Vagus nerve stimulation promotes generalization of conditioned fear extinction and reduces anxiety in rats

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ABSTRACT

Background: Exposure-based therapies are used to treat a variety of trauma- and anxiety-related disorders by generating successful extinction following cue exposure during treatment. The development of adjuvant strategies that accelerate extinction learning, improve tolerability, and increase efficiency of treatment could increase the efficacy of exposure-based therapies. Vagus nerve stimulation (VNS) paired with exposure can enhance fear extinction, in rat models of psychiatric disorders, and chronic administration of VNS reduces anxiety in rats and humans. Objective: We tested whether VNS, like other cognitive enhancers, could produce generalization of extinction for stimuli that are not presented during the extinction sessions, but are associated with the fear event.
Methods: Male Sprague Dawley rats underwent auditory fear conditioning with two easily discriminable auditory stimuli. Following fear conditioning, extinction training consisted of exposure to only one of the conditioned sounds. Half of the rats received VNS and half received sham stimulation during with sound presentations. VNS effects on anxiety were examined in a separate study where VNS was administered prior to testing on the elevated plus maze.
Results: Sham stimulated rats given 20 presentations of a conditioned stimulus (CS) during the extinction session showed performance that was matched to VNS-treated rats given only 4 presentations of the CS. Despite comparable levels of freezing to the presented CS, only the VNS-treated rats showed a significant decrease in freezing to the CS that was not presented. VNS-induced generalization of extinction was observed only when the two sounds were paired with footshock within the same fear conditioning session; VNS did not promote generalization of extinction when the two sounds were conditioned on different days or in different contexts. On the anxiety test, VNS administration significantly increased time spent in the open arms of the elevated plus maze.
Conclusion: These results provide evidence that VNS can promote generalization of extinction to other stimuli associated with a specific fear experience. Furthermore, non-contingent VNS appears to reduce anxiety. The ability to generalize extinction and reduce anxiety makes VNS a potential candidate for use as an adjunctive strategy to improve the efficacy and tolerability of exposure-based therapies.

Introduction

Exposure-based therapies are used to treat maladaptive fears and compulsions that are brought about by traumatic events [1–8].

The approach is designed to extinguish conditioned fear; a process that depends upon the suppression of old learned associations by the formation of new associations [9–11]. Unfortunately, drop out, non-response, and relapse rates are high [12]. Impaired extinction memory retention has been observed in individuals who suffer from posttraumatic stress disorder [13–19]. Such impairments may contribute to the development of trauma-related disorders, and may interfere with progress in therapy.

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Another challenge of exposure-based therapies is that it is not feasible to expose patients to every thought or sensory stimulus that may remind them of the trauma. Frequently, patients cannot recall significant portions of the traumatic event [20,21]. An ideal adjunct to exposure-based therapies would enhance extinction for the cues presented during therapy as well as trauma-related cues that are not presented during therapy. Encouraging recent findings indicate that some memory consolidation-enhancing methods, such as mild stress or administration of the NMDA partial agonist D-cycloserine (DCS), promote such generalization of extinction [22,23]. However, results of studies of cognitive enhancers as adjuncts are mixed [24]. Some researchers consider the use of pharmacological cognitive enhancers during exposure-based therapy risky due to the potential for reinforcing learned negative associations with reminders of the trauma, or with the therapy itself, when patients experience a severe anxiety response [25]. Optimal adjuvant treatments would promote generalization of extinction and concurrently reduce anxiety to improve treatment efficacy and tolerability.

Vagus nerve stimulation (VNS) paired with specific stimuli has emerged as a strategy to enhance memory consolidation [26,27] as well as consolidation of extinction memory in healthy rats [28–30]. Additionally, VNS administration paired with exposure to conditioned cues accelerates the extinction of conditioned fear and attenuates reinstatement in a rat model of PTSD [31]. VNS could also increase tolerability during exposure-based therapies as chronic administration of VNS reduces anxiety in rats [32,33] and humans [34]. These results suggest that VNS, which is FDA approved for epilepsy and treatment-resistant depression in humans, may be a promising adjunct to exposure-based therapies. In previous studies of VNS effects on extinction, VNS was paired with presentation of only a single conditioned cue. Given the memory-enhancing effects of VNS, the present experiments were designed to determine whether VNS-enhanced extinction could produce generalization of extinction. Based on evidence that chronic VNS reduces anxiety, we also tested whether the VNS parameters used to enhance the consolidation of memory and extinction can reduce anxiety.

Materials and methods

Animals

All procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of the University of Texas at Dallas. Eighty-eight male Sprague-Dawley rats (Charles River) weighing 225–250 g on arrival were housed on a 12 h light/dark cycle (lights on at 7:00 a.m.) with access to food and water *ad libitum*. Before the start of every experiment, rats were handled for five days, 5 min per day.

VNS and sham surgery

Surgery protocols are described in detail elsewhere [35]. In brief, rats were anesthetized with isoflurane (2% at an oxygen flow rate of 600–800 ml/min). The left vagus nerve was located at the cervical level and isolated from other tissue. The left vagus nerve was selected to avoid descending stimulation effects on the sinoatrial node. Central activation from the left vagus nerve is bilateral [36]. The cuff was placed around the nerve and secured in place with a suture. The platinum-iridium wires were tunneled subcutaneously behind the ear to the top of the head and connected to the Omnetics connector which was affixed to the skull using acrylic, to make the headcap. Cessation of breathing was used to test for correct implantation and effectiveness of the VNS cuff; following

implantation, while under anesthesia, current (0.8 mA, 1 s) was applied through the cuff electrode and breathing rate was visually monitored. If cessation of breathing was not observed, the cuff was adjusted or replaced until cessation of breathing was achieved with stimulation. For sham rats, surgery was conducted in the same manner but the cuff electrode was not implanted. During sham surgery, the vagus nerve was isolated from the other tissue and an Omnetics conector was affixed to the skull. Rats were given one week to recover following surgery.
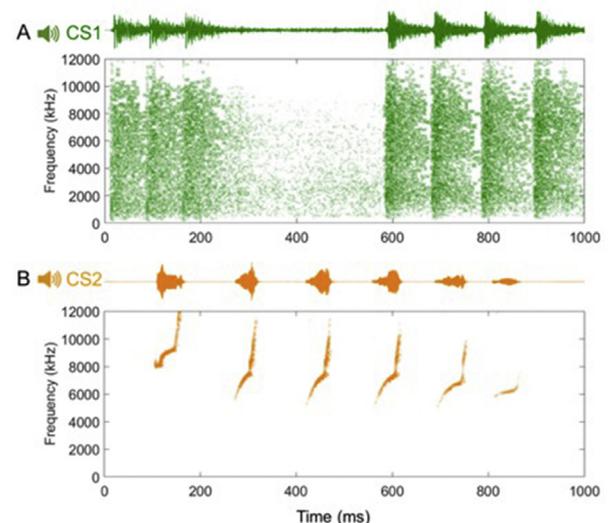
Extinction generalization

Auditory fear conditioning (AFC) consisted of two discrete, complex auditory stimuli that were paired with a footshock. The stimuli: machine gunfire (CS1) and a marmoset vocalization (CS2), were selected because they activate the auditory cortex more broadly and naturally than pure tones, and our previous work determined that they are perceptually distinct and discriminable [37,38]. Spectrograms of these cues show distinct differences in frequency bandwidth, frequency modulation, rise time, and decay time. Each stimulus was presented at 70 dB (Fig. 1).

Stimuli interleaved during AFC (co-conditioned)

Experiment 1 was designed to determine whether VNS promotes generalization of extinction across auditory cues. Thirty-four rats underwent auditory fear conditioning (AFC) where two discriminable stimuli (CS1 and CS2) were presented during the same conditioning session. Both CS1 and CS2 were coupled with footshocks. Only one of the stimuli was presented during the extinction treatment day but both were presented during test sessions (Fig. 4a).

Two days of AFC were conducted in Context A (electric grid floor). During AFC, CS1 and CS2 were randomly interleaved with an interstimulus interval (ISI) between 120 and 240 s. Four presentations of each CS coupled with a footshock (0.8 mA, 1 s) were administered on each day of AFC. As in our previous studies [27–30], we aimed to minimize timing predictability by administering the

Fig. 1. Spectrograms of each conditioned stimulus used during AFC. A. Spectrogram for CS1 (machine gun fire). B. Spectrogram for CS2 (marmoset vocalization). Spectrograms for CS1 and CS2 were used in generalization experiments, as they are easy to discriminate given they have distinct differences in frequency bandwidth, frequency modulation, rise time, and decay time. Duration of stimuli were also different, CS1 lasted 50 s while CS2 lasted 39 s.

footshock at a random time during each CS presentation. Twenty-four hours following Day 2 of AFC, rats were given a conditioned fear response test (CFRT) to determine whether fear conditioning was equivalent across the two cues and across the two groups before VNS. The CFRT was carried out in Context B (peppermint smell, black Plexiglas floor), where each CS was presented four randomly interleaved times, without reinforcement. Videos were recorded via webcam and the amount of time spent freezing during presentations of each CS was scored by two experimenters who were blind to treatment conditions, and used as the measure of conditioned fear. On the following day, rats were given extinction training in Context B, where only one CS was presented and paired with either VNS or sham stimulation (Presented CS); the other CS was not presented on extinction treatment day (Non-Presented CS). The CS selected as the Presented CS was counter-balanced. VNS-treated rats received four presentations of the CS that overlapped with VNS (VNS + Extinction group; n = 14). The parameters used for VNS were the same as those used to enhance memory consolidation and the consolidation of extinction of conditioned fear [28–31] and they are within the range of stimulation parameters approved by the FDA for seizure prevention in humans [33]. For seizure prevention, a 30 s train of VNS is given every 5 min throughout the day and night. Here, the "paired" VNS was delivered at an intensity of 0.4 mA, with a pulse width of 100 μs and frequency of 20 Hz for a single 30 s train, starting 150 ms before the onset of each 30-sec tone [29]. We previously found that 20 presentations of the CS paired with sham stimulation and four presentations of the CS paired with VNS generate equivalent levels of extinction [30]. To compare generalization, the Presented CS was extinguished to the same degree across groups by giving sham-treated rats 20 presentations of the CS paired with sham stimulation (Extended Extinction group; n = 14). To assess any effects of VNS alone on extinction, a third group of rats were subjected to the same AFC and CFRT procedures but remained in the home cage on extinction day and received equal amounts of VNS in lieu of extinction (VNS Alone group; n = 6). Twenty-four hours later, levels of conditioned fear to Context B and to each CS were tested again by placing all rats into Context B, recording baseline freezing to the context for 5 min, then presenting rats with four randomly interleaved presentations of each CS. The CFRT session was recorded by webcam and baseline freezing to Context B, freezing during CS1, and freezing during CS2 were scored by two blind experimenters. The CFRTs could allow for extinction learning because they involve four non-reinforced exposures to both stimuli on each test day. However, we find a significant effect when only four of the exposures are paired with VNS [28–31]. Although we have not observed locomotor or other performance effects that may obscure measurement of conditioned fear, taking the CFRT before and after VNS treatment prevents mistaking of immediate performance disturbances for VNS effects on extinction. Testing retention 24 h later allows for a measure of VNS effects on the consolidation of the extinction memory. Furthermore, with this approach, it is unlikely that the VNS serves as a safety signal because fear is measured in the absence of VNS.

Stimuli separated by time during AFC

To determine if VNS led to generalization of extinction for cues learned on separate days, we performed Experiment 2 where CS1 was coupled with a footshock and, 24 h later, CS2 was coupled with a footshock (Fig. 5a). Experiment 2 was preregistered on Open Science Framework (osf.io/wk7re). Twenty rats were subjected to two days of AFC in Context A (neutral smell, electric grid floor). On Day 1 of AFC, rats were presented with only CS1, which was presented 8 times coupled with a footshock (0.8 mA, 1 s). On Day 2, CS2 was presented 8 times coupled with a footshock (0.8 mA, 1 s). Twenty-four hours

following AFC, rats underwent a pre-extinction CFRT in Context B (peppermint smell, black acrylic floor). Rats were presented with four unreinforced presentations of CS1. On the following day, rats underwent another pre-extinction CFRT where this time they were presented with four unreinforced presentations of CS2. Both CFRT days were recorded with a Logitech camera. Percent of time spent freezing during presentations of CS1 and CS2 were scored by two blind experimenters and used as the measure of conditioned fear. On Day 5, rats underwent extinction to either CS1 or CS2 paired with either sham stimulation or VNS (counter-balanced across groups). Extinction was performed the same way as in Experiment 2; the CS was presented four times if paired with VNS (VNS + Extinction group, n = 10), or 20 times if paired with sham stimulation (Extended Extinction group, n = 10). Twenty-four hours after the extinction treatment session, rats were placed into Context B where freezing to the context was recorded for 5 min, then four presentations of each CS were randomly interleaved. This post-extinction CFRT session was recorded and freezing to Context B, CS1, and CS2 were scored by two blind experimenters and used as a measure of conditioned fear.

Stimuli separated by context during AFC

To determine if VNS led to generalization of extinction for cues learned in different contexts, we performed Experiment 3 where AFC to CS1 occurred in Context A, and AFC to CS2 occurred in Context C within 1 h (Fig. 6a). Experiment 3 was preregistered on Open Science Framework (osf.io/wk7re). Sixteen rats were subjected to AFC for CS1 in Context A (neutral smell, shock grid floor) and AFC for CS2 in Context C (peppermint smell, shock grid floor, different light position, different room). On the first day of AFC, CS1 was coupled with a footshock (0.8 mA, 1 s) four times, and then rats were returned to their home cages. After 15 min in the home cage, rats were transferred into Context C where CS2 was coupled with a footshock four times. On Day 2, rats were again placed into Context C and exposed to four presentations of CS2 coupled with a footshock and then, after 15 min, transferred to Context A and exposed to four presentations of CS1 coupled with a footshock. Twenty-four hours later, rats underwent a pre-extinction CFRT to CS1 in Context A, where CS1 was presented four times without reinforcement. Rats were returned to their home cages for 15 min then transferred to Context C where they underwent another pre-extinction CFRT to CS2, where CS2 was presented four times without reinforcement. Test sessions were recorded by a Logitech camera and freezing to each CS was scored by two experimenters who were blind to treatment conditions. On Day 4 rats underwent extinction to CS1, in Context A, paired with VNS or sham treatment. Extinction was performed the same way as in Experiments 2 and 3; VNS-treated rats received four presentations of CS1 paired with VNS (VNS + Extinction group; n = 8) whereas sham-treated rats received 20 presentations of CS1 (Extended Extinction group; n = 8). Twenty-four hours following the extinction session, rats were given a post-extinction CFRT for each CS. Rats were first placed in Context A, where baseline freezing to the context was recorded for 5 min, then CS1 was presented four times without reinforcement and freezing behavior was recorded. After 15 min in the home cage, rats were transferred to context C where baseline freezing to the context was recorded for 5 min, then four unreinforced presentations of CS2 were given. Test sessions were recorded by a Logitech camera and freezing to context A, context C, CS1, and CS2 were scored by two blind experimenters as measures of conditioned fear.

VNS effects on anxiety

To determine if the parameters of VNS given during extinction were sufficient to reduce anxiety, we administered VNS or sham

stimulation to eighteen rats prior to testing on the elevated plus maze (EPM). In order to prevent a possible neophobia effect before EPM testing, rats were given a single 30 s train of VNS or sham stimulation per day, spread across four days. Rats were connected to the AM systems stimulator using the previously described parameters (20 Hz, 0.4 mA, 30 s, 100μs pulse width), however stimulation was not triggered in response to an event. Rather, a single non-contingent train of VNS was given in the home cage. Sham-treated rats were connected to the stimulator in the same way as VNS-treated rats but did not receive stimulation. On Day 4, rats underwent testing on the EPM 10 min after being disconnected from the stimulator.

The EPM was used to measure anxiety [39]. Rats were placed onto an elevated plus-shaped maze (10.0 cm wide, 50.0 cm long, elevated 55.0 cm off the floor), with walls (30.0 cm tall) on two opposing arms and no walls on the other opposing arms. Time spent in the open arms, time spent in the closed arms, and time spent in the center of the maze were recorded during the 10-min test. An entry into an arm was scored when the rat's full body, excluding the tail, was in the arm at one time. All behavior was recorded and scored using AnyMaze video tracking software. Time spent in the open arms was used as a measure of risk taking, and the percent of the total time spent moving was taken as a control measure of general locomotion.

Statistical analyses

Data for extinction generalization experiments were analyzed using a two-factor repeated measures ANOVA with a Greenhouse-Geisser correction followed by a Tukey's post hoc test for multiple comparisons. Context freezing was analyzed using a one-way ANOVA with a Tukey's post hoc test for multiple comparisons or an unpaired samples *t*-test. Data for VNS effects on anxiety were analyzed using an unpaired samples *t*-test.

Statistically significant effects were defined as those with p values that were <0.05. All error bars represent standard error of the mean. Individual data points are represented on each graph as circles. Criteria for exclusion of rats from the analysis was performance ≥2 standard deviations away from the mean on any task. Six rats were excluded from analysis for failure to express conditioned fear following AFC (freezing less than 50% to the CS after tone-shock couplings). Exclusion of these rats did not alter results.

Results

VNS + Extinction leads to generalization of extinction for co-conditioned stimuli

VNS paired with exposure promotes extinction of a fear conditioned stimulus. Here, we tested the hypothesis that VNS, like other cognitive enhancers, would promote generalization of extinction for multiple fear-related stimuli learned within a session. In Experiment 1, rats underwent AFC to two distinct auditory stimuli interleaved during the fear conditioning sessions. All rats exhibited similar levels of conditioned fear to both CS1 and CS2 at the CFRT given prior to extinction training (Pre-Extinction). Rats underwent either VNS + Extinction (n = 14), where a train of VNS was paired with presentations of one of the conditioned cues a total of four times and the other CS was not presented, or Extended Extinction (n = 14) where the conditioned cue was presented without VNS a total of 20 times and the other CS was not presented. The CFRT measured 24 h after the Extinction Treatment session was compared across the two groups. A two-factor repeated measures ANOVA indicated a significant effect of treatment group across days (F(3, 64) = 47.00 p = 3.6 × 10^{-8}). Following 20 presentations of

either CS1 or CS2 paired with sham stimulation, Extended Extinction rats showed a reduction in conditioned fear to the CS presented during extinction (Presented CS) versus Pre-Extinction freezing (p = 2.3 × 10^{-4}). Four presentations of CS1 or CS2 paired with VNS was also sufficient to reduce freezing to the Presented CS in VNS + Extinction rats versus Pre-Extinction freezing (p = 3.2 × 10^{-4}) (Fig. 2b).

Extended Extinction rats showed no reduction in conditioned fear to the CS that was not presented during extinction (Non-Presented CS) versus Pre-Extinction freezing (p = 0.15). In contrast, VNS + Extinction rats showed a significant reduction in freezing during exposure to the Non-Presented CS when compared to Pre-Extinction freezing to the same CS (VNS + Ext vs. Pre-Extinction, p = 2.1 × 10^{-4}) (Fig. 2c). This indicates that VNS + Extinction led to generalization of extinction whereas Extended Extinction did not.

VNS is likely to enhance fear extinction by engaging neuromodulatory networks to support extinction-related plasticity. However, it is possible that VNS simply reduces fear or anxiety leading to reduced time spent freezing in response to either CS. To determine if VNS administration had a non-extinction-specific effect, such as a general reduction in expression of fear, a matched amount of VNS was administered in the home cage in lieu of extinction (VNS Alone, n = 6). A two-tailed *t*-test indicated no significant effect of VNS Alone (VNS Alone vs. Pre-Extinction freezing, t (11) = 1.8 p = 0.16), demonstrating that VNS Alone was not sufficient to reduce conditioned fear to either CS (Fig. 2d). These findings are consistent with previous results and indicate that VNS reduces conditioned fear by enhancing fear extinction [28].

Since VNS accelerates extinction, it is possible that reduced freezing to the Non-Presented CS is due to enhanced extinction of Context B. To determine if VNS-dependent generalization of extinction was due to enhanced learning that Context B is safe, baseline freezing to Context B was measured for 5 min prior to any CS presentation during the Post-Extinction CFRT. A one-way ANOVA indicated a significant effect across groups (F(2, 31) = 19.21 p = 3.7 × 10^{-6}). However, VNS + Extinction and Extended Extinction demonstrated equivalent freezing to context (p = 0.68) (Fig. 2e). This similarity indicates that the VNS effect on extinction generalization cannot be explained by VNS enhancement of extinction to Context B. VNS Alone rats showed elevated freezing to the extinction context, likely because they were left in the homecage instead of undergoing extinction training in Context B. Together, these findings indicate that VNS does not reduce conditioned fear to the Non-Presented CS through an enhancement of context extinction, or a general VNS-induced reduction in fear expression. Note that the comparable freezing to context in VNS + Extinction and Extended Extinction animals is consistent with the hypothesis that VNS enhances and generalizes extinction because the time in the extinction context was five times greater for Extended Extinction rats on the extinction treatment day.

Experiment 2: VNS-induced generalization of extinction does not extend to stimuli that are conditioned on separate days.

To determine if fear memories that were acquired at different times were susceptible to VNS-induced generalization of extinction, we coupled CS1 alone with a footshock during the first AFC session and then coupled CS2 alone with a footshock 24 h later. As in Experiment 1, an extinction session consisted of only one CS given with VNS (n = 10) or sham stimulation (n = 10). A two-factor repeated measures ANOVA indicated a significant effect of group across days (F(2, 37) = 26.65 p = 6.8 × 10^{-7}). All rats exhibited similar levels of conditioned fear to both CS1 and CS2 at the Pre-Extinction CFRT. Following 20 presentations of either CS1 or CS2 paired with sham stimulation, Extended Extinction rats showed a reduction in freezing in response to the Presented CS versus Pre-
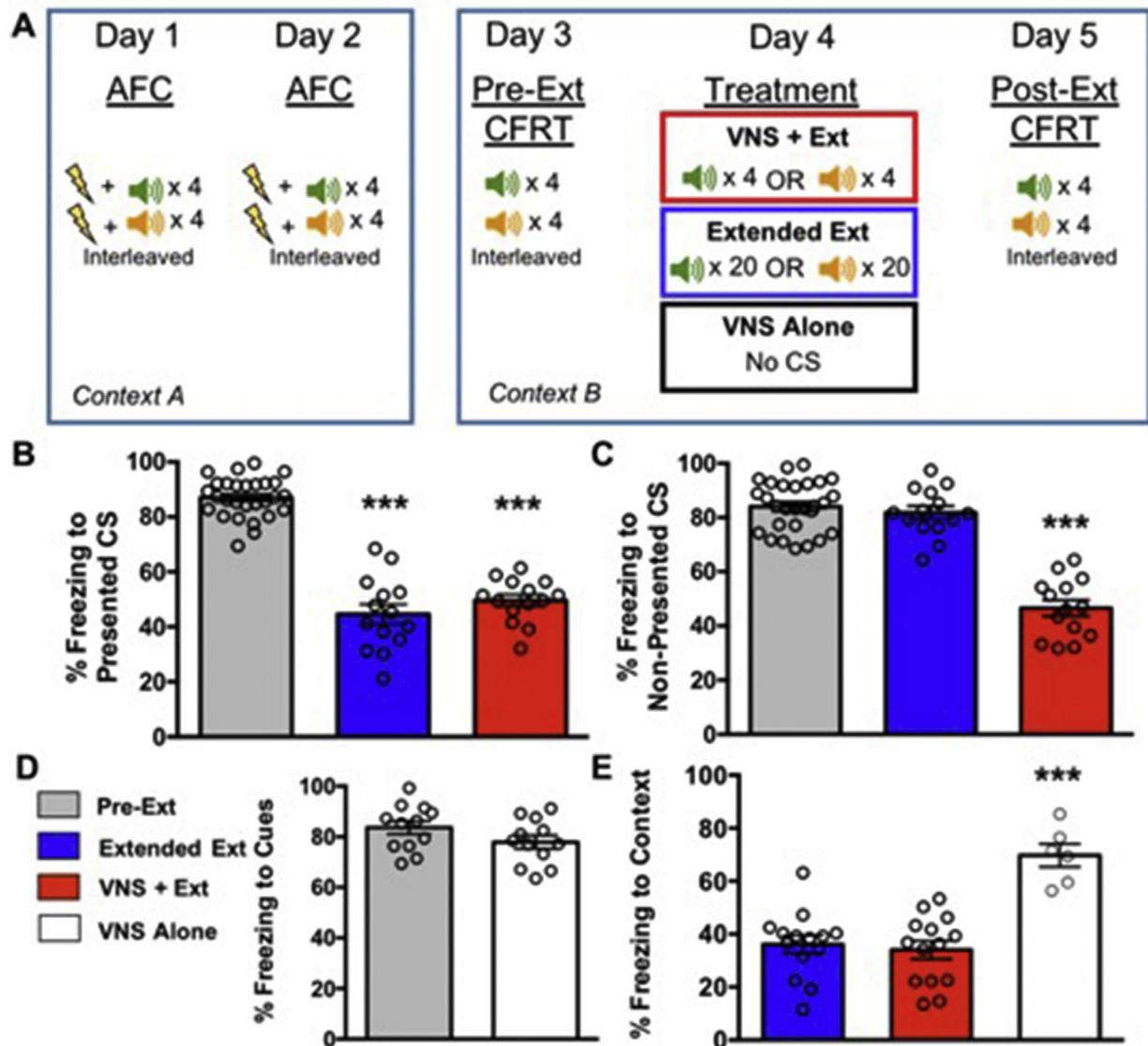
Fig. 2. VNS + Extinction leads to generalization of extinction for both the CS that was presented and the CS that was not presented during extinction training. A. Timeline for AFC, conditioned fear response testing, and extinction treatment. AFC was administered across two days where both CS1 and CS2 were presented and paired with footshocks on each day. The order of CS presentation was random and interleaved such that each rat was administered 4 presentations of each CS on each day. Following AFC, rats underwent a pre-extinction conditioned fear response test (CFRT) where they were presented with four presentations of each CS to measure conditioned fear. Twenty-four hours later, rats underwent treatment consisting of either: 20 extinction trials of only CS1 or only CS2, paired with sham stimulation (Extended Extinction), 4 extinction trials of only CS1 or only CS2, paired with VNS (VNS + Extinction), or no exposures to either CS but equivalent amounts of VNS in the home cage (VNS Alone). A day later, rats underwent another CFRT to assess levels of conditioned fear to both stimuli. **B. Extended Extinction and VNS + Extinction rats show equal reduction in conditioned fear to the Presented CS.** Following 20 unreinforced presentations of a CS, Extended Extinction rats showed reduced freezing in response to the CS versus freezing during Pre-Extinction ($p = 2.3 \times 10^{-4}$). VNS + Extinction rats also showed reduced freezing in response to the Presented CS versus the Pre-Extinction ($p = 3.2 \times 10^{-4}$). **C. Only VNS + Extinction leads to generalization of extinction for the Non-Presented CS.** Extended Extinction did not show reduced fear in response to the Non-Presented CS versus the Pre-Extinction freezing response ($p = 0.15$). In contrast, VNS + Extinction rats showed a reduction in freezing response to presentation of the Non-Presented CS versus the Pre-Extinction freezing response, even though it was never presented during extinction ($p = 2.1 \times 10^{-4}$). **D. VNS Alone does not lead to extinction of either CS.** Following VNS in the home cage in lieu of extinction, VNS Alone rats showed no reduction in conditioned fear for either CS versus the Pre-Extinction CFRT ($p = 0.16$). **E. Context extinction cannot explain VNS + Extinction generalization.** There is no difference in baseline fear to the context between Extended Extinction and VNS + Extinction groups, rats spend equal amounts of time freezing to the context during the Post-Extinction CFRT ($p = 0.68$). VNS Alone rats show elevated freezing to the context versus Extended Extinction ($p = 2.1 \times 10^{-3}$) and VNS + Extinction rats ($p = 3.8 \times 10^{-3}$).

Extinction freezing ($p = 7.1 \times 10^{-3}$). Four presentations of CS1 or CS2 paired with VNS (VNS + Extinction) was also sufficient to significantly reduce freezing to the Presented CS versus Pre-Extinction freezing ($p = 4.4 \times 10^{-3}$). The degree of extinction was comparable to that observed with Extended Extinction (Fig. 3b).

Extended Extinction rats did not show a reduction in conditioned fear to the Non-Presented CS versus Pre-Extinction freezing ($p = 0.12$), similar to results of Experiment 1 with interleaved AFC. However, in contrast with the results from Experiment 1,

administration of VNS during extinction was not sufficient to reduce freezing to the Non-Presented CS (VNS + Ext freezing vs. Pre-Extinction freezing: $p = 0.29$) (Fig. 3c).

An unpaired *t*-test indicated no significant difference in freezing to the context between Extended Extinction and VNS + Extinction ($t(9) = 0.59$ $p = 0.56$) (Fig. 3d). Taken together, these findings suggest that VNS does not promote generalized extinction of conditioned fear when the original fear learning does not occur at the same time.
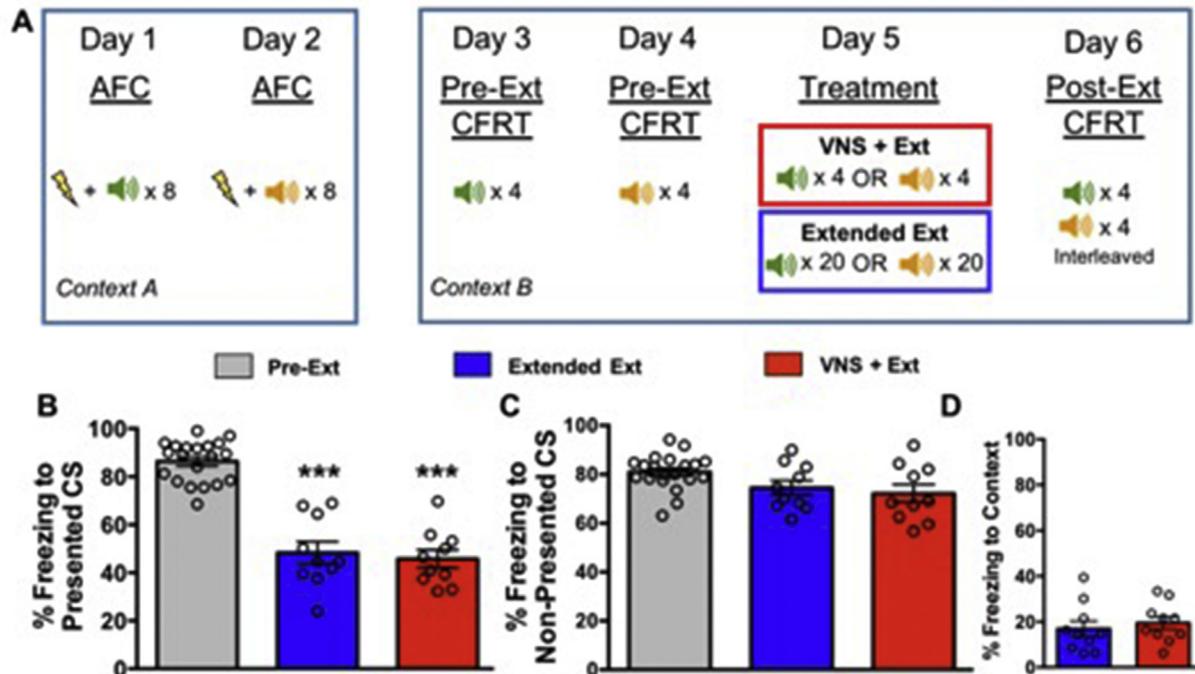
Fig. 3. Separating CS1 and CS2 conditioning by 24 h blocks the VNS effect on generalization. A. Timeline for AFC, CFRT, and extinction treatment. AFC was administered across two days where CS1 was presented on 1 day and CS2 was presented 24 h later. A CFRT to CS1 was administered on day 3 where CS1 was presented four times without reinforcement to measure conditioned fear to CS1. The next day, a CFRT was administered to CS2 where CS2 was presented four times without reinforcement to measure conditioned fear to CS2. Twenty-four hours later, rats underwent extinction consisting of either: 20 extinction trials of CS1 or CS2 paired with sham stimulation (Extended Extinction), or 4 extinction trials of CS1 or CS2 paired with VNS (VNS + Extinction). Following extinction, a Post-Extinction CFRT was administered where four presentations of each CS were presented and randomly interleaved to assess conditioned fear to each CS following extinction. **B. Extended Extinction and VNS + Extinction rats show equal reduction in conditioned fear to the Presented CS.** Following 20 presentations of the Presented CS, both Extended Extinction and VNS + Extinction rats showed reduced freezing to the Presented CS ($p = 7.1 \times 10^{-3}$ vs Pre-Extinction) and ($p = 4.4 \times 10^{-3}$ vs. Pre-Extinction), respectively. **C. Neither Extended Extinction rats or VNS + Extinction rats show reduced freezing to the Non-Presented CS.** After extinction training, freezing to the Non-Presented CS was not reduced for Extra Extinction rats ($p = 0.12$). In contrast to Fig. 1, freezing to the Non-Presented CS was not reduced in ($p = 0.29$). **D. Baseline freezing to the context is not different between groups.** Extended Extinction and VNS + Extinction rats spent equivalent amounts of time freezing to the extinction context during the 5 min prior to CS presentation at the Post-Extinction CFRT ($p = 0.55$).

Experiment 3: VNS-induced generalization of extinction does not extend to stimuli that are conditioned in separate contexts.

We next tested whether fear memories learned during a single session but across two contexts were susceptible to VNS-induced generalization of extinction. We paired CS1 with footshocks in Context A and then, on the same day, paired CS2 with footshocks in Context C. During extinction, only CS1 was presented in Context A, paired with VNS (n = 8) or sham stimulation (n = 8). A two-factor repeated measures ANOVA indicated a significant effect of group across days ($F_{(2, 29)} = 19.16$ $p = 5.0 \times 10^{-6}$). All rats exhibited similar levels of conditioned fear to both CS1 and CS2 at the Pre-Extinction CFRT. Similar to Experiment 1 using interleaved AFC and Experiment 2 using separated AFC, following 20 presentations of CS1 paired with sham stimulation, Extended Extinction rats showed a reduction in conditioned fear to the Presented CS ($p = 2.3 \times 10^{-3}$). Four presentations of CS1 paired with VNS was also sufficient to reduce freezing to the Presented CS in VNS + Extinction rats versus Pre-Extinction freezing ($p = 1.2 \times 10^{-3}$) (Fig. 4b).

After AFC in two distinct contexts, Extended Extinction rats did not show a reduction in conditioned fear for the Non-Presented CS (Pre-vs. Post-Extinction freezing; $p = 0.38$), as seen in Experiments 1 and 2. When AFC was separated by time, VNS + Extinction rats did not show reduced conditioned fear of the Non-Presented CS ($p = 0.40$) (Fig. 4c). Unpaired t-tests indicated no significant difference in freezing to the Extinction Context between Extended Extinction and VNS + Extinction ($t_{(7)} = 0.53$ $p = 0.60$) (Fig. 4d) or in freezing to the Non-Extinction context ($t_{(7)} = 0.67$ $p = 0.52$)

(Fig. 4e). These findings suggest that VNS does not generalize extinction of conditioned fear when the original fear learning does not happen in the same context.

Experiment 4: VNS reduces anxiety.

Previous research indicates that chronic VNS is anxiolytic [34]. We aimed to see if the VNS given during extinction training could produce an anxiolytic effect. We administered VNS or sham stimulation prior to testing on the EPM. In order to prevent VNS-related neophobia on the day of EPM testing, rats were given one non-contingent stimulation in their home cage/day for four days. Ten minutes following the single, non-contingent stimulation, rats were disconnected from the stimulator. On the fourth day, rats were placed on the EPM for 10 min immediately after being disconnected from the stimulator. During the EPM session, time spent in the open arms, time spent in the closed arms, and total time spent moving were recorded. An unpaired t-test indicated a significant increase in time spent in the open arms in VNS-treated rats versus sham ($t_{(16)} = 6.35$ $p = 3.1 \times 10^{-3}$). Total time spent moving was not different between groups, indicating no gross locomotor effects. These results indicate that a 30-sec train of VNS can reduce anxiety within minutes.

Discussion

Exposure-based therapies are founded on the premise that unreinforced exposure to conditioned cues leads to extinction of learned associations [13]. Exposure therapy is used to treat disorders such as PTSD, phobia, obsessive-compulsive disorder, and
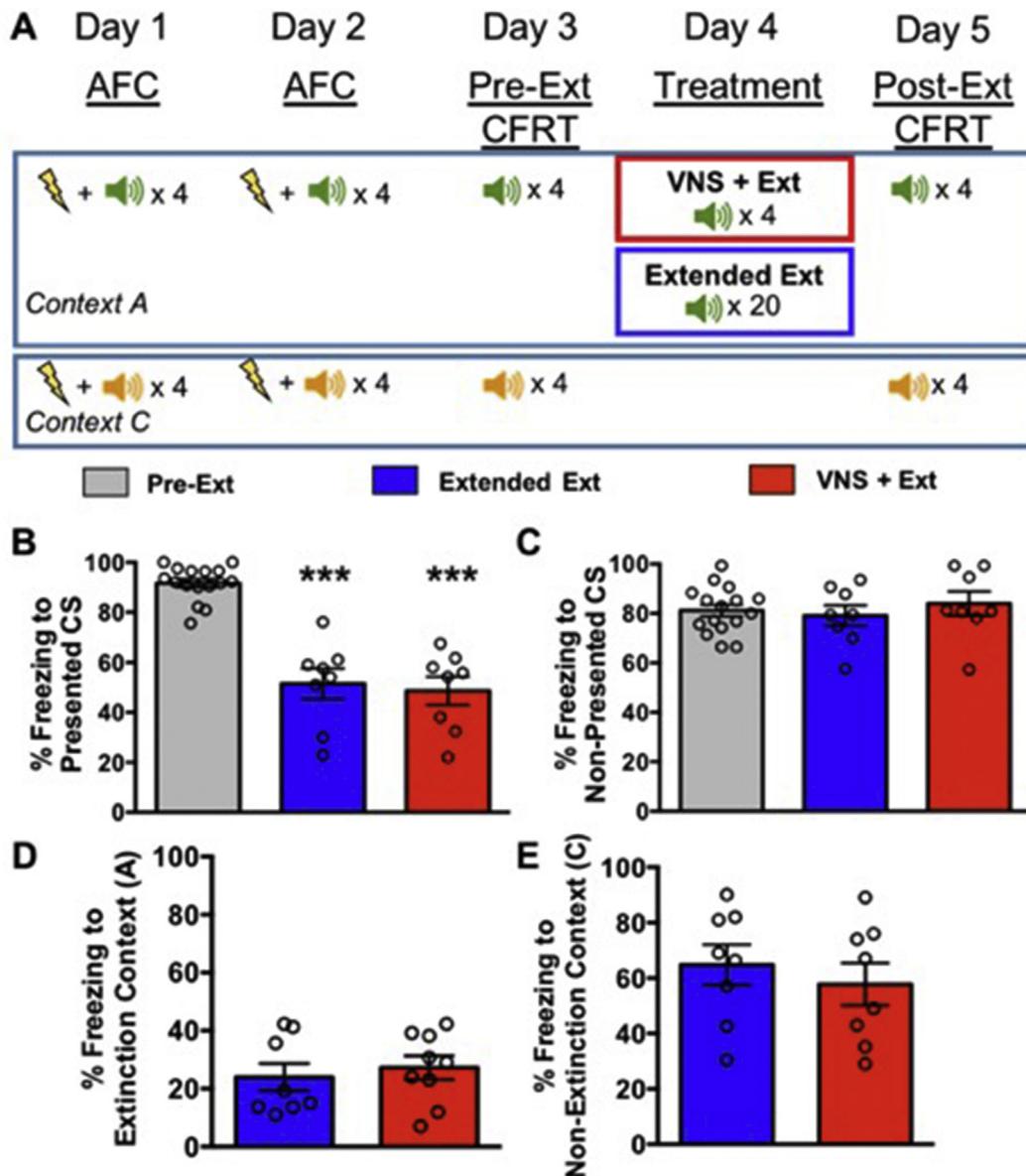
Fig. 4. Separating conditioning of CS1 and CS2 by context blocks the VNS + Extinction effect on generalization. A. Timeline for AFC, CFRT, and extinction treatment. AFC was administered across two days, where CS1 was presented only in Context A, and CS2 was presented only in Context C, but both CS1 and CS2 were presented during the same session. Twenty-four hours after AFC, a Pre-Extinction CFRT was administered for CS1 in Context A, where CS1 was presented four times without reinforcement. On the same day, a Pre-Extinction CFRT was also administered to CS2 in Context C, where CS2 was presented four times without reinforcement. Following the Pre-Extinction CFRT day, rats were subjected to extinction treatment consisting of either: 20 presentations of CS1 in Context A paired with sham stimulation (Extended Extinction), or 4 presentations of CS1 in Context A paired with VNS (VNS + Extinction). Twenty-four hours following extinction, a Post-Extinction CFRT was given to CS1 in Context A, and then on the same day a Post-Extinction CFRT was given for CS2 in Context C. **B. Extended Extinction and VNS + Extinction rats show equal reduction in conditioned fear to the Presented CS.** Following extinction of CS1 in Context A, Extended Extinction and VNS + Extinction rats showed reduced conditioned fear to the Presented CS versus Pre-Extinction ($p = 2.3 \times 10^{-3}$) and ($p = 1.2 \times 10^{-3}$), respectively. **C. Neither Extended Extinction rats or VNS + Extinction rats show reduced freezing to the Non-Presented CS.** Freezing to the Non-Presented CS was not reduced after Extinction training for Extended Extinction rats ($p = 0.38$). Similar to Fig. 2, freezing to the Non-Presented CS was also not reduced for VNS + Extinction rats versus Pre-Extinction ($p = 0.40$). **D. Baseline freezing to the extinction context is not different between groups.** Extended Extinction and VNS + Extinction rats spent an equal amount of time freezing to the extinction context during the 5 min prior to presentation of CS1 during the Post-Extinction CFRT ($p = 0.53$). **E. Baseline freezing to the non-extinction context is not different between groups.** Extended Extinction and VNS + Extinction rats spent an equal amount of time freezing to the non-extinction context during the 5 min prior to CS2 presentation during the Post-Extinction CFRT ($p = 0.62$).

addiction. Unfortunately, these therapies show a high incidence of non-response, dropout, and relapse [12]. Various explanations have been offered, including the observation that patients with anxiety-related disorders show impairments in recall of extinction learning [15–19]. Furthermore, it is not feasible to expose patients to every thought or sensory stimulus that may remind them of the trauma. Some recent findings indicate that methods that enhance memory consolidation, such as mild stress or administration of the NMDA partial agonist D-cycloserine (DCS), promote generalization of extinction to reminders that are not presented during therapy [22,23]. Therefore, other tools for enhancement of memory consolidation could be useful adjuvants for exposure-based therapies. In order to improve treatment efficacy and reduce relapse, an ideal adjunct would promote extinction to the totality of cues associated with the trauma experience while reducing anxiety to improve tolerability.
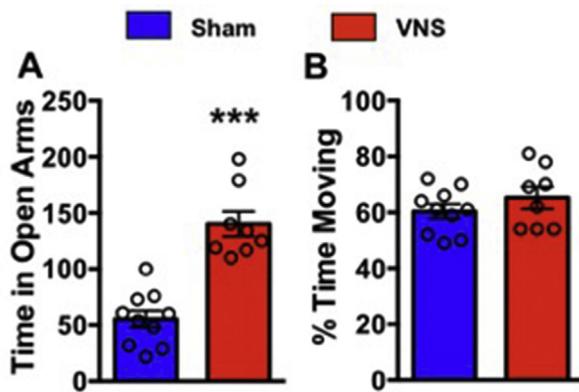
Fig. 5. VNS reduces anxiety. Rats were given one non-contingent train of VNS or sham stimulation in their home cage. Ten minutes later, they were removed from their home cage and placed on the elevated plus maze (EPM). VNS-treated rats spent significantly more time in the open arms of the EPM when compared to sham-treated rats ($p = 2.1 \times 10^{-3}$), indicating a VNS-induced reduction in anxiety. The percent of time spent moving during EPM testing was similar between groups, indicating no general locomotor effect.

VNS has emerged as a potential strategy to promote neural plasticity, enhance memory, and reduce conditioned fear when delivered coincidentally with cue presentation during fear extinction [29–31], and chronic VNS has anxiolytic effects [32]. We tested whether VNS, like other cognitive enhancing strategies, would promote generalization of extinction and accelerate extinction to multiple fear-related cues in rats. We also tested whether the amount of VNS given to promote extinction was sufficient to reduce anxiety.

The results of these studies provide evidence that VNS paired with exposure to the CS can enhance the consolidation of extinction of conditioned fear, not only for the CS that is paired with VNS, but also for another CS that is associated with the same fear experience. VNS-enhanced extinction generalized to a conditioned cue that was acquired during the same fear conditioning session, indicating that VNS-paired extinction training specifically enhances extinction of learned associations acquired within a session. VNS effects on extinction generalization cannot be explained by enhanced learning of the extinction context, as VNS + Extinction and Extended Extinction rats showed equivalent reductions of freezing responses to the extinction context. Both groups froze less in the context than rats given VNS in lieu of extinction (VNS Alone), indicating that both groups extinguished fear of the context to an equivalent degree, despite the fact that the Extended Extinction group spent more time in the context during the extinction session. VNS Alone rats did not show extinction of conditioned fear of the presented auditory cue or the context, nor did they show generalization of extinction, suggesting that the VNS-induced generalization effect is not due to a general VNS-related reduction in freezing, fear, or anxiety. Taken together, these results indicate that the VNS effects on freezing responses are due to the generalization of extinction of conditioned fear.

Importantly, when presentations of each stimulus occurred on different days, or in different contexts, VNS-enhanced extinction did not promote generalization. This finding suggests that VNS does not act to reduce all conditioned fear. Because fear is an adaptive biological process that protects individuals from danger [40], it is important for trauma sufferers to extinguish conditioned fear of cues that are associated with maladaptive fears without interfering with all learned fears. Memories must be bound by time and place to be susceptible to extinction generalization. Interestingly, optogenetic studies indicate that neuronal ensembles for similar experiences that happen closely in time can overlap. However, following the passage of time or the changing of context, memories are represented by separate neuronal ensembles, indicating that memory engram cells are timing and context-specific [41–43]. The present findings indicate that VNS affects memories that are bound by time, place, and neuronal ensemble, when the effects of Extended Extinction are more limited.

The results of the present investigation do not explain why Extended Extinction does not lead to the same generalization effect as VNS + Extinction. Presenting five times as many presentations of the CS during extinction paired with sham stimulation (Extended Extinction) is sufficient to generate similar levels of freezing to the Presented CS compared to VNS + Extinction. However, Extended Extinction rats do not show generalization of extinction for the Non-Presented CS. Taken together, these findings indicate that there is a qualitative difference in VNS-influenced extinction and extinction achieved following five times as many exposures paired with sham stimulation, despite the comparable levels of freezing to the Presented CS. The degree of memory displayed at the CFRT does not reveal all of the facets of extinction memory, such as how long-lasting or broadly-reaching it is. In addition to facilitating extinction of conditioned fear, previous studies demonstrate that VNS-enhanced extinction is less susceptible to reinstatement of conditioned fear [31], indicating that the neural plasticity supporting VNS-enhanced extinction memory is more rapid and robust. Here, we report evidence that VNS-enhanced extinction is also more broadly tuned. Generalization of learning is seen with other memory enhancing treatments such as administration of histone deacetylase inhibitors [44]. Similarly, generalization of extinction is seen with other methods that enhance memory consolidation, such as mild stress and administration of D-cycloserine [22,23]. VNS can enhance memory consolidation in rats and humans and it also facilitates experience-dependent plasticity [23,24,27,45–54]. We have observed similarities and differences in plasticity related to VNS-enhanced and Extended Extinction [28,30]. For example, VNS administration during extinction training predisposes field potentials in the basolateral complex of the amygdala (BLA) to LTP following high-frequency stimulation of the infralimbic region (IL) of the prefrontal cortex, whereas the same stimulation of the IL did not produce LTP in the BLA of animals given Extended Extinction training with sham stimulation [30]. Follow-up work examined the potential of a VNS-induced effect on meta-plasticity that could set the stage for these alterations in LTP. VNS-induced molecular changes include a reduction in expression of the activity-regulated cytoskeleton-associated protein (Arc) in the BLA [28]. These VNS-induced effects indicate that there can be lasting synaptic and behavioral changes that are not achieved with extra training.

In brain regions involved in fear extinction, administration of VNS alters levels of neuromodulators important for learning and memory. VNS rapidly activates the locus coeruleus [48] and increases levels of norepinephrine in the amygdala [46], and intra-BLA infusions of norepinephrine enhance the consolidation of extinction memory in rats [55]. Similarly, a 2007 study showed that a higher intensity VNS (2 mA) administered in 30 s trains every 5 min for 3 h increased levels of brain-derived neurotrophic factor (BDNF) in the prefrontal cortex [56]. BDNF is important for memory consolidation and fear extinction [57,58]. BDNF engages downstream effectors and pathways related to plasticity, including Arc expression [59], which is altered following VNS-paired extinction [28]. Both chronic (30 s on every 5 min for 14 days) and acute (30 s on every 5 min for 2 h) VNS increase phosphorylation of several sites on the BDNF receptor, tyrosine receptor kinase B (TrkB) [60]. Furthermore, multiple studies have demonstrated that infusion of BDNF into the prefrontal cortex is sufficient to generate extinction of fear, even without training [61,62]. Thus, it is possible that BDNF

contributes to generalization of extinction seen in response to VNS + Extinction, but not Extended Extinction. However, the duration of acute VNS that affected BDNF or its receptor was greater than the duration of stimulation used in the current studies. Furthermore, VNS alone did not promote extinction. Thus, if four 30 s trains of VNS increases BDNF, that BDNF is not sufficient to produce the observed generalization of extinction.

Administration of VNS prior to anxiety testing produced anxiolytic effects. We found that VNS given in the home cage immediately before testing on the EPM increased time spent in the open arms. The anxiolytic effect of VNS cannot explain the generalization of extinction because rats were given equivalent amounts of VNS when fear conditioning occurred on different days and in different contexts, yet VNS-treated rats maintained a fear response to the CS that was not presented during extinction trials. Furthermore, VNS alone was not sufficient to reduce freezing in response to conditioned cues. However, evidence that VNS reduces anxiety indicates promise for VNS as an adjunct to exposure-based therapies, where dropout and non-compliance rates are high [12], and avoidance behavior is a major symptom. The unique combination of memory consolidation enhancing and anxiety reducing effects gives VNS an advantage over currently available pharmacological adjuncts. Though some research involving currently available cognitive enhancers shows promise for use during exposure-based therapies [22], they do not improve tolerability and they may reinforce associations of aversive anxiety responses with reminders of the trauma. In contrast, options like benzodiazepines that reduce anxiety interfere with memory consolidation that is required for extinction of conditioned fear [52,53]. Strategies that can both enhance memory consolidation and reduce anxiety would be ideal because they would enhance the consolidation of extinction memory while improving tolerability of exposure-based therapy. VNS appears to provide this unique combination (Fig. 6).

Here, we found that VNS paired with exposure to a conditioned stimulus led to generalization of extinction of stimuli that co-occurred during AFC, whereas Extended Extinction training did not. VNS effects were not explained by enhanced contextual extinction or a direct effect of VNS on the expression of fear. Co-occurrence of stimuli during AFC was necessary for extinction generalization, indicating that VNS influenced extinction in an experience-dependent manner, only for conditioned cues that were associated with each other in both time and context. We have found that VNS produces a rapid and robust extinction of conditioned fear in rats [28–31]. This extinction enhancement could overcome extinction impairments seen in patients with anxiety-related disorders. VNS could also be effective in increasing tolerability because chronic administration of VNS reduces anxiety in rats [33] and humans [33], and we found here that VNS also has an immediate anxiolytic effect. Additionally, we report here that VNS can lead to generalization of extinction, which may make exposure-based therapies more efficient and prevent cue-induced relapses

elicited by reminders not addressed during exposure-based therapy. These results indicate that VNS, which has been used in tens of thousands of patients with drug-resistant epilepsy [63], has promise as an adjunct treatment to enhance the efficacy and improve the tolerability of exposure-based therapies.

Conflicts of interest

Acknowledgments

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Fig. 6. Dual benefits of VNS. Cognitive enhancers show promise as adjuncts to exposure-based therapies; however, most pharmaceutical enhancers of cognition do not improve tolerability of the therapy by reducing anxiety. Anxiolytic drugs may improve tolerability but they are not effective, possibly because they interfere with the consolidation of new extinction memories. However, VNS enhances memory consolidation and accelerates extinction, and it also reduces anxiety. This unique combination of effects suggests that VNS may offer a desirable alternative to drugs as an adjunct therapy.

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