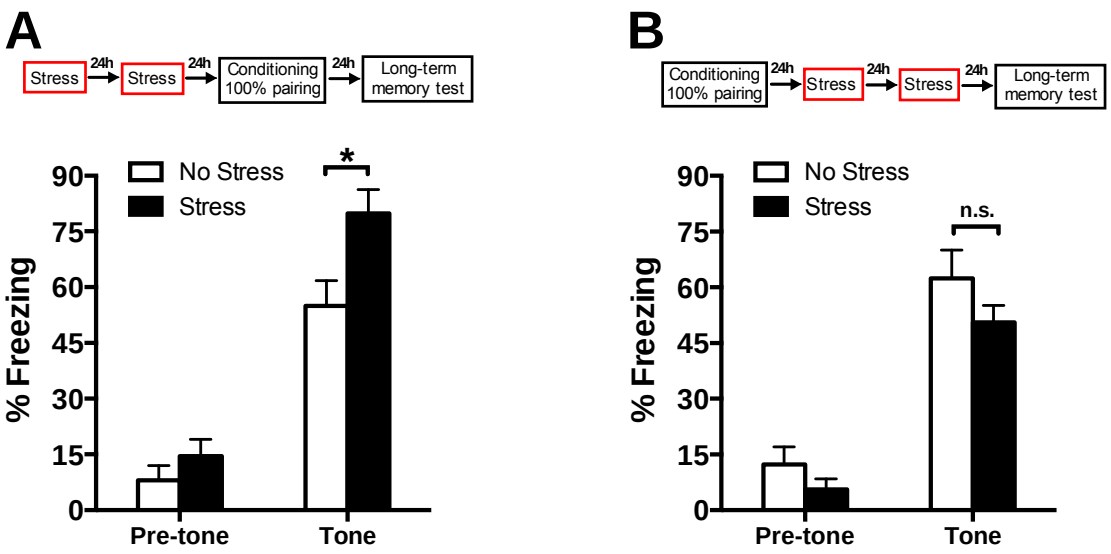

Figure S7. Prior stress enhances fear to unambiguous cues. (**A**) Prior stress enhances tone-elicited freezing in a conditioning paradigm with a tone-footshock contingency of 100%, (**B**) but no facilitation was observed when repeated stress followed conditioning (*n* = 8-10/group). Data are means ± 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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