EFFECTS OF VAGUS NERVE STIMULATION ON EXTINCTION OF CONDITIONED FEAR

by

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I dedicate this work to my mom, Stacey Tiller. Thank you for giving me all the tools needed to accomplish this.

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EFFECTS OF VAGUS NERVE STIMULATION ON EXTINCTION OF CONDITIONED FEAR

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Extinction of conditioned fear is the basis for exposure-based therapies that are used to treat disorders including posttraumatic stress disorder (PTSD), obsessive compulsive disorder (OCD), and specific phobias. Though exposure-based therapies are the best evidence-based treatment for anxiety-related disorders, nonresponse and dropout rates are still as high as fifty percent. Failure during or after treatment could stem from generalized extinction impairments seen in patients with PTSD, OCD, and specific phobias. Adjuvant treatments that could enhance extinction of conditioned fear, and improve treatment tolerability would be useful in improving treatment outcomes for patients with anxiety-related disorders. Vagus nerve stimulation (VNS) has been safely used in tens of thousands of patients for epilepsy and treatment resistant depression since FDA approval in 1997. VNS enhances memory consolidation in rats and in humans, and can also enhance extinction of conditioned fear in healthy rats. Here, we expand findings regarding VNS and fear extinction. We found that VNS can enhance extinction of fear in a rat model of PTSD that is resistant to extinction, as well as reduce the incidence of relapse and reverse PTSD-like symptoms including avoidance and heightened anxiety more than one week following treatment.

We also found that VNS can generate more broad extinction learning by promoting generalization of extinction of conditioned fear. Though VNS is anxiolytic, our findings also indicate that the mechanism by which VNS enhances extinction of conditioned fear is not through a reduction in anxiety. Taken together, our findings indicate that VNS could be a useful adjunct for exposure-based therapies to improve treatment outcomes by enhancing extinction, and improve tolerability by reducing anxiety.

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CHAPTER 1

INTRODUCTION

Extinction learning has been studied since Pavlov's 1927 observations that previously learned associations can be modified with additional training[1]. Extinction of conditioned fear is the process by conditioned fear responses are reduced following repeated exposure to the conditioned stimulus without reinforcement. Extinction is not memory erasure, rather a competing memory trace is created to suppress the fear memory[2-4].

Today, successful extinction learning is the goal of exposure-based therapies that are used to treat anxiety-related disorders such as: PTSD[5-7], OCD[8-10], and specific phobias[11-12]. During exposure-based therapies, patients are exposed to cues that they find threatening in a safe place. If therapy is successful, patients can learn that previously fear-evoking cues are no longer threatening. Although exposure-based therapies are considered the best evidence-based practice for treating anxiety-related disorders [13], non-response and dropout rates are still as high as fifty percent[14]. One potential reason for this resistance to treatment could be the underlying extinction memory impairment seen in patients with disorders such as PTSD[15]. PTSD patients show impairments in extinction of conditioned fear both for cues related to the trauma[16-18], as well as for fears acquired in controlled laboratory studies[15, 19]. Extinction impairments are also seen in disorders such as OCD[20] and specific phobias[21]. These disorders are then treated with repeated opportunities for extinction, which are unsuccessful given the impairments in the ability to extinguish conditioned fears.

Since many individuals with anxiety-related disorders still experience symptoms following treatment, efforts have been made to develop therapies that can be used in conjunction with

exposure-based therapies to increase effectiveness. Considering extinction retention is deficient in many types of anxiety-related disorders and exposure-based therapies rely on extinction learning, pharmacological tools which enhance the consolidation of new memories have been investigated as potential adjuncts to exposure-based therapies. For example, administration of D-Cycloserine (DCS) has been shown to enhance memory consolidation[22], and has even shown promise clinically as it was enhanced extinction of conditioned fear in patients with OCD and social anxiety disorder[23-25]. However, literature on the effectiveness of DCS and other memory enhancing drugs has been mixed[26]. Some studies do not report augmentation of exposure-based therapy following DCS administration[27-28]. Additionally, some clinicians even consider the use of memory enhancing drugs for the treatment of anxiety-related disorders to be risky; these pharmacological manipulations lack temporal specificity and run the risk of enhancing the association of anxiety experienced during therapy rather than enhancing extinction learning[29]. Research has also investigated the use of anxiolytic drugs as adjuncts to exposure based therapies. Many anxiolytic medications impair memory consolidation[30-32], and are thus counterproductive in exposure-based therapies where successful extinction is the goal, as new learning is required for fear extinction[4]. New therapeutic strategies that could enhance extinction learning during exposure-based therapies while allowing temporal precision would be useful in the treatment of anxiety-related disorders with extinction memory deficits. Any extinction-enhancing adjunct treatment could be even more valuable if it had the added benefit of reducing anxiety, which could act to improve tolerability and compliance, thus reducing dropout rates. Vagus nerve stimulation (VNS) has been FDA-approved for safe use in humans since 1997 for the prevention of seizures and since 2005 for treatment-resistant depression. Since approval,

VNS has been used safely in tens of thousands of patients[33]. Stimulations of the vagus can be delivered with temporal precision, and stimulations as short as 0.5 sec are effective across various treatment paradigms. In addition to having clinical utility for epilepsy and depression, VNS also increases experience-dependent plasticity. Pairing VNS with a tone or a motor movement expands stimulus-specific cortical representations in the auditory or motor cortices respectively[34-37]. VNS can also enhance memory consolidation in rats and in humans[38-39]. Consistent with evidence that VNS can accelerate consolidation and promote cortical plasticity, our lab found that VNS accelerates the rate of extinction learning in healthy rats[40], and promotes plasticity in brain circuitry critical for extinction[41].

Based on the exciting findings that VNS can accelerate extinction learning and produce lasting changes in extinction circuitry, the projects in this dissertation aim to elucidate additional VNS effects on extinction, including effects in a rat model of pathological fear. In Chapter 2, we outline the single prolonged stress (SPS) rat model of PTSD. The SPS model was developed by Dr. Isreal Liberzon in 1997, and has since been cited and used in hundreds of research papers[42-43]. In our review, we discuss the numerous ways that SPS-treated rats display similarities to PTSD patients. In addition to displaying PTSD-like symptoms including: heightened anxiety, elevated startle, and social withdrawal, SPS-treated rats show impaired extinction of conditioned fear. We then aimed to assess whether VNS could enhance extinction learning in this model of pathology that shows resistance to extinction, similar to many anxiety-related disorders.

In Chapter 3, we investigate whether or not VNS can accelerate extinction in the SPS rat model of PTSD, where SPS-treated rats show resistance to fear extinction. Based on evidence from our lab that VNS enhances extinction and experience-dependent plasticity[40-41], we hypothesized that

administration of VNS during extinction would accelerate extinction even in a rat model of pathology. We utilized the SPS paradigm and then subjected rats to auditory fear conditioning and extinction training paired with VNS or sham stimulation. Findings indicate that VNS-treated rats show accelerated extinction, even following SPS. Following extinction, we investigated whether VNS can make extinction memories more permanent and reduce relapse. We found that VNS protected against reinstatement in rats subjected to SPS. Additionally, we assessed additional PTSD-like symptoms one week following the completion of extinction training to determine the effects of enhanced extinction on PTSD-like symptoms in SPS-treated rats. We found that VNS treatment during extinction was sufficient to reverse PTSD-like symptoms seen in SPS-treated rats even a week after VNS completion. The findings of Chapter 3 indicate that VNS paired with extinction in SPS-treated rats accelerates the rate of extinction, makes extinction more permanent, and reduces general PTSD-like symptoms one week later.

In Chapter 4, we investigated the ability of VNS to make extinction more broad in healthy rats. Based on evidence indicating tools that enhance memory consolidation could generate generalized extinction[44-45], we hypothesized that VNS could promote generalization of extinction to multiple fear-related stimuli. We examined whether VNS could enhance extinction for multiple stimuli associated with a single fear event. We gave interleaved auditory fear conditioning where two easily discriminable auditory stimuli were paired with a footshock, then we administered VNS- or sham-paired extinction training where only one of the stimuli was presented. We found that pairing VNS during extinction of one CS also led to extinction of the CS not presented during extinction training; sham-paired extinction did not produce generalization of extinction. We also found that these effects on generalization were not due to a

general VNS-induced reduction in freezing, nor due to VNS-enhanced extinction of the context, nor due to counter-conditioning, nor due to VNS-induced reduction in general anxiety. Additionally, effects of VNS on generalization of extinction were attenuated when fear conditioning for each CS was not interleaved. Separating fear conditioning to each stimulus by context or by time prevented VNS-induced generalization of extinction. These findings indicate that VNS can make extinction more broad by promoting generalization of extinction for stimuli that co-occur during fear conditioning.

Given that successful extinction is the goal of exposure-based therapies, and that current pharmaceutical adjuncts fail to provide symptom relief for a large percentage of patients, the development of new adjuvant therapies is necessary. Administration of VNS accelerates extinction to make learning more rapid, more permanent, and more broad. VNS also generates a reduction in PTSD-like symptoms more than one week following the completion of treatment, which could improve tolerability of treatment. All of these results taken together indicate that VNS could be a useful adjuvant treatment for use with exposure-based therapies to treat disorders such as PTSD, OCD, and specific phobias.

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CHAPTER 2

USING THE SINGLE PROLONGED STRESS MODEL TO EXAMINE THE PATHOPHYSIOLOGY OF PTSD

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Abstract

The endurance of memories of emotionally arousing events serves the adaptive role of minimizing future exposure to danger and reinforcing rewarding behaviors. However, following a traumatic event, a subset of individuals suffers from persistent pathological symptoms such as those seen in posttraumatic stress disorder (PTSD). Despite the availability of pharmacological treatments and evidence-based cognitive behavioral therapy, a considerable number of PTSD patients do not respond to the treatment, or show partial remission and relapse of the symptoms. In controlled laboratory studies, PTSD patients show deficient ability to extinguish conditioned fear. Failure to extinguish learned fear could be responsible for the persistence of PTSD symptoms such as elevated anxiety, arousal, and avoidance. It may also explain the high non-response and dropout rates seen during treatment. Animal models are useful for understanding the pathophysiology of the disorder and the development of new treatments. This review examines studies in a rodent model of PTSD with the goal of identifying behavioral and physiological factors that predispose individuals to PTSD symptoms. Single prolonged stress (SPS) is a frequently used rat model of PTSD that involves exposure to several successive stressors. SPS rats show PTSD-like symptoms, including impaired extinction of conditioned fear. Since its development by the Liberzon lab in 1997, the SPS model has been referred to by more than 200 published papers. Here we consider the findings of these studies and unresolved questions that may be investigated using the model.

Introduction

The focus of this *Frontiers in Pharmacology* Research Topic is the neural mechanisms of memory. Memory is a fundamental process in all animals, as it allows survival and success

through learned adaptive behaviors. However, some highly stressful experiences can lead to maladaptive fear, anxiety, and protracted periods of suffering like in Posttraumatic Stress Disorder (PTSD). A hallmark symptom of this condition is re-experiencing the traumatic event, suggesting that the problem lies in the mechanisms controlling storage and expression of the traumatic memories. In this mini-review, we will discuss prospective research studies performed in animals to uncover clues about how traumatic experiences can lead to the pathophysiology of PTSD. We also outline some current limitations, knowledge gaps, and areas that require further investigation.

Single-Prolonged Stress

Single prolonged stress (SPS) is a frequently used rat model of PTSD. Since its initial description 20 years ago[1], the SPS procedure has been referred to by over 200 peer reviewed studies. Although it is called a "single" prolonged stress, the procedure is comprised of successive, multi-modal stressors (Figure 2.1). The prolonged stress begins with a two-hour immobilization period that is immediately followed by a forced-swim experience, lasting twenty minutes, and then a brief loss of consciousness induced by ether exposure. After recovery, rats remain undisturbed for seven days [1]. In some cases, they are socially isolated (individually housed) during this period[2]. When rats undergo auditory or contextual fear conditioning 7 days after this procedure, they demonstrate impaired retention of extinction learning and the conditioned fear response persists longer than it does with fear conditioning alone[2]. This approach can be useful for modeling PTSD-like symptoms because those who experience multiple traumas, or a trauma early in life, are more susceptible to developing PTSD following a later traumatic event[3-5].

Precisely how a previous trauma predisposes individuals to the development of PTSD remains unknown. The first trauma or traumas may simply make an individual more anxious, in general, or more sensitive to future stressors. Alternatively, a previous stressor may set the brain up to acquire, store, or retrieve traumatic memories differently, going forward. Some researchers have hypothesized that an impairment in the recall of fear extinction learning may be an underlying cause of PTSD symptoms[6-7]. The SPS rat model provides an opportunity for testing these hypotheses.

Effects of SPS on Behavior and the Brain

Many findings suggest that SPS produces behavioral and physiological symptoms that are similar to those observed in PTSD[1, 8-10]. Examples of behavioral effects of SPS are illustrated in Figure 2.1. SPS rats demonstrate sleep abnormalities[11] enhanced anxiety[12-13], arousal[14], and fear learning[15-16] as well as impaired spatial and recognition memory, social interaction[8, 17] and fear extinction[2, 16]. Most changes are observed seven days, but not one day, after exposure to the SPS procedure, suggesting that behavioral and cellular changes promoted by SPS are time-dependent[10, 18-19]. Although it has been demonstrated that partial SPS does not generate extinction impairments[20], the critical features of the SPS procedure for development of a PTSD-like phenotype remain unclear. For example, the passage of time alone may be sufficient for an incubation or sensitization effect following the SPS procedure, or a second stressful experience may be necessary to produce cumulative effects on behavior. In Figure 2.1, behavioral effects of SPS are categorized by the time of testing, i.e., whether testing occurred after SPS, SPS + 7 days (with or without social isolation), or SPS + 7 days + an additional stressor. Though there are variations in some SPS procedures (i.e., social isolation versus group housing), many studies

report consistent SPS effects. For example, social isolation during the quiescent period [21] and group housing during the quiescent period[22] both produced an enhancement in contextual fear conditioning following SPS.

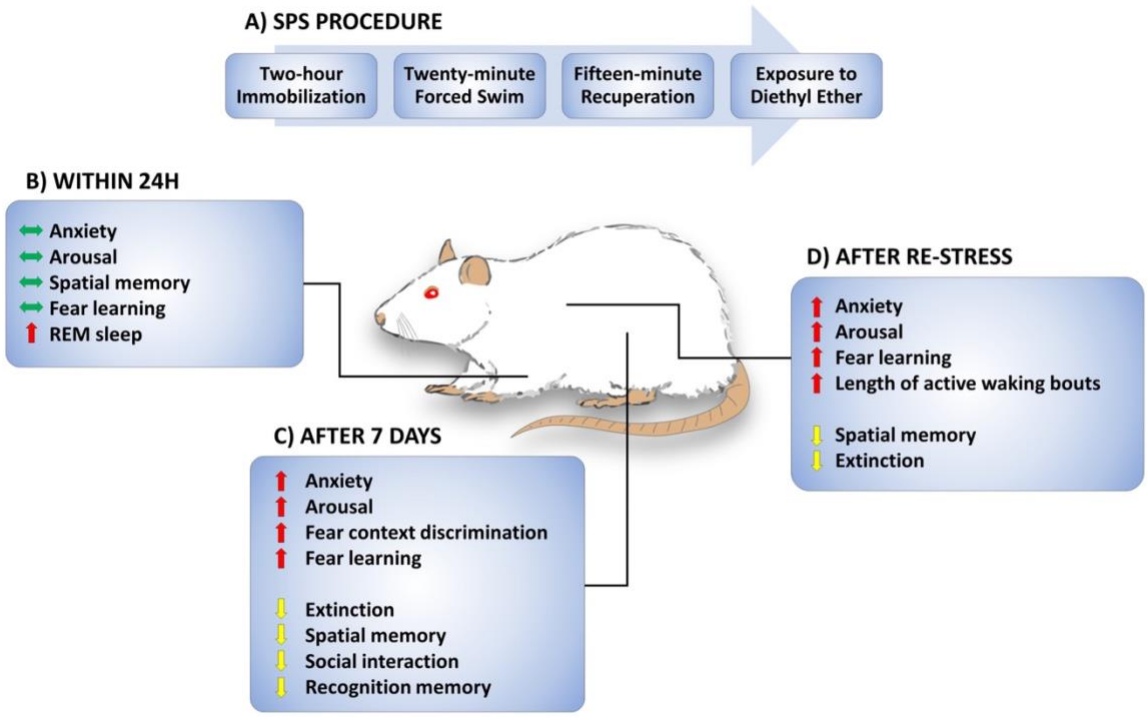

Figure 2.1. Single prolonged stress (SPS) procedure and SPS-induced behavioral changes.

A. Timeline of SPS procedure. Rats are subjected to a two-hour immobilization period, followed immediately by a 20-minute forced swim. Rats are given a brief period of recuperation and then subjected to diethyl ether until anesthetized. B) Behavioral changes one day later. Anxiety, arousal, spatial memory, and fear learning are described to be unchanged, while sleep disturbances are already observed. C) Behavioral changes one week later. Anxiety, arousal, fear context discrimination and fear learning are increased. On the other hand, extinction, spatial memory, social interaction, and recognition memory are impaired following the full length of SPS. D) Behavioral changes in SPS rats following re-stress. Increased anxiety, arousal, fear learning and abnormal active waking bouts is described. Extinction and spatial memory are impaired following re-stress. Green, red and yellow arrows mean *no change*, *increase* and *decrease*, respectively.

Impaired Extinction of Conditioned Fear

One explanation for the persistence of fear, anxiety, avoidance, and re-experiencing symptoms in PTSD is that some individuals have strong traumatic memories that are less susceptible to extinction. Indeed, some studies of PTSD patients show enhanced conditioned fear[23-25], and several animal studies demonstrate an enhancement in contextual fear conditioning following SPS[8, 15-16]. However, others have reported extinction impairments despite normal acquisition of conditioned fear[7, 10-11, 26-27]. Using skin conductance responses as a measure of conditioned fear, Milad and colleagues found that PTSD patients showed normal fear conditioning and within-session extinction, but poor retention of extinction on later tests[6-7]. In rats, prior exposure to the SPS procedure impaired extinction of both cued[2, 16, 28] and contextual fear conditioning[2, 29-30], whereas acquisition of conditioned fear and extinction within a session were not affected[2, 20]. Given the evidence that within-session conditioning and retrieval are normal, these findings suggest that consolidation of the extinction memory is impaired in human PTSD patients and in SPS rats. Neurobiological changes that could contribute to impairments in behavior and fear extinction are discussed below (Table 2.1).

Hippocampus

The hippocampus plays a role in storing fear memories and in mediating stress responses[31-32]. Not surprisingly, the hippocampus is highly sensitive to chronic stress[32]. This is confirmed by functional magnetic resonance imaging (fMRI) studies demonstrating that PTSD patients have a smaller hippocampal volume than healthy controls[33-34], although some research suggests that a lower hippocampal volume may represent a risk factor for PTSD[35]. These findings indicate that reduced hippocampal function might be associated with resistant memory impairments in PTSD.

Table 2.1. Cellular changes in three key areas controlling memory and emotionality after single prolonged stress (SPS) model of PTSD.

Different from behavioral changes, expression of receptors and other proteins, as well as neurotransmitters, are observed within hours after SPS. Mechanisms controlling neuroendocrine responses, memory and emotion show a full profile of disruption after 7 days of incubation or after subsequent stress experience. CaM = calmodulin, CaMKII = Ca2+/calmodulin-dependent protein kinase II; CB1 = cannabinoid receptor 1; pERK = phosphorylated extracellular signal-regulated kinase; BDNF = brain-derived neurotrophic factor; NPY = Neuropeptide Y; LTP/LTD = long-term potentiation/depression; NMDA = ionotropic glutamate receptor; IL-6 = Interleukin 6; pPKB = phosphorylated protein kinase B; TrkB = tyrosine receptor kinase B; 5-HT2C receptor = serotonin receptor. * = measured 1h after SPS; ** One study reported decrease.

HIPPOCAMPUS	**EFFECT**	**REFERENCES**
WITHIN 24h	⬆ Apoptosis	(Li et al., 2010; Liu et al., 2010; Han et al., 2013)
	⬇ Glucocorticoid receptor	(Liberzon et al., 1999a; Zhe et al., 2008)
	⬇ Mineralocorticoid receptor	(Liberzon et al., 1999a; Zhe et al., 2008)
	⬇ LTP/LTD*	(Kohda et al., 2007)
AFTER 7 DAYS	⬆ Apoptosis	(Li et al., 2010)
	⬆ Glucocorticoid receptor**	(Zhe et al., 2008; Knox et al., 2012b; Eagle et al., 2013)
	⬆ CB1 receptor	(Zer-Aviv and Akirav, 2016)
	⬆ NMDA receptor	(Yamamoto et al., 2008)
	⬆ Oxytocin receptor binding	(Liberzon and Young, 1997)
	⬆ IL-6	(Liu et al., 2016)
	⬇ Glucocorticoid receptor	(Ganon-Elazar and Akirav, 2013)

	⬇ Mineralocorticoid receptor	(Zhe et al., 2008)
	⬇ LTP	(Kohda et al., 2007)
AFTER RE-STRESS	⬆ BDNF	(Takei et al., 2011)
	⬆ TrkB	(Takei et al., 2011)
	⬆ Autophagy	(Wan et al., 2016)
	⬆ Protein kinase M zeta	(Ji et al., 2014)
	⬆ Apoptosis	(Wang et al., 2012)
	⬆ Glucocorticoid receptor	(George et al., 2015)
	⬆ Glycine transporter	(Iwamoto et al., 2007)
	⬆ Muscarinic receptor	(Brand et al., 2008)
AMYGDALA	**EFFECT**	**REFERENCES**
WITHIN 24h	⬆ Apoptosis	(Xiao et al., 2015)
	⬆ pERK	(Liu et al., 2010)
	⬆ CaM	(Xiao et al., 2009)
	⬇ Glucocorticoid receptor	(Han et al., 2014)
	⬇ Mineralocorticoid receptor	(Han et al., 2014)
AFTER 7 DAYS	⬆ Glucocorticoid receptor	(Ganon-Elazar and Akirav, 2013)
	⬆ CaM	(Xiao et al., 2009)
	⬆ Oxytocin	(Liberzon and Young, 1997)
	⬆ 5-HT2C receptor	(Harada et al., 2008)

	⬆ Apoptosis	(Liu et al., 2010)
	⬆ pERK	(Liu et al., 2010)
	⬆ CB1 receptor	(Zer-Aviv and Akirav, 2016)
	⬆ Neuropeptide Y	(Cui et al., 2008)
	⬇ CaMKII	(Xiao et al., 2009)
AFTER RE-STRESS	⬆ Norepinephrine	(Lin et al., 2016a)
	⬆ CB1 receptor	(Zer-Aviv and Akirav, 2016)
	⬇ Dopamine	(Lin et al., 2016a)
PREFRONTAL CORTEX	**EFFECT**	**REF**
WITHIN 24h	⬆ Mineralocorticoid receptor	(Zhang et al., 2012)
	⬆ CaM	(Wen et al., 2012)
	⬆ Apoptosis	(Li et al., 2013)
	⬆ Caspases	(Zhang et al., 2016)
AFTER 7 DAYS	⬆ Caspases	(Wen et al., 2012)
	⬆ Glucocorticoid receptor	(Knox et al., 2012b; Ganon-Elazar and Akirav, 2013)
	⬆ pERK	(Wen et al., 2016; Wen et al., 2017)
	⬆ CaM	(Wen et al., 2012)
	⬆ Apoptosis	(Wen et al., 2016; Wen et al., 2017)

	⬇ Mineralocorticoid receptor	(Zhang et al., 2012)
	⬇ CaMKII	(Wen et al., 2012)
	⬇ Glutamate	(Knox et al., 2010; Perrine et al., 2016)
AFTER RESTRESS	⬆ Glucocorticoid receptor	(Ganon-Elazar and Akirav, 2013; George et al., 2015)
	⬆ CB1 receptor	(Zer-Aviv and Akirav, 2016)
	⬇ CaMKII	(Wen et al., 2012)
	⬇ Norepinephrine efflux	(Lin et al., 2016b)
	⬇ Dopamine efflux	(Lin et al., 2016b)
	⬇ Glutamate	(Perrine et al., 2016)

To our knowledge, no studies have examined the effect of the SPS procedure on hippocampal volume, however, the hippocampus has been the subject of many investigations. Enhanced apoptosis, a phenomenon involved in programed cell death that results in morphological changes, is observed in the hippocampus shortly after SPS, and persists after the undisturbed phase, and after a subsequent stressor[36-39]. Re-stress after SPS also enhances autophagosomes and autophagy-related markers[40]. Likewise, studies using the SPS model show evidence of enhanced oxidative stress and inflammation [41]. For example, IL-6, malondialdehyde, NOX2, and 4-hydroxynonenal contribute to apoptotic cell death in the hippocampus following SPS[13, 36, 38-39]. Balance and expression of GR and MR receptors is disrupted in the hippocampus of SPS rats. Thus, while decreased expression of GR and MR is observed shortly after SPS[42-43], increased

expression of these receptors is observed after a week or after re-stress[20, 28, 43-44]. Synaptic plasticity-related mechanisms are also influenced by SPS. Both LTP and LTD are decreased after SPS[8], while NMDA receptor expression is enhanced[29]. In a recent study using c-Fos expression, Knox and colleagues found that SPS disrupted the inhibition of ventral hippocampal activity during extinction retrieval as well as the functional connectivity within the dorsal hippocampus during extinction learning[10].

Amygdala

The amygdala is also involved in the control of fearful states and learning of emotional experiences. Imaging studies have revealed that PTSD patients show exaggerated amygdala activity in response to trauma-related cues or unrelated arousing stimuli and during new fear learning[45-47], supporting the notion that enhanced amygdala activity could be involved in impaired extinction learning or generalization of fear responses.

Studies using the SPS model demonstrate changes in the amygdala starting a day after the procedure (Table 2.1). Increased apoptosis and downstream signals, like phosphorylated extracellular signal–regulated kinases, glucose-regulated protein 78 (GRP78) and caspase 3, 9 and 12 expression were observed in the amygdala one day after SPS, and some reached peak levels 7 days later[37, 48-49], suggesting that SPS-induced morphological and connectivity changes may precede the behavioral and memory deficits observed after the 7-day period. Potentiated-fear learning following SPS was paralleled by an early decrease in GR and MR receptors in the amygdala, as well as by blunted LTP and decreased colocalization of GR and MR receptors one week later[8, 12]. Intracellular calcium levels are changed shortly after SPS and the effect persists for one week[50]. Acute changes in calmodulin (CaM) and calcium-CaM kinase II (CaMKII), two

messengers involved in Ca^{2+} homeostasis and signaling processes related to learning and memory, were up- and downregulated, respectively, within one day of SPS[50], indicating that SPS disrupts fundamental mechanisms of cell signaling, which may lead to amygdala hyperactivity, enhanced fear expression and impaired extinction of conditioned fear.

Prefrontal cortex

Inhibition of amygdala hyperactivity and cognitive flexibility are important prefrontal cortex functions that are implicated in PTSD susceptibility and symptoms[51-53]. This notion is supported by functional imaging studies showing a reduced activity of the medial prefrontal cortex and anterior cingulate cortex in PTSD patients during presentation of trauma-related and non-related aversive stimuli[52-54]. Moreover, the volume of the ventromedial prefrontal cortex and the anterior cingulate cortex is reduced in individuals with PTSD[51, 55-56]. Abnormal morphological changes in the pathway from the anterior cingulate cortex to the amygdala was also found in PTSD patients[57], suggesting that a series of changes in the normal control of the fearful states or behavioral flexibility by the frontal cortex may be involved in the pathophysiology of PTSD. Evidence for similar changes in the prefrontal cortex of rats submitted to the SPS model remains sparse. As in the hippocampus and amygdala, neuronal apoptosis and dysregulation of autophagic activity in the prefrontal cortex appears one day after SPS[17, 58-59]. Elevated levels of protein kinase RNA-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1) in the endoplasmic reticulum (ER), glucose-regulated protein (GRP) 94 and apoptosis-related caspase-12 are involved in the persistent apoptotic profile seen one week after SPS[58, 60-62]. Unbalanced control of calcium indicates that intracellular messengers controlling neuronal excitability are disrupted following SPS[62]. This is

corroborated by studies showing decreased levels of glutamate in the prefrontal cortex one week after SPS or re-stress[63-64]. The concentration of MRs is elevated one day after SPS[65], while GR expression is enhanced one week later and after re-stress[20, 28, 66], indicating temporally distinct disturbances in stress-related systems.

Decreased volume and integrity of prefrontal sub-regions have been reported in PTSD patients[67-68]. Similarly, SPS disrupts normal activity of the infralimbic region of the medial prefrontal cortex before re-stress[10], suggesting that SPS could predispose the prefrontal cortex to dysfunctional activity during fear learning and/or subsequent extinction trials. However, since different regions of the prefrontal cortex control distinct aspects of fear learning and extinction, additional studies are needed for a better understanding about changes that can be predisposing factors or consequences of the trauma.

Effects of SPS on HPA-axis

Early research on the pathophysiology of PTSD identified a decrease in cortisol levels[69]. Later studies demonstrated that administration of low doses of dexamethasone produced suppression of plasma cortisol, indicating that the hypothalamus-pituitary-adrenal cortex (HPA) axis may become sensitive to negative feedback in PTSD patients[70]. Similarly, enhanced suppression of the HPA-axis is observed in rats 7 days after SPS[1, 18]. The data currently available suggest that the enhanced glucocorticoid negative feedback observed in SPS may be linked to overexpression of GR and a reduced expression of MR in key areas mediating activity of the HPA-axis during stress[18, 43-44].

Changes in the HPA-axis may contribute to PTSD symptoms by interfering with extinction of conditioned fear. For example, exogenous administration of stress-levels of cortisol can impair

the retrieval of long-term memories[71], but the same treatment enhances consolidation of new memories[72-73]. These findings suggest that SPS-induced enhanced suppression of the HPA-axis may have the opposite effect, perpetuating the fear memory by facilitating retrieval of the traumatic memory and impairing consolidation of extinction memory[74]. However, a few studies have dissociated GR upregulation and extinction impairments in the SPS model. A significant increase in GR expression was observed in the hippocampus and prefrontal cortex 7 days after partial SPS (e.g. forced swimming and ether exposure) that did not impair extinction of conditioned fear[20]. These results indicate that glucocorticoid receptor expression must reach a threshold in order to interfere with the consolidation of extinction, or, there is another SPS-related change that influences the extinction of conditioned fear. Consistent with the view that enhanced suppression of the HPA-axis and the resulting decrease in circulating glucocorticoids predisposes animals to the PTSD phenotype, Keller and colleagues[16] found that inhibition of corticosterone synthesis prior to fear conditioning exacerbated the extinction impairment in SPS rats.

Taken together, these findings indicate that the SPS model is a useful tool for studying the role of the HPA-axis in PTSD. Future studies should examine the full extent of HPA-axis changes, including the evaluation of SPS effects on circulating glucocorticoid levels. Further studies may be designed to determine whether HPA-axis dysfunction is a predisposing factor or a consequence of traumatic experience.

Limitations

Many PTSD-like effects have been identified in rats exposed to SPS. However, seemingly subtle deviations in the procedure may have significant consequences on behavior and physiology[20]. In

this review, we have sorted the behavioral and physiological consequences of SPS by the time of testing. Some effects are transient, and some emerge after 7 days or a re-stress experience, suggesting that the effects of SPS are time- and experience-dependent. Variations on SPS parameters can be utilized to identify factors producing maladaptive fear and arousal states. Future studies are needed to determine the relative contributions of the passage of time and stress experience to these SPS-related changes.

Here, we also describe evidence that extinction impairments are a common feature of PTSD and the rat SPS model of PTSD. A major caveat is that human females are two times more likely to develop PTSD following a traumatic event[75], yet SPS-induced deficits in extinction are only seen in male rats. In one study that investigated sex differences in the SPS model, Keller et al[76] demonstrated that SPS affects GR expression in the dorsal hippocampus in females, but extinction retention deficits were observed only in males, suggesting that female rats are more resilient to the memory extinction effects of SPS. Such differences may be indicative of a sexually divergent response to conditioned fear. Emerging evidence indicates that female rats express fear by darting rather than freezing[77], indicating that reliance on freezing as a single measure of fear may be misleading.

Conclusions

Although we have focused on factors contributing to extinction impairments, the SPS model can be used to investigate hypotheses about the biological causes of other debilitating symptoms such as social withdrawal, heightened anxiety, elevated startle response, hypervigilance, and sleep disturbances. Though the SPS model is a useful tool to study the PTSD symptomatology, additional studies are needed to examine sex differences, the timing of onset and persistence of

symptoms, as well as the features of the SPS procedure that are necessary for the development of PTSD-like symptoms. Given the understanding that all models have limitations, it is encouraging to note that several other animal models demonstrate extinction impairments and PTSD-like symptoms[78-82]. Utilization of multiple animal models of PTSD and meticulous examination of PTSD-like symptoms will be critical to unfold the pathophysiology of PTSD, and lead to novel and efficient therapeutic strategies.

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CHAPTER 3

EFFECTS OF VAGUS NERVE STIMULATION ON EXTINCTION OF CONDITIONED FEAR AND POSTTRAUMATIC STRESS DISORDER SYMPTOMS

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Abstract

Exposure-based therapies help patients with posttraumatic stress disorder (PTSD) to extinguish conditioned fear of trauma reminders. However, controlled laboratory studies indicate that PTSD patients do not extinguish conditioned fear as well as healthy controls, and exposure therapy has high failure and dropout rates. The present study examined whether vagus nerve stimulation (VNS) augments extinction of conditioned fear and attenuates PTSD-like symptoms in an animal model of PTSD. To model PTSD, rats were subjected to a single prolonged stress (SPS) protocol, which consisted of restraint, forced swim, loss of consciousness, and one week of social isolation. Like PTSD patients, rats subjected to SPS show impaired extinction of conditioned fear. The SPS procedure was followed, one week later, by auditory fear conditioning (AFC) and extinction. VNS or sham stimulation was administered during half of the extinction days, and was paired with presentations of the conditioned stimulus (CS). One week after completion of extinction training, rats were given a battery of behavioral tests to assess anxiety, arousal, and avoidance. Results indicated that rats given SPS one week prior to AFC (PTSD model) failed to extinguish the freezing response after eleven consecutive days of extinction. Administration of VNS reversed the extinction impairment and attenuated reinstatement of the conditioned fear response. Delivery of VNS during extinction also eliminated the PTSD-like symptoms, such as anxiety, hyperarousal, and social avoidance for more than one week after VNS treatment. These results provide evidence that extinction paired with VNS treatment can lead to remission of fear and improvements in PTSD-like symptoms. Taken together, these findings suggest that VNS may be an effective adjunct to exposure therapy for the treatment of PTSD.

Introduction

Posttraumatic stress disorder (PTSD) affects 22.4 million Americans and can develop following highly stressful experiences such as combat or sexual assault[1]. Although most individuals who have traumatic experiences exhibit transient symptoms of stress, approximately 30% of these individuals suffer from symptoms for longer than one month and meet the criteria for diagnosis of PTSD[1]. According to the fifth edition of the Diagnostic and Statistical Manual (DSM-5) an individual may be diagnosed with PTSD after experiencing or witnessing trauma in addition to presenting the following symptoms: re-experiencing the trauma (i.e., experiencing emotional or physical distress in response to reminders of the trauma); avoidance of trauma-related stimuli; negative affect, including loss of interest in enjoyable activities; and heightened startle and arousal. Symptoms must persist for more than one month and cause significant social or occupational dysfunction[2]. The prevalence of PTSD is greater in individuals who have experienced multiple traumatic events, suggesting that earlier stressors predispose individuals to the development of PTSD following a traumatic event later in life[3-5].

Exposure-based therapies are considered the gold standard of treatment for PTSD[6]. The goal of exposure-based therapies is to replace conditioned associations of the trauma with new, more appropriate associations. These therapies are based on Pavlov's observations that learned associations can be modified with extinction training[7]. Despite their demonstrated therapeutic efficacy, exposure based therapies for PTSD have high nonresponse and dropout rates[8-10]. PTSD patients appear to be resistant to exposure-based therapies because of a generalized extinction deficit[11-14]. Further, PTSD patients are impaired in their ability to extinguish conditioned fears

that are acquired in controlled laboratory studies[12, 15-16]. Adjuvant treatments that improve the consolidation of extinction learning may improve the effectiveness of exposure-based therapies. Vagus nerve stimulation (VNS) was approved by the Federal Food and Drug Administration for the prevention of seizures in patients with drug-resistant epilepsy in 1997. Considering that VNS can enhance memory consolidation in rats and humans[17-18], we hypothesized that administration of VNS during extinction training could enhance consolidation of an extinction memory. We recently reported that VNS enhanced consolidation of fear extinction following auditory fear conditioning (AFC) and promoted synaptic plasticity in the brain circuitry underlying extinction memory[19-20]. These findings suggest that VNS might also be effective in enhancing extinction memory in a rat model of PTSD. To investigate this possibility, we used the single prolonged stress (SPS) procedure in rats, which models successive traumatic events and increases susceptibility for PTSD-like symptoms following fear conditioning[21]. Like PTSD patients, rats subjected to SPS and fear conditioning exhibit impaired extinction of conditioned fear[21-22]; this impairment is specifically seen during consolidation of extinction[23]. Rats subjected to SPS show behaviors that can be compared to PTSD symptoms including: re-experiencing the trauma, elevated anxiety, arousal, and avoidance[21-24]. Here, we investigated the effects of pairing VNS with exposure to the conditioned stimulus (CS) during extinction on the conditioned fear response and on other PTSD-like symptoms in rats subjected to SPS. The findings suggest that VNS enhances extinction and attenuates reinstatement of fear. Furthermore, VNS administration during extinction is associated with a reduction in PTSD-like symptoms one week later.

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. Male Sprague-Dawley rats (Taconic, Hudson, NY) weighing 225-250 g on arrival were housed on a 12 h light/dark cycle (lights on at 7:00 am) with access to food and water *ad libitum*. Only male rats were used as previous results indicate that female rats are not susceptible to the extinction impairment produced by SPS [24]. Criteria for exclusion of rats from the analysis was performance $\geq$ 2 standard deviations away from the mean on any task.

To investigate the effects of VNS on extinction and reinstatement of conditioned fear, twenty-two rats were subjected to the SPS procedure (see "Rat model of PTSD" subheading below)[21], followed one week later by AFC. Rats subjected to the SPS procedure and AFC were referred to as "PTSD model" rats. Extinction training began 24 hours after AFC. The full course of extinction consisted of 11 days of exposure to the CS without reinforcement. During extinction, odd numbered days (Extinction Days 1, 3, 5, 7, 9, and 11) were used as tests of conditioned fear; four presentations of the CS were administered and conditioned fear to the CS was measured. On even numbered days (Extinction Days 2, 4, 6, 8, and 10), VNS or sham stimulation was administered and temporally paired with the four CS presentations. Fourteen of the PTSD model rats were given VNS during extinction, and 8 were given sham stimulation (Figure 3.1a). Twenty-four rats were given the same AFC and extinction without the SPS procedure. These rats were referred to as "AFC alone" rats. Of these, 14 were given VNS during extinction, and 8 were given sham stimulation during extinction (Figure 3.1b). Twenty-four hours after Extinction Day

11, rats were given a single reminder footshock. Reinstatement was tested the following day by measuring freezing in response to the conditioned cue.

To test the effects of VNS on PTSD-like symptoms, separate rats were given AFC with SPS (PTSD model rats n=16) or without SPS (AFC alone rats n=16) and exposed to the same 11 days of extinction. Eight rats from each group were given VNS during extinction and 8 were given sham stimulation. Seven to ten days after completion of all 11 Extinction Days, rats were tested on a battery of behavioral tests to examine generalized anxiety, arousal, avoidance, and social interaction (Figure 3.1). The order of test administration was counterbalanced to control for potential interactions. These rats were not given a reminder shock or reinstatement test.

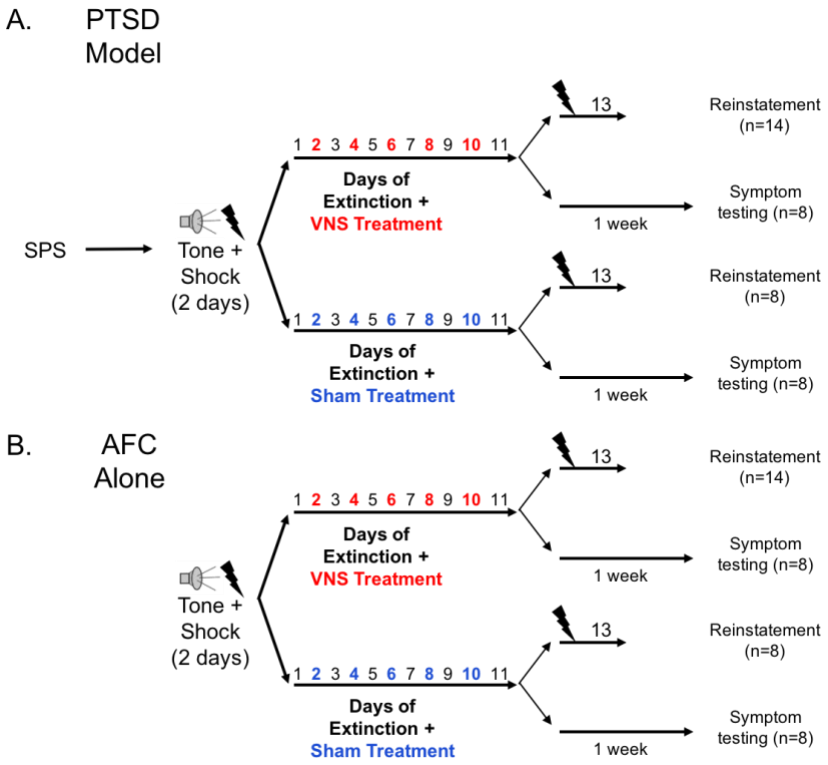

Figure 3.1. Timeline for extinction, reinstatement, and behavioral assays.

A. Protocol for PTSD model rats. Rats underwent the SPS procedure followed by two days of AFC and 11 days of extinction, 5 were paired with VNS (red) or sham stimulation (blue) on alternate (even) days. Freezing in the presence of the CS was used as a measure of conditioned fear. Conditioned fear was measured on the odd days that fell between VNS or sham stimulation days. Following extinction, some rats underwent a reinstatement trial where they received one unsignaled footshock. The day after the footshock, these rats were given another day of extinction to measure conditioned fear. The remaining rats were tested on a battery of behaviors to measure PTSD symptoms one week after the end of extinction. The order of the tests was counterbalanced. B. Protocol for AFC alone rats. Rats in the AFC alone group underwent the same protocol for AFC and extinction, however these rats were not subjected to the SPS procedure.

Cuff electrode preparation

Cuff electrodes were prepared as previously described[25]. In brief, platinum-iridium wire electrodes were affixed to biocompatible micro-renathane cuffs (1.25mm inner diameter, 2.5mm outer diameter, 4.0mm long). Omnetics four-pin connectors were used to connect the VNS cuff to an AM Systems stimulator. Two of the connector pins made contact with the platinum-iridium wires in the cuff in order to deliver stimulation to the vagus nerve.

Surgical implantation of cuff electrode

Surgery protocols are described in detail elsewhere[19-20, 25]. 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 stimulation effects on the sinoatrial node. Central activation from the left vagus nerve is bilateral[26]. 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 (0.8mA, 1 second) was

applied through the cuff to test for cessation of breathing. If cessation of breathing was not observed, the cuff was adjusted or replaced. For sham rats, surgery was conducted in the same manner to isolate the vagus nerve, but the rats were not implanted with a cuff. Animals were given one week to recover following surgery.

Rat model of PTSD

Procedures for SPS were adapted from methods developed by Liberzon and colleagues[21]. In brief, rats were restrained for two hours in a plastic cone. Immediately after restraint, rats were forced to swim in a tank of water (22.0-inch diameter, 20°C) for 20 minutes. Following a 15-minute recuperation period, rats were placed in a desiccator and exposed to diethyl ether vapor (Sigma) until they became anesthetized and unresponsive. They were immediately returned to their home cages and left socially isolated for one week.

Auditory fear conditioning (AFC)

On the first day of AFC, rats were exposed to 4 pre-tones (four 9kHz tones, lasting 30 seconds, at an intensity of 70dB, administered without any reinforcement) to assess baseline freezing to the tone. Immediately after the pre-tones, rats received 8 tones coupled with a footshock (1 second, 0.4mA). The tones were presented at a random inter-stimulus interval (ISI) of between 120 and 240 seconds. Each shock was administered at a randomized time during the last 25 seconds of the 30 second tone presentation. Twenty-four hours later, rats underwent a second day of AFC consisting of eight more tone-shock pairings administered in the same way as the previous day, excluding the pre-tones. All AFC took place in Context A (electric grid floor, no olfactory cue). To compare acquisition of conditioned fear between AFC alone rats and PTSD model rats, sessions were recorded and scored by two researchers blind to the treatment

conditions. Freezing during the tone, defined as the cessation of movement aside from breathing[27], was used as a measure of conditioned fear. We chose this AFC protocol to compare findings in the PTSD model to what we have observed in normal rats using a similar protocol. In this previous study, we found that 8 tone-shock pairings per day for 2 days, with unpredictable shock-timing during the 30 second tone produced conditioned fear that was not fully extinguished after 11 days of extinction in sham-treated controls[19].

Extinction Days

Twenty-four hours after both days of AFC, rats underwent 11 days of extinction in Context B. Context B consisted of the same conditioning chamber, but contained a distinct Plexiglas insert to change the texture of the floor and the addition of an odor (peppermint oil). Each day of extinction consisted of four presentations of the CS (tone) in the absence of any reinforcement (shock). Based on evidence that VNS can enhance memory consolidation, extinction was carried out over several days to allow for consolidation of the extinction memory, based on evidence that VNS can enhance memory consolidation. This extended protocol for extinction more closely resembles a clinical timeline for individuals with PTSD who would undergo multiple days of exposure-based therapies. Two observers who were blind to the treatment groups measured the percent of time spent freezing during each 30-second tone, which was recorded as the measure of conditioned fear. Freezing times below 10 percent of the total 120 seconds of tone exposure was considered remission of fear[19]. During extinction, rats initially respond to being connected to the stimulator, this occurs in both sham-treated and VNS-treated rats. This response is typically present only during the first extinction session. To avoid potential performance effects of VNS or sham stimulation, conditioned fear responses were measured only on alternate, non-stimulation

days (Extinction Days 1, 3, 5, 7, 9, and 11) when the rats were not connected to the stimulator (Figure 3.1). Although we have not systematically measured unintended effects of VNS, we have noticed that both sham- and VNS-treated rats occasionally attend to the connector after it is attached to the headcap. This variable behavior could interfere with the conditioned response. Additionally, measuring conditioned fear on days when rats were not receiving VNS or sham stimulation made it less likely that interoceptive state-dependent effects of VNS could serve as a safety signal, and provided an opportunity to observe the effect of VNS on consolidation of extinction memory.

Vagus nerve stimulation (VNS) and sham stimulation

Treatment with VNS or sham stimulation was given during extinction on even numbered days (Extinction Days 2, 4, 6, 8, and 10). To administer stimulation, an AM systems stimulator was connected to the cuff connector on the headcap via a 25.0cm long PVS multiconductor cable (Cooner Wire). Stimulation started 150.0ms before the onset of each tone and then continued for the duration of the tone. VNS was given at a frequency of 20Hz, an intensity of 0.4mA for 30 seconds with a 100μs pulse width. Sham-treated rats were connected to the stimulator in the same way as VNS-treated rats, but did not receive stimulation.

Reinstatement

Following the completion of extinction, reinstatement of conditioned fear was tested. Twenty-four hours after the 11th day of extinction, rats (N=44) were placed in Context A and given one unsignaled footshock (unconditioned stimulus) delivered for one second at 0.4 mA intensity, in the absence of the tone (CS). Rats remained in Context A for five minutes after the footshock. To observe the reaction to the reinstatement shock, sessions were recorded and scored by two

researchers blind to the treatment conditions. Freezing was recorded during the entire 5-minute observation period. Twenty-four hours after administration of the unconditioned stimulus, rats were exposed to the CS in Context B, to test for reinstatement of fear.

Elevated Plus Maze (EPM)

To test generalized anxiety, rats were placed on the central part of an elevated plus-shaped maze (10.0cm wide, 50.0cm long, 55.0cm off the floor) with walls (30.0cm tall) on two opposing arms and no walls on the other opposing arms. During a ten-minute test, time spent in the open arms, time spent in the closed arms, and time spent in the center of the maze were recorded. Rats were considered to be in an arm when all four paws were in that arm at one time. All behavior was recorded and scored by two blind researchers. Time spent in the open arms and entries into the open arms were taken as a measure of risk taking[28]. Percent of total time spent moving was taken as a control measure of general locomotion.

Acoustic Startle Response

Before startle behaviors were measured, rats were placed into the apparatus for 5 minutes to habituate them to the cage. On the following day, rats were placed into the same 20x20x20 cm^3 wire-mesh cage centered on a startle platform (Lafayette Instrument Co.) that used a piezoelectric transducer to generate a continuous record of the rat's activity. Startle responses were elicited by 50.0ms bursts of white noise at 95dB sound pressure level. Each rat was subjected to 20 presentations of the startle stimulus with an ISI of 180 seconds. The waveform of each response served as the measure of the startle response.

Marble Burying

To test novel object avoidance, rats underwent a marble burying task. Rats exposed to a noxious object in their homecage will vigorously bury that object, a phenomenon known as defensive burying[29]. This avoidance behavior can be seen in rats following fear conditioning with non-salient, novel objects[30]. This defensive burying of novel objects is sensitive to anxiolytic treatments and is used to measure anxiety and avoidance behavior[31]. Following habituation to the novel bedding, rats were individually placed into a cage that was identical to their homecage with BioFresh nitrocellulose comfort bedding (3.0cm deep). Fifteen identical, shiny marbles were placed in three rows in the rear third of the cage. After 10 minutes, the number of marbles buried was counted and recorded as a measure of novel stimulus avoidance. Only marbles that were more than 2/3 covered by bedding were considered buried. Percent of marbles buried = (number marbles buried/number of marbles present) * 100.

Social Interaction

A three-chamber social interaction task was used to assess social behaviors. The apparatus consisted of three equal-sized chambers: the nonsocial zone, the social zone, and the center. The nonsocial zone contained a small wire cage that was empty and sealed; the social zone contained an identical wire cage with a stimulus rat inside. The stimulus rat was matched in size and sex to the experimental rat. The experimental rat was placed into the center of the apparatus, facing the nonsocial zone, and allowed to explore for ten minutes. Interactions of the experimental rat with the stimulus rat, time spent in the nonsocial zone, time spent in the social zone, and time spent in the center were recorded. A rat was considered to be in a zone of the apparatus only when all four paws were in that zone at once. All behavior was recorded and scored by two experimenters

who were blind to treatment conditions. The sociability index (time spent in the social zone – time spent in the nonsocial zone) / (time spent in the social zone + time spent in the nonsocial zone) was used to indicate a preference to interact with or avoid the stimulus rat.

Data analysis

Data were analyzed using a two-way repeated measures ANOVA or a one-way ANOVA, with a Greenhouse-Geisser correction followed by a Tukey's post hoc test for multiple comparisons or a Holm-Bonferroni sequential correction test for non-independent samples. Statistically significant effects were defined as those with p values that were <0.05. All error bars represent standard error of the mean.

Results

VNS administration during exposure to the conditioned stimulus enhanced extinction and reduced reinstatement of conditioned fear.

We modeled PTSD by combining SPS with AFC one week later. A two-factor repeated measures ANOVA indicated a significant effect of group across days ($F(18,246)= 4.764$ $p<0.0001$). Although animals with and without SPS exposure demonstrated comparable levels of conditioned fear following AFC, SPS treatment resulted in significantly higher levels of freezing in response to the CS after eleven consecutive days of extinction (Figure 3.2a, sham). Without VNS, the PTSD model rats did not reach remission of conditioned fear (<10% freezing to the CS). Administration of VNS treatment during five out of the eleven Extinction Days led to remission of CS-evoked freezing behavior in PTSD model rats (Figure 3.2a, VNS). By Extinction Day 5, PTSD model rats given VNS showed decreased freezing versus rats given sham stimulation ($p<0.01$). This effect continued until the completion of treatment: Extinction

Day 7 ($p<0.0001$); Extinction Day 9 ($p<0.0001$); Extinction Day 11 ($p<0.0001$). This supports the hypothesis that VNS treatment can enhance extinction of conditioned fear in an animal model of PTSD.

A single reminder of the unconditioned stimulus (reinstatement footshock on Day 12) was sufficient to increase freezing to the CS when presented 24 hours later. In PTSD model rats given sham stimulation, the level of conditioned fear returned to that observed before any extinction; freezing behavior on Extinction Day 13 was not significantly different from Extinction Day 1 ($p>0.05$). This result indicates that a single stressor is sufficient to restore strong fear behavior in PTSD model rats despite a long period of extinction. The addition of VNS during extinction prevented the reinstatement of conditioned fear observed in PTSD model rats. Freezing behavior in PTSD model rats given VNS was dramatically reduced on Extinction Day 13 compared to Extinction Day 1 ($p<0.00001$) (Figure 3.2a). This result indicates that VNS during extinction makes PTSD model rats resilient to stress-induced relapse.

To compare the fear demonstrated by the PTSD model rats, we examined fear in rats that underwent AFC in the absence of SPS (AFC alone rats). These rats exhibited remission of fear behavior (≤10% freezing) and resistance to reinstatement (Figure 3.2b, sham); freezing behavior in sham-treated rats was dramatically reduced on Extinction Day 13 compared to Extinction Day 1 ($p<0.00001$). These results indicate that the stability and degree of fear extinction is substantially different between PTSD model rats and AFC alone rats, as previously reported[21-23]. Still, in rats that received AFC alone, VNS during extinction accelerated extinction of conditioned fear (Figure 3.2b). On average, VNS-treated rats reached remission of fear two days earlier than sham-treated rats ($p<0.01$). In rats given AFC alone, VNS treatment reduced

freezing versus sham on Extinction Day 5 ($p<0.001$) and Extinction Day 7 ($p<0.05$). Freezing following reinstatement was not different between the sham- and VNS-treated rats ($p>0.05$).

Prior to VNS- or sham-paired extinction, rates of acquisition of conditioned fear between AFC alone rats and PTSD model rats were examined (Figure 3.3a). A two-factor repeated measures ANOVA indicated a significant effect of group across tones for acquisition of conditioned fear ($F(7, 168)= 2.473$ $p<0.05$). All rats show a significant effect of time, as levels of freezing increase as the number of tone-shock pairings increases ($F(7, 168)=41.36$ $p<0.0001$). A Holm-Bonferroni sequential correction revealed a significant difference between PTSD model rats and AFC alone rats only at tones 9 and 10 ($p<0.01$). PTSD model rats showed a deficit in fear retention versus AFC alone rats ($p<0.01$) at the start of the second day of fear conditioning. This could be explained by evidence that SPS impairs consolidation, but has no learning effect within a session[23]. This effect was not present during other AFC tones or at the start of extinction training (Extinction Day 1).

A one-way ANOVA revealed no significant effect of freezing following the reinstatement shock ($F(2.582, 8.94)=0.522$ $p>0.05$). All rats showed similar levels of conditioned fear immediately following reinstatement shock (Figure 3.3b), indicating the shock was equally aversive to all rats. However, 24 hours later there was a significant difference between PTSD model rats given sham stimulation and all other rats ($p<0.0001$).

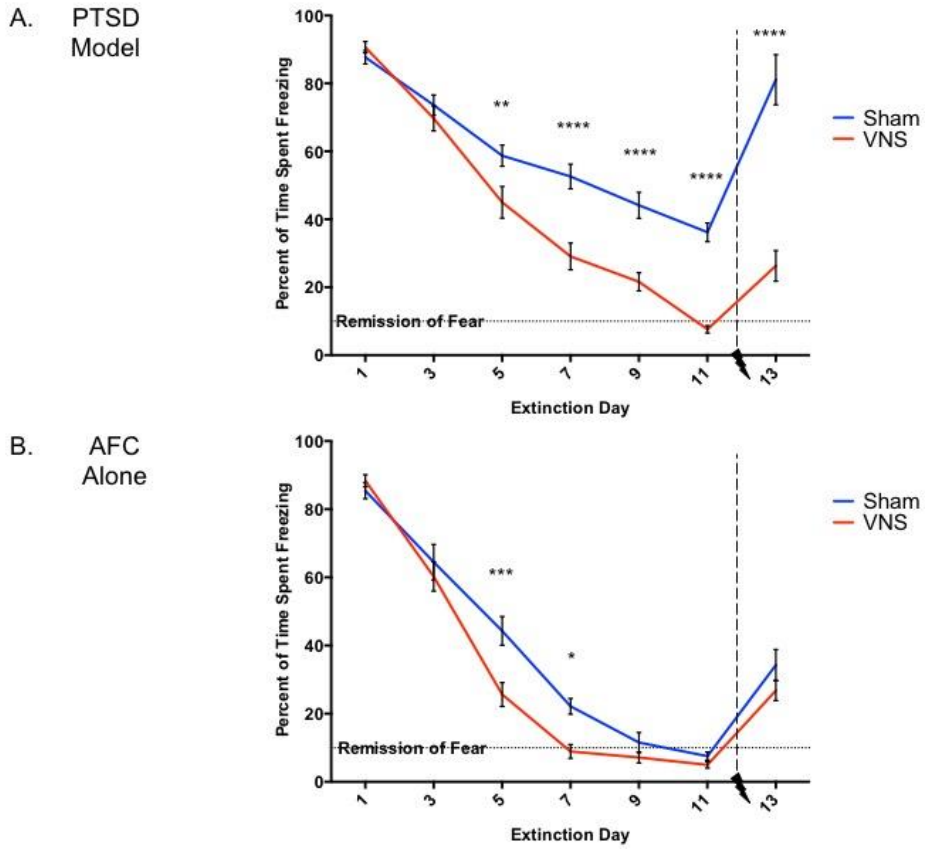

Figure 3.2. Conditioned fear responding across Extinction Days.

A. VNS treatment reverses maladaptive fear seen in PTSD model rats. Following eleven consecutive days of extinction, rats exposed to the SPS procedure one week before AFC (PTSD model rats) did not reach remission of fear. A single, unpaired reminder of the unconditioned stimulus was sufficient to reinstate conditioned fear to the level of freezing measured on the first day of extinction in the PTSD model. PTSD model rats given VNS reached remission of fear by the end of treatment. VNS also attenuated the reinstatement of conditioned fear. Freezing in rats given VNS was significantly reduced compared with freezing on the first day of extinction. PTSD model rats given VNS showed significantly less fear than sham-treated rats on Extinction Day 5 (**$p<0.01$) and Extinction Days 7, 9, and 11 (****$p<0.0001$). PTSD model rats given VNS showed reduced reinstatement of conditioned fear vs. sham-treated rats (****$p<0.0001$). B. VNS treatment accelerates extinction in control rats subjected to AFC alone. All rats that underwent AFC alone reached remission of fear by the end of extinction. VNS led to more rapid remission of fear than sham stimulation. VNS-treated rats showed reduced freezing vs. sham-treated rats on Extinction Day 5 (***$p<0.001$) and Extinction Day 7 (*$p<0.05$). AFC alone rats alone did not show complete reinstatement of conditioned fear following a reminder of the unconditioned stimulus.

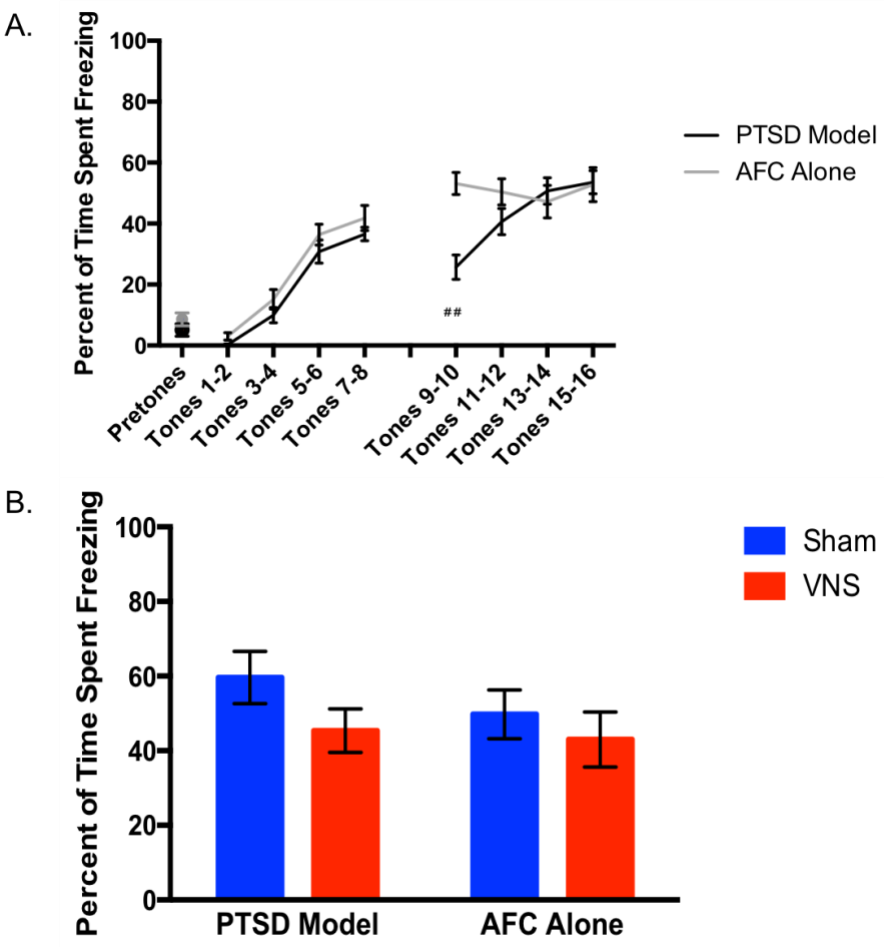

Figure 3.3. AFC Alone rats and PTSD model rats respond similarly to footshock.

A. Acquisition of conditioned fear is similar between groups. Prior to VNS- or sham-paired extinction, rats underwent two days of AFC. On the first day of AFC, rats were exposed to four pretones to assess baseline freezing to the tone, pretone freezing was similar between groups. Acquisition of conditioned fear was similar between all rats on these days, however, PTSD model rats show a reduction in freezing at tones 9 and 10 versus AFC alone rats (##$p<0.01$). This effect is no longer present on subsequent tones. B. Following reinstatement shocks, rats show similar levels of freezing. Following a reinstatement shock in Context A, rats from all groups show comparable levels of freezing ($p>0.05$).

A total of two AFC alone rats (1 Sham and 1 VNS) met the exclusion criteria. These rats were not included in analysis because they did not exhibit conditioned fear following AFC (freezing behavior was < 2 standard deviations away from the mean).

VNS administration during extinction sessions reduced PTSD-like symptoms.

Elevated Plus Maze

To test the effect of VNS on general anxiety, rats were tested on the elevated plus maze (Figure 3. 4a-b). A one-way ANOVA revealed a significant effect across groups ($F(2.430, 17.01)=17.26$ $p<0.0001$). PTSD model rats given sham stimulation spent less time in the open arms than AFC alone rats given sham stimulation ($p<0.05$), and made fewer entries into the open arms ($p<0.01$), indicating that anxiety was elevated in the PTSD model rats. VNS treatment reversed this effect, in that PTSD model rats given VNS during the extinction sessions spent more time in the open arms ($p<0.01$), and made more entries into the open arms versus PTSD model rats given sham stimulation ($p<0.001$). PTSD model rats given VNS during extinction sessions spent a similar amount of time in the open arms as AFC alone rats ($p>0.05$). VNS treatment also increased time spent in the open arms ($p<0.05$) and entries into the open arms in AFC alone rats ($p<0.05$). These results demonstrate that VNS treatment during extinction reduced general anxiety one week after treatment. Total locomotion was not different in PTSD model rats versus AFC alone rats, and administration of VNS did not affect total locomotion (Figure 3.4c).

Acoustic Startle Response

To test for hyperarousal, rats underwent an acoustic startle response test. VNS treatment during extinction reduced startle responses in PTSD model rats and in AFC alone rats (Figure 3.4d). A one-way ANOVA indicated a significant effect across groups ($F(1.40, 9.80)=6.980$ $p<0.05$). Startle amplitudes prior to habituation (the first 15 startle bursts) were similar in PTSD model rats given sham stimulation and sham-treated AFC alone rats ($p>0.05$). PTSD model rats that received VNS during extinction showed a reduction in startle amplitude versus sham-treated rats

(p<0.05). VNS also decreased startle amplitude versus sham-treated AFC alone rats (p<0.05). These results demonstrate that although SPS did not increase startle responses, VNS was still effective in reducing startle amplitude.

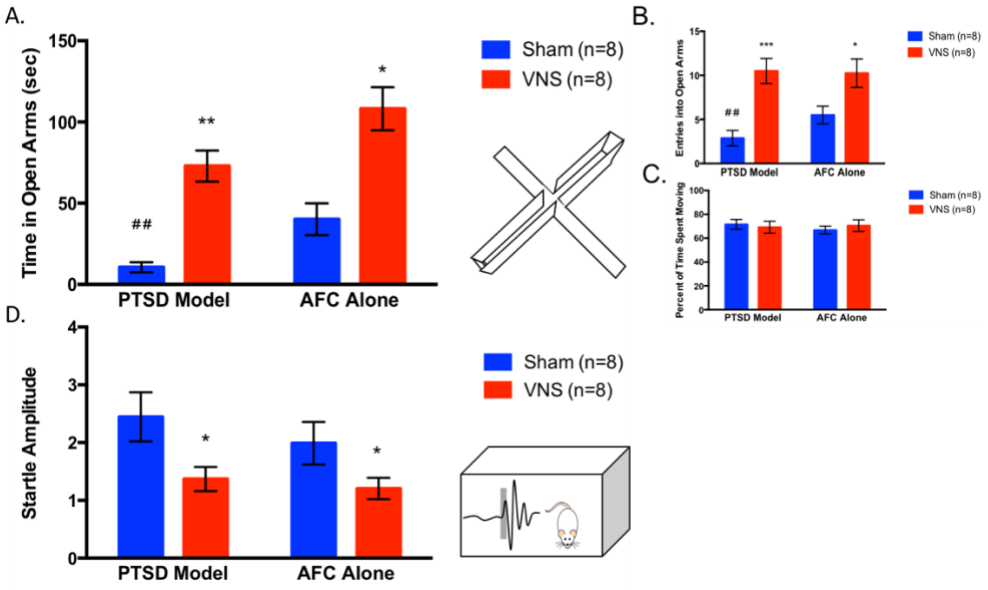

Figure 3.4. VNS treatment reversed PTSD-like symptoms of anxiety and exaggerated startle.

A. VNS administration during extinction reduced anxiety. One week after the completion of extinction, PTSD model rats spent less time in the open arms of the maze than AFC alone rats (##p<0.01), indicating heightened anxiety. VNS during extinction reversed this effect in PTSD model rats and also decreased anxiety in AFC alone rats. VNS-treated rats spent more time in the open arms vs. sham-treated rats in the PTSD model (**p<0.01) and in the control group that underwent AFC alone (*p<0.05) B. VNS treatment increased entries into the open arms. Similar to time spent in the open arms, PTSD model rats showed a reduced number of entries into the open arms versus AFC alone rats (##p<0.01). Administration of VNS during extinction reversed this effect and increased entries into the open arms in PTSD model rats (*** p<0.001) and in AFC alone rats (*p<0.05). C. Total locomotion was not affected. Total time spent moving in the elevated plus maze was similar between groups (p>0.05). D. VNS treatment reduced startle responses. Following extinction, there was no difference in PTSD model rats vs. AFC alone rats. Administration of VNS during extinction reduced startle amplitudes in PTSD model rats vs. sham (*p<0.05) and in rats that underwent AFC alone vs. sham (*p<0.05).

Marble Burying

To test for avoidance of novel objects, rats were tested on a marble burying task. VNS treatment during extinction reduced avoidance in PTSD model rats (Figure 3.5a). A one-way ANOVA indicated a significant effect across groups $F(2.694, 18.86)=6.622$ $p<0.01$. PTSD model rats given sham stimulation buried more marbles than AFC alone rats ($p<0.01$). PTSD model rats given VNS during extinction buried fewer marbles than those given sham stimulation ($p<0.05$), and buried a similar number of marbles as AFC alone rats ($p>0.05$). This indicates that VNS treatment during extinction reduced novel avoidance behavior in PTSD model rats.

In AFC alone rats, there was no difference between VNS and sham stimulation ($p>0.05$).

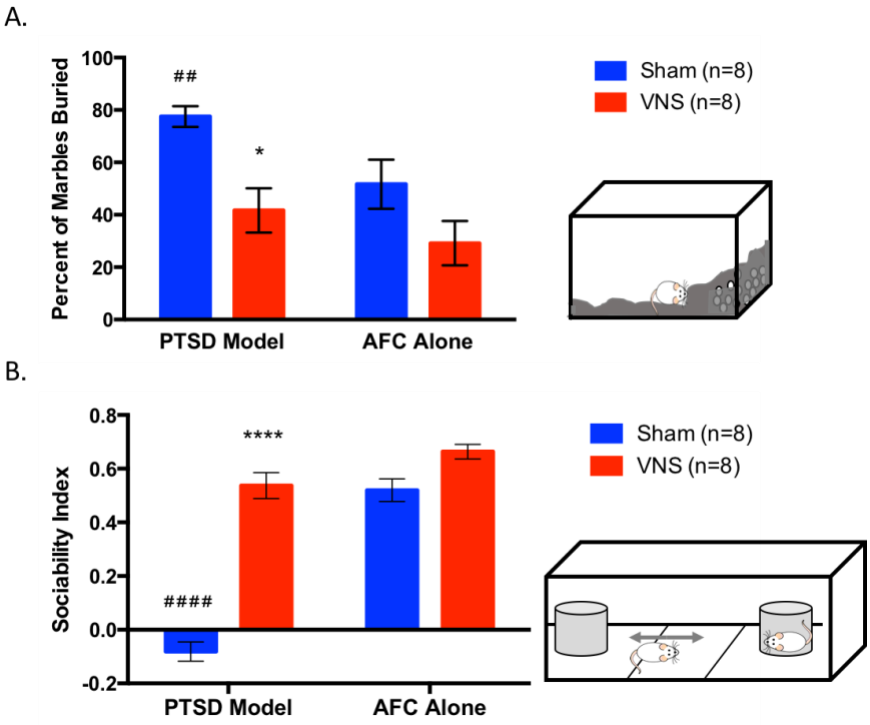

Figure 3.5. VNS during extinction decreases novel avoidance and social withdrawal behavior.

A. VNS reduced novel object avoidance in PTSD model rats. One week following extinction, PTSD model rats showed increased marble burying vs. AFC alone rats (##$p<0.01$). VNS during extinction reversed this effect PTSD model rats (*$p<0.05$ vs. sham). There was no difference in

AFC alone rats. B. VNS increased social interaction. PTSD model rats given sham treatment showed diminished social interaction vs. AFC alone rats (#### $p<0.0001$). Administration of VNS during extinction reversed this effect and increased social interaction (****$p<0.0001$ vs. sham). There was no difference between VNS and sham stimulation in AFC alone rats.

Social Interaction

To test whether VNS during extinction could reverse abnormal social interactions characteristic of PTSD, rats were evaluated using a social interaction test. PTSD model rats showed social withdrawal. VNS during extinction reversed the social withdrawal and restored normal social behavior (Figure 3.5b). A one-way ANOVA indicated a significant effect across groups $F(2.632, 18.42)=59.44$ $p<0.0001$. PTSD model rats given sham stimulation had a negative sociability index, indicating the typical preference to interact with the stimulus rat was deficient. The sociability index for the PTSD model rats was significantly lower than that of AFC alone rats ($p<0.00001$). This was reversed by VNS; PTSD model rats given VNS had a higher sociability index than those given sham stimulation ($p<0.0001$), and the sociability index was not significantly different from that of AFC alone rats. These results show that VNS treatment during extinction improved social interaction in PTSD model rats. There was no significant difference between social interaction indexes of VNS- versus sham stimulation-treated AFC alone rats.

Taken together, these results demonstrate that anxiety-related behavior in PTSD model rats is qualitatively and quantitatively distinct from that of AFC alone rats.

Discussion

The SPS rat model of PTSD shares important characteristics with PTSD in human subjects. For clinical diagnosis of PTSD, patients must exhibit symptoms from each of the four criteria for more than one month[2]. PTSD patients show extinction impairments that may be responsible for

the persistence of fear and anxiety symptoms. Here, we observed that exposure to SPS (restraint, swim stress, loss of consciousness, and social isolation) seven days prior to fear conditioning makes rats susceptible to impaired extinction of the fear response. This is consistent with previous observations of extinction impairments in the SPS model[22-23]. However, we found that SPS combined with subsequent AFC lead to a fear response to the CS even after 11 days of extinction. PTSD model rats also showed significantly higher reinstatement, a measure of relapse, following a reminder of the unconditioned stimulus. One of the symptom clusters of PTSD is intrusion symptoms, such as distress and re-experiencing after exposure to traumatic reminders. The present findings indicate that the animal model of PTSD demonstrates resistance to extinction learning and re-experiencing the trauma (inferred from the freezing response) in the presence of reminders of the trauma.

Alterations in arousal and reactivity, such as exaggerated startle responses make up the second symptom cluster. One-week after completion of extinction, PTSD model rats showed heightened anxiety on an elevated plus maze, but the acoustic startle response was not significantly different in PTSD model rats and AFC alone rats. PTSD patients demonstrate hypervigilance and exaggerated startle responses. It is possible that auditory fear conditioning alone increases the acoustic startle response, obscuring the effect of multiple stressors. For example, the acoustic startle response is potentiated in fear-conditioned rats and humans when it is tested in the presence of conditioned cues[32-33]. Avoidance is another symptom cluster that is described in the DSM-5. PTSD model rats showed an increase in the novel avoidance task of marble burying. The fourth symptom cluster of PTSD is negative alterations in cognition or mood, such as social withdrawal and persistent negative emotions. Social interaction scores were significantly lower

for PTSD model rats than they were for AFC alone rats. In fact, AFC alone rats showed a strong preference for the social zone in the social interaction test, whereas PTSD model rats did not show a preference at all for the social zone over the non-social zone. These findings provide evidence of social withdrawal and less engagement in normal activities in the PTSD model. Taken together, these findings suggest that the SPS model of PTSD shows many behaviors that resemble PTSD symptoms, and may be useful in the study of the effects of traumatic events on the brain and behavior.

VNS administration during extinction reversed the extinction impairment observed in PTSD model rats, and VNS improved symptoms from each PTSD symptom cluster including: re-experiencing fear, elevated anxiety, arousal, avoidance, and social withdrawal. PTSD model rats continued to exhibit a freezing response to the CS after eleven consecutive days of extinction. Others have shown enhancement in conditioned fear following contextual fear conditioning in the SPS model[34]. The extinction impairment in PTSD model rats reported here cannot be explained by a conditioning enhancement, as PTSD model rats do not show an enhancement in conditioning, in fact they show a temporary deficit in fear retention during tones 9 and 10. VNS administration during extinction reversed the extinction impairment in the rat PTSD model. Like AFC alone rats, PTSD model rats given VNS during extinction demonstrated remission of conditioned fear. This reduction in conditioned fear was also observed following reinstatement. A single reminder of the unconditioned stimulus was sufficient to fully reinstate conditioned fear in PTSD model rats, but VNS treatment during extinction prevented this relapse. These findings suggest that VNS may facilitate progress in exposure therapy by enhancing extinction of conditioned fear and reducing relapse.

While persistent fear in the presence of reminders of the trauma is a well-recognized PTSD symptom, generalized anxiety, hyperarousal, and avoidance behaviors can also be disabling. The administration of VNS during extinction reduced anxiety and avoidance behavior one week later, on tasks that did not involve the CS. The observation that VNS treatment reduced avoidance of novel stimuli and startle responses, and increased exploratory and social behavior in PTSD model rats suggests that this adjuvant therapy can improve pathological behaviors that are not directly related to specific trauma cues.

Extinction is the goal of exposure-based therapies and VNS enhances extinction. However, the mechanisms by which VNS enhances extinction are not yet known. VNS enhances memory consolidation[17-19] and alters the release of neuromodulators into the brain that may promote experience-dependent plasticity[20, 35-39]. Pairing VNS with an auditory stimulus alters auditory cortical maps while pairing VNS with motor learning modulates maps in the motor cortex, indicating that the nature of the plasticity is driven by the training that is paired with VNS[40-44]. We recently reported that VNS promotes plasticity in the pathway from the infralimbic area of the prefrontal cortex to the basolateral complex of the amygdala in rats[20, 45]. Humans with PTSD exhibit reduced activation of the ventromedial prefrontal cortex and increased activation of the amygdala[46-47]. Additionally, extinction impairments, like those observed in rats exposed to SPS, are associated with decreased prefrontal cortical control over amygdala activity[48-49]. VNS-enhancement of consolidation of the extinction memory via facilitation of plasticity in this circuitry could be responsible for successful extinction following VNS in PTSD model rats.

VNS may also enhance extinction by inhibiting activity of the sympathetic nervous system[50-51]. The vagus nerve is sometimes referred to as the “vagal brake” as activation of the vagus nerve

activates the parasympathetic system and slows heart rate following the sympathetic stress response[52]. One study showed that chronic VNS reduced a measure of anxiety in rats[53], and another suggested that chronic VNS improved scores on the Hamilton Anxiety Scale in human patients suffering from treatment-resistant depression[54]. It is possible that an immediate VNS-induced reduction in anxiety contributes to VNS-driven extinction by interfering with the sympathetic response to the CS, thus breaking the association of the CS with fear. In addition, a total of 20 trains of VNS administered over the course of 11 days may be sufficient to produce lasting anxiolytic effects, as has been observed following chronic VNS. Such a long-lasting anxiolytic effect would explain the reduction of general PTSD-like symptoms in VNS-treated rats. However, it is not likely that a general and lasting anxiolytic effect is responsible for VNS-driven remission of fear as unpaired administration of VNS did not enhance extinction of conditioned fear in a previously reported study[19].

These findings demonstrate that VNS treatment can reverse extinction impairments and provide benefits across a variety of symptoms in a rat model of PTSD. Extinction of conditioned fear in nonhuman animals is frequently used as a preclinical model of exposure therapy[13, 55-57]. The present findings suggest that VNS may be an effective adjunct to exposure therapy. Since VNS has been used in tens of thousands of patients with drug-resistant epilepsy[58] and delivery during exposure therapy requires considerably less stimulation, VNS may be safely used to enhance extinction in the treatment of PTSD and other disorders that show improvements with exposure-based therapies.

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CHAPTER 4

VAGUS NERVE STIMULATION PROMOTES GENERALIZATION OF CONDITIONED FEAR EXTINCTION IN RATS

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Abstract

Exposure-based therapies aim to generate successful extinction for specific cues presented during treatment and are used to treat a variety of anxiety-related disorders. The development of adjuvant treatments that enhance exposure-based therapies to promote generalization of extinction and generate more broad extinction learning could decrease the incidence of relapse and lead to better treatment outcomes. Vagus nerve stimulation (VNS) paired with exposure can enhance fear extinction, even in rat models of psychiatric disorders. However, the ability of VNS to broaden extinction and generalize extinction to multiple conditioned stimuli associated with a fear experience remains untested. Rats underwent auditory fear conditioning with two easily discriminable auditory stimuli. Following fear conditioning, extinction training consisted of exposure to only one of the sounds paired with VNS or sham stimulation. Delivery of VNS during extinction led to enhanced extinction for both conditioned stimuli, whereas extended extinction led to reduced fear for only the cue presented during training. This generalization effect was not due to an enhancement in context extinction, counter conditioning, or an anxiolysis. VNS-induced generalization of extinction was observed only when fear conditioning for the two stimuli were conducted within the same fear conditioning trial. These results provide evidence that VNS can promote generalization of extinction to a stimulus associated with a specific fear experience, while leaving non-related fear memories intact. The ability to generalize extinction makes VNS a potential candidate for use as an adjunctive strategy to supplement the benefits of exposure-based therapies.

Introduction

Extinction of conditioned fear is the process by which new learned associations suppress previously learned fear associations[1-3]. Extinction learning is the goal of exposure-based therapies, in which patients are exposed to cues that they find threatening while in a safe environment until they learn that the cues no longer signal threat. Exposure-based therapies are used to treat anxiety-related disorders including posttraumatic stress disorder (PTSD)[4-6], obsessive compulsive disorder (OCD)[7-9], and specific phobias[10-11]. Although exposure-based therapies are considered the best evidence-based practice[12], nonresponse rates and dropout rates can be as high as fifty percent[13].

Patients with anxiety-related disorders often are fearful of a variety of cues related to a traumatic experience. Exposure-based therapies promote cue-specific extinction. For example, exposure-based therapy for PTSD patients relies on patients to recount the stimuli present during the trauma. However, frequently PTSD patients exhibit memory impairments for aspects of the trauma[14-15] as a result, remission of fear to all cues is implausible. In addition, patients with anxiety-related disorders often show extinction deficits, as is the case in PTSD[16-19], OCD[20], and specific phobias[21]. Given the incomplete extinction of all fear-related cues and deficits in extinction of conditioned fear seen in many anxiety-related disorders, as well as the propensity for relapse following treatment, efforts are being made to develop adjuvant therapies that can be used in conjunction with exposure-based therapies to enhance and broaden the extinction of conditioned fear. Adjuvant treatments would have additional benefits if the strategy were able to boost extinction of cues related to a fear experience without the explicit presentation of each cue during therapy.

Promotion of more broad extinction that leads to a reduction of conditioned fear to stimuli beyond the specific cues presented during extinction is considered generalization of extinction. Recent work suggests that generalization of extinction can occur following treatment in patients with specific phobias[22]. Furthermore, methods that enhance memory consolidation, such as mild stress and D-cycloserine administration both promote generalization of extinction[23-24].

We found that vagus nerve stimulation (VNS), a treatment emerging as a viable treatment for neurological disorders including stroke, tinnitus, and epilepsy[25-27] can enhance extinction of conditioned fear in rats with adaptive fear[28-30], and reverses extinction impairments and attenuates reinstatement in a rat model of PTSD[31]. Furthermore, VNS administration during extinction training reversed other PTSD-like symptoms, including generalized anxiety and avoidance behavior[31].

These findings indicate that VNS can enhance extinction of conditioned fear by both accelerating the rate of learning and by promoting more complete extinction that is less prone to relapse. Given that VNS enhances memory consolidation, we tested the hypothesis that VNS promotes generalization of extinction. Here, we report evidence that VNS enhances the generalization of extinction of fear conditioned cues. We show that this effect on generalization is not due to a VNS-induced enhancement of context extinction, nor VNS-induced counter-conditioning. Although we find that administration of VNS is anxiolytic, the enhancement of extinction is independent of the anxiolytic effect.

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. One hundred and sixty-six 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 am) with access to food and water *ad libitum*. Before the start of every experiment, rats were handled for five days, five minutes per day. Criteria for exclusion of rats from the analysis was performance $\geq$ 2 standard deviations away from the mean on any task. Six rats were excluded from analysis (one from Experiment 1, two from Experiment 4, one from Experiment 5, and two from Experiment 6) for failure to express conditioned fear following auditory fear conditioning (freezing less than 50 percent to the CS). Exclusion of these rats had minimal effects on statistical comparisons.

Surgical implantation of cuff electrode

Surgery protocols are described in detail elsewhere[29-30, 32]. 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 [33]. 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 (0.8mA, 1 second) was applied through the cuff to test for cessation of breathing. If cessation of breathing was not observed, the cuff was adjusted or replaced. For sham rats, surgery was conducted in the same manner to isolate the vagus nerve, but the rats were not implanted with a cuff. Animals were given one week to recover following surgery.

Conditioned Stimuli (CS)

For generalization experiments, rats were subjected to auditory fear conditioning (AFC) paired with two complex auditory stimuli. The two stimuli chosen were machine gunfire (CS1) and a marmoset vocalization (CS2). These two stimuli were selected because they activate the auditory cortex more broadly and naturally than pure tones, and our previous work determined that rats can readily discriminate them (Figure 4.1). Spectrograms of these cues show distinct differences in frequency bandwidth, frequency modulation, rise time, and decay time. Each stimulus was presented at 70dB.

In the experiment that tested the effects of anxiety reduction on extinction, the conditioned stimulus was a 30-second long 9kHz tone presented at 70db - the same conditioned stimulus that was used in our previous studies[29-31].

Vagus nerve stimulation (VNS) and sham stimulation

Treatment with VNS or sham stimulation was given during extinction training and paired with exposure to a single CS during extinction sessions (Experiments 1, 3, 5, and 6). To administer stimulation, an AM systems stimulator was connected to the cuff connector on the headcap via a 25cm long PVS multiconductor cable (Cooner Wire). During extinction, each stimulation was paired with an exposure to the CS and started 150.0ms before the onset of each tone and then

continued for 30 seconds. VNS was given at a frequency of 20Hz, an intensity of 0.4mA for 30 seconds with a 100μs pulse width. Sham-treated rats were connected to the stimulator in the same way as VNS-treated rats, but did not receive stimulation.

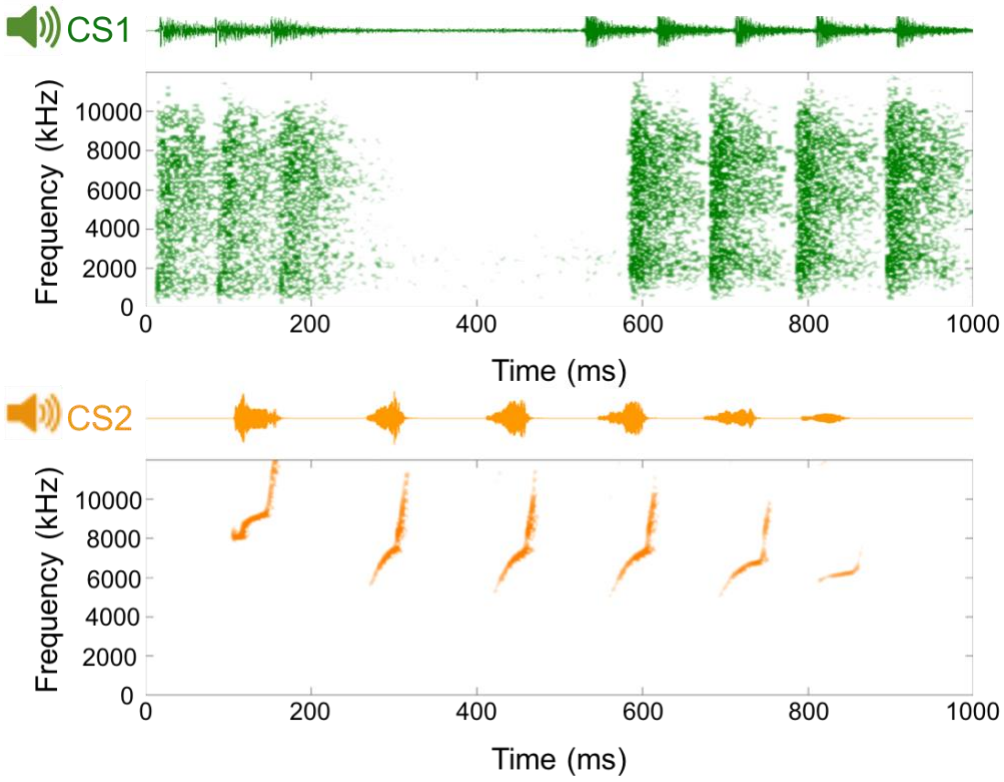

Figure 4.1. Spectrograms of each conditioned stimulus used during AFC.

A. Spectrogram for CS1 (machine gun fire). B. Spectrogram for CS2 (marmoset vocalization). C. Spectrogram for CS3 (pure 9kHz tone). 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. CS3 was used to test the effects of peripheral vagal blockade on VNS enhancement in conditioned fear extinction.

For stimulation given in the home cage, (Experiment 2) rats received the same number of stimulations as VNS given during extinction. Rats were connected to the AM systems stimulator in the same way as previously described, however stimulation was not triggered in response to an event, rather VNS (20Hz, 0.4mA, 30 seconds, 100μs pulse width) 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.

For stimulation given in the conditioned place preference apparatus (Experiment 2), VNS and sham was administered the same way as the non-contingent home cage stimulations described above, however rather than taking place in the rat's home cage, the stimulations were delivered in either side of a conditioned place preference apparatus (see *Conditioned Place Preference* section).

Conditioned place preference (CPP)

The conditioned place preference (CPP) task was used to measure potential preferences or aversions to VNS administration[34]. Rats were placed into a CPP apparatus made of two enclosed black sides (25.0cm by 30.0cm) separated by a white compartment (25.0cm by 12.0cm) with doors to each black compartment (8.0cm by 8.0cm). One of the black compartments had a smooth acrylic floor, where the other had a honeycomb cut acrylic floor to make them distinct. Rats were allowed to wander freely during preference tests which were recorded via webcam. Time spent in each black compartment and time spent in the center were measured using AnyMaze video tracking software and were used to determine if there was a preferred side. Time spent moving served as a measure of general locomotion.

Methyl-scopolamine Administration

Methyl-scopolamine (methyl-scop) is a peripherally acting muscarinic acetylcholine receptor antagonist that does not cross the blood brain barrier[35]. Low doses of methyl-scop block peripheral vagal function, while leaving the central vagal activity intact[36]. Methyl-scop (Sigma) or vehicle (saline) were administered I.P. at a dose of 0.1mg/kg. In experiment 2, methyl-scop

and vehicle were administered 15 minutes prior to EPM testing. In experiment 3, methyl-scop and vehicle were administered 15-minutes prior to VNS or sham stimulation.

Elevated plus maze (EPM)

The elevated plus maze (EPM) was used to measure anxiety[37]. Rats were placed onto an elevated plus-shaped maze (10.0cm wide, 50.0cm long, elevated 55.0cm off the floor), with walls (30.0cm 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 ten-minute test. To be considered in an arm, the rat's four paws needed to be in that 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.

Experimental design

Experiment 1: Interleaved AFC

Experiment 1 was designed to test the effects of VNS on extinction of conditioned fear of both the stimulus given during extinction, as well as a stimulus not presented during extinction. To test this, 34 rats underwent auditory fear conditioning (AFC) to CS1 and CS2. Both CS1 and CS2 were paired with footshocks, and then only one of the stimuli was presented during extinction to test the effect of VNS on extinction of the CS not presented during extinction (Figure 4.2a).

On the first day of testing, before the administration of footshocks, rats were subjected to two presentations of each CS to assess their baseline (pre-conditioning) freezing to each CS. Immediately after, rats were subjected to the first of two days of AFC in context A (neutral

smell, electric grid floor). During AFC, the CS1 and CS2 were interleaved and presented randomly with an ISI between 120 and 240 seconds. Four presentations of each CS paired with a footshock (0.8mA, 1 second) were administered on each day of AFC. As in our previous studies (refs), we aimed to minimize timing predictability by administering the footshock at a random time during each 30-second CS presentation. Twenty-four hours following day 2 of AFC, rats were given a conditioned fear response test (CFRT) 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 freezing to each CS was scored by two blind experimenters as a 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; the other CS was not presented on extinction day. The CS presented during extinction was counterbalanced. VNS-treated rats received four presentations of the CS paired with VNS (VNS + Extinction group; n=14). Since VNS enhances extinction learning, sham-treated rats require five times more presentations of the CS to reach levels of extinction comparable to VNS-treated rats[30]. Therefore, sham-treated rats received 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 remained in the home cage and received four trains of VNS in the absence 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 five minutes, then presenting rats with four randomly interleaved presentations of the two CSs. The CFRT session was recorded by webcam and freezing to context B, CS1, and CS2 were scored by two blind experimenters.

Experiment 2: Effects of VNS on CPP

Experiment 2 was conducted to determine whether administration of VNS produced hedonic or aversive effects. Following habituation to the CPP apparatus, 12 rats were subjected to an initial preference test to measure baseline preference to either side of the apparatus when they were allowed to freely wander between sides. Video recordings were captured by webcam and time spent in each of the sides was measured (see *Conditioned Place Preference* section). Twenty-four hours later, rats underwent five consecutive days of VNS and sham stimulation in the CPP apparatus. Rats were designated to receive VNS in either the smooth side or the honeycomb side of the apparatus, this was counterbalanced. On each of the five days, rats received VNS in their designated VNS-side, and sham stimulation in their designated sham-side, the order of stimulation was counterbalanced on each day. Following VNS and sham treatment, rats underwent another preference test to assess any preference for the side where VNS or sham stimulation were administered.

To ensure that the protocol described above could drive place preference, we repeated the same protocol in 6 naïve rats. However, instead of giving VNS or sham-stimulation in the CPP apparatus, rats were given a reward of FrootLoops in one side or no reward in the other side. The side with the FrootLoops reward was counterbalanced. After habituation, the initial preference test, and 5 consecutive days of consuming FrootLoops in one section of the CPP apparatus, rats were given a test to assess any preference for the side of the CPP apparatus where they received the food reward.

Experiment 3: Effects of peripheral vagal blockade on VNS-reduction in anxiety

Experiment 3 was designed to test the effects of peripheral vagal activation on the VNS-induced reduction in anxiety. Rats underwent three days where a single train of non-contingent VNS or sham stimulation was delivered in their home cage to habituate them to stimulation. On day 4, rats were injected with either saline or methyl-scop 15 minutes prior to stimulation leading to the following groups: Sham-Saline (n=9); Sham-Methyl-scop (n=9); VNS-Saline (n=9); VNS-Methyl-scop (n=9). After the injection, rats were given one non-contingent stimulation in the home cage and then they underwent testing on the elevated plus maze (see *Elevated Plus Maze* section).

Experiment 4: Effects of anxiety reduction on VNS-paired extinction

Experiment 4 was designed to test the contribution of anxiety reduction on VNS-paired extinction (Figure 4.4a). Auditory fear conditioning was carried out as in Experiment 1, however a single CS (30-second 9kHz tone) was paired with footshock. On the first day of AFC, the CS was presented twice alone prior to any tone-shock pairings to assess baseline freezing to the tone, then the CS was presented eight times and paired with a footshock (0.4mA, 1 second) each time. On day 2, the CS was presented eight times paired with a footshock. Twenty-four hours following the completion of AFC, rats underwent a pre-extinction CFRT where the CS was presented four times in the absence of any reinforcement. This test session was recorded via webcam and scored by two blind reviewers as a measure of conditioned fear. On day 4, 15 minutes prior to beginning extinction training, rats were injected with either methyl-scop or vehicle. Four presentations of 9kHz were paired with VNS or sham stimulation leading to the following four groups: Sham-Saline (n=8); Sham-Methyl-scop (n=8); VNS-Saline (n=9); VNS-

Methyl-scop (n=9). Twenty-four hours following extinction, rats were subjected to a post-extinction CFRT where the CS was presented four times in the absence of reinforcement. This test session was recorded via webcam and scored by two blind reviewers as a measure of conditioned fear.

To determine if generalization of extinction could occur for cues learned on separate days, we performed Experiment 5 where CS1 was paired with a footshock, and twenty-four hours later, CS2 was paired with a footshock. To determine if generalization of extinction could occur for cues learned in different contexts, we performed Experiment 6 where AFC to CS1 occurred in context A, and AFC to CS2 occurred in context C within one hour.

Experiment 5: AFC separated by time

Experiment 5 was preregistered on Open Science Framework (osf.io/wk7re) and designed to test the contribution of timing during fear learning on generalization of fear extinction (Figure 4.6a). Twenty rats were subjected to two days of AFC in context A (neutral smell, electric grid floor). On the first day of AFC, rats were presented with only CS1. On day 1, CS1 was presented twice prior to any footshock to assess baseline freezing to the tone, then CS1 was presented 8 times paired with a footshock (0.8mA, 1 second). Twenty-four hours later, after allowing consolidation, rats were presented with only the CS2. On day 2, CS2 was presented twice prior to any footshock to assess baseline freezing to the tone, then CS2 was presented 8 times paired with a footshock (0.8mA, 1 second). Twenty-four hours following AFC, rats underwent a pre-extinction CFRT in context B (peppermint smell, black acrylic floor) where they 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 via Logitech camera and freezing to the CS was scored by two blind experimenters as the measure of conditioned fear. On day 5, rats underwent extinction to either CS1 *or* CS2 paired with either sham stimulation or VNS. The chosen extinction CS counterbalanced. Extinction was performed the same way as in experiment 2; the extinction CS was presented four times if paired with VNS (VNS + Extinction group), or 20 times if paired with sham stimulation (Extended Extinction group) in order to reach comparable levels of extinction. Twenty-four hours following extinction, levels of conditioned fear to context B and to each CS were tested again during a post-extinction CFRT. Rats were placed into context B where baseline freezing to the context was recorded for five minutes, then four presentations of each CS were randomly interleaved. This session was recorded and freezing to context B, CS1, and CS2 were scored by two blind experimenters.

Experiment 6: AFC separated by context

Experiment 6 was preregistered on Open Science Framework (osf.io/wk7re) and was designed to test the contribution of the context in which fear learning took place on generalization of fear extinction (Figure 4.7a). Rats were subjected to AFC for CS1 in context A (neutral smell, shock grid floor) and AFC to CS2 in context C (peppermint smell, shock grid floor, different lighting, different room). On the first day of AFC, rats were exposed to two presentations of CS1 in context A, prior to any footshock, to assess baseline freezing to the sound. Immediately after, CS1 was paired with a footshock (0.8mA, 1 second) four times, and then rats were returned to their home cages. After 15 minutes in their home cages, rats were transferred into context C where they were CS2 was presented twice, prior to any footshock, in order to assess baseline freezing to the sound. Immediately after, CS2 was paired with a footshock four times. On day 2,

rats were placed into context C and rats were exposed to four presentations of CS2 paired with a footshock, and then after 15 minutes, transferred to context A and exposed to four presentations of CS1 paired 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 minutes 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 via webcam and freezing to the 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 stimulation. 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) whereas sham-treated rats received 20 CS1 presentations (Extended Extinction group). Twenty-four hours following the extinction session, CFRT was tested. Rats were first placed in context A where baseline freezing to the context was recorded for five minutes, then CS1 was presented four times without reinforcement and freezing behavior was recorded. After 15 minutes in the home cage, rats were transferred to context C where baseline freezing to the context was recorded for five minutes, then four unreinforced presentations of CS2 were given. Test sessions were recorded via webcam and freezing to context A, context C, CS1, and CS2 were scored by two blind experimenters as measures of conditioned fear.

Statistical Analyses

Data for generalization experiments (Experiments 1, 5, and 6) 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 unpaired t-tests. Data for peripheral vagal blockade experiments (Experiments 2 and 3) were analyzed using a one-way repeated measures ANOVA with a Greenhouse-Geisser correction followed by a Tukey's post hoc test for multiple comparisons. Data for CPP (Experiment 4) were analyzed using a paired sample 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.

Results

Experiment 1: VNS + Extinction leads to generalization of extinction for co-conditioned stimuli

VNS paired with exposure promotes extinction of a single fear conditioned stimulus. Here, we tested the hypothesis that VNS-dependent extinction would generalize to multiple co-occurring stimuli. 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 Pre-Extinction CFRT. 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, or Extended Extinction (n=14) where the conditioned cue was presented without VNS a total of twenty times. The extinguished cue was counter-balanced within each group. A two-factor repeated measures ANOVA indicated a significant effect of treatment group across days ($F(3, 64)=47.00$ $p=3.6\times10^{-8}$). 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 (Extinguished CS) versus Pre-Extinction (Extended Extinction vs. Pre-Extinction, $p=2.3\times10^{-4}$). Four presentations of CS1 or CS2 paired with VNS was also sufficient

to reduce freezing to the Extinguished CS in VNS + Ext rats versus Pre-Extinction (VNS + Ext vs. Pre-Extinction, $p=3.2x10^{-4}$) (Figure 4.2b).

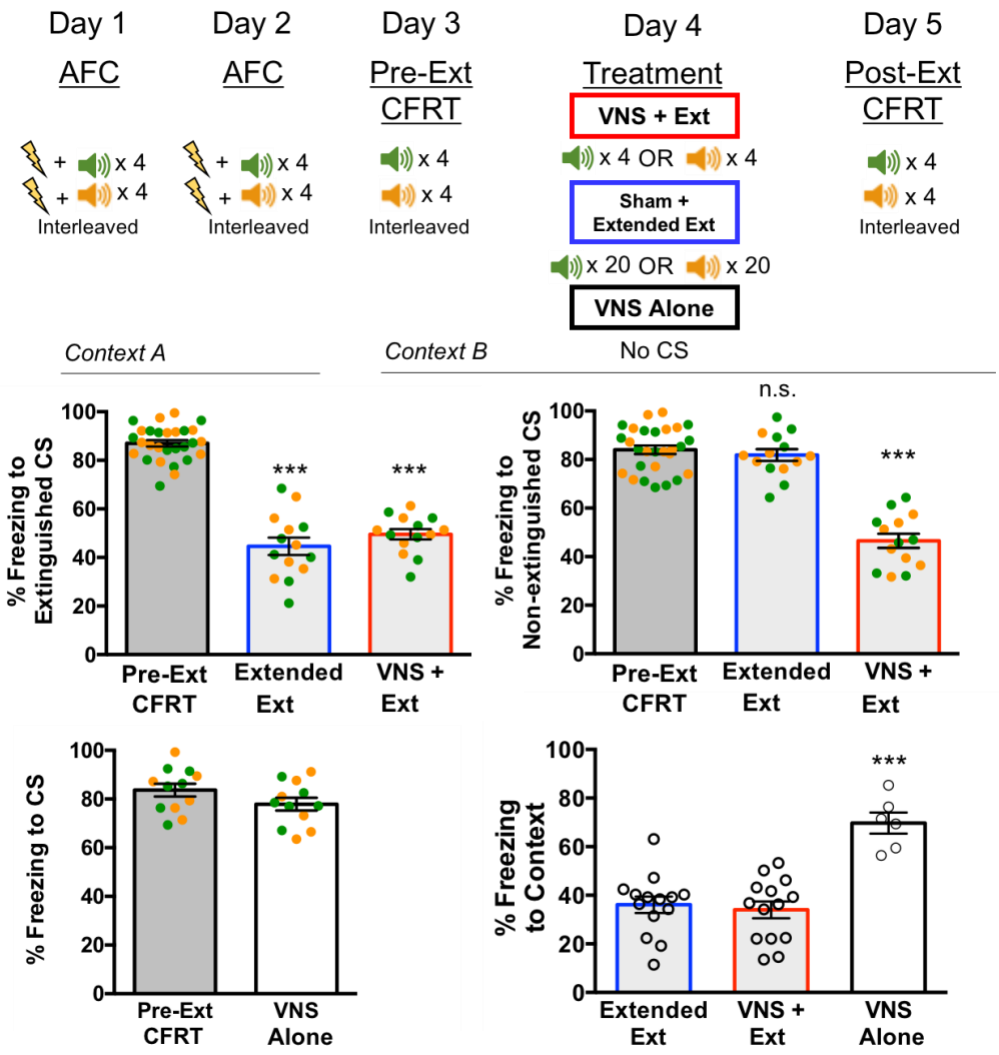

Figure 4.2. VNS + Extinction, but not Extended Extinction or VNS Alone leads to generalization of extinction for both the Extinguished CS and the Non-extinguished CS.

A. Timeline for AFC, conditioned fear response testing (CFRT), 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 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 CS1 or CS2 paired with sham stimulation (Extended Extinction), 4 extinction

trials of CS1 or CS2 paired with VNS (VNS + Extinction), or no extinction trials but equivalent amounts of VNS in the home cage (VNS Alone). On extinction day, only one of the stimuli was presented. A day later, rats underwent another CFRT to assess levels of conditioned fear following extinction to both stimuli. B. Extended Extinction and VNS + Extinction rats show equal reduction in conditioned fear to the Extinguished CS. The Pre-Extinction CFRT shows rats from both groups, as levels of freezing to each CS were equivalent across all groups. The Extinguished CS was counterbalanced, there was also no difference in fear acquisition or expression to CS1 (green dots), or CS2 (orange dots). Following 20 presentations of the Extinguished CS, Extended Extinction rats show reduced freezing to the CS versus the Pre-Extinction CFRT ($p=2.3x10^{-4}$). VNS + Extinction rats also show reduced freezing to the Extinguished CS versus the Pre-Extinction CFRT ($p=3.2x10^{-4}$). Importantly, Extended Extinction and VNS + Extinction reach equivalent levels of extinction for the Extinguished CS ($p=0.77$). C. Only VNS + Extinction leads to generalization of extinction for the Non-extinguished CS. Extended Extinction rats show no reduction in freezing versus the Pre-Extinction CFRT for the Non-extinguished CS ($p=0.15$). In contrast, VNS + Extinction rats showed a reduction in conditioned fear versus the Pre-Extinction CFRT for the Non-extinguished CS even though it was never presented during extinction ($p=2.1x10^{-4}$). 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. Extinction of the context cannot explain VNS + Extinction generalization. If VNS + Extinction simply enhanced the association that context B was unreinforced, that could explain why there was a reduction in freezing to both conditioned stimuli. However, there is no difference between baseline fear to the context between Extended Extinction and VNS + Extinction, rats spend equal amounts of time freezing to the context prior to the Post-Extinction CFRT ($p=0.68$). VNS Alone rats show elevated freezing to the context versus Extended Extinction ($p=2.1x10^{-3}$) and VNS + Extinction rats ($p=3.8x10^{-3}$), likely because these rats underwent VNS in the home cage and were not exposed to the context during extinction.

Extended Extinction rats showed no reduction in conditioned fear to the CS not presented during extinction (Non-Extinguished CS) versus Pre-Extinction (Extended Extinction vs. Pre-Extinction $p=0.15$). In contrast, VNS + Ext rats showed a significant reduction in freezing in the presence of the Non-Extinguished CS versus Pre-Extinction (VNS + Ext vs. Pre-Extinction, $p=2.1x10^{-4}$) (Figure 4.2c). This indicates that VNS + Extinction led to generalization of extinction where fear to both cues was diminished whereas Extended Extinction did not.

VNS is believed to enhance fear extinction by engaging neuromodulatory networks during exposure to support experience-dependent plasticity. However, it is possible that VNS acts to

reduce fear independent of extinction. To determine if VNS administration had a non-extinction-specific effect, 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, $t(11)=1.8$ $p=0.16$), demonstrating that VNS Alone was not sufficient to reduce conditioned fear to either CS versus Pre-Extinction (Figure 4.2d). These findings are consistent with previous studies and indicate that VNS reduces fear by enhancing fear extinction[29].

To determine if VNS-dependent generalization of extinction was due to enhanced extinction of the context in which extinction took place, baseline freezing to context B was measured for five minutes prior to any CS presentation during the Post-Extinction CFRT. A one-way ANOVA indicated a significant effect across groups ($F(2, 31) =19.21$ $p=3.7x10^{-6}$). VNS + Extinction and Extended Extinction demonstrate equivalent freezing to context (VNS + Ext vs. Extended Ext, $p=0.68$) (Figure 4.2e). This demonstrates that VNS effects on extinction generalization cannot be explained by a VNS enhancement of extinction to the context. VNS Alone rats showed elevated freezing to the context, consistent with the absence of cued extinction in this context. Together, these findings indicate that VNS does not reduce conditioned fear to the Non-Extinguished CS via an enhancement of context extinction.

Experiment 2: VNS does not induce CPP

To examine the potential of VNS to serve as a rewarding CS that replaces negative CS-US association (counter conditioning), a CPP was used to assess potential hedonic effects of VNS. VNS was given in one compartment of the CPP apparatus and sham stimulation was given in the other compartment, each rat served as its own control and received both VNS and sham

stimulation. Rats showed no baseline preference for either side of the apparatus. VNS presentation did not increase preference. Paired samples t-tests indicated no significant differences across VNS or sham stimulated sides of the CPP apparatus during either the Initial Preference Test or during the Post-Stimulation Preference Test. Following VNS administration in one compartment of the apparatus and sham stimulation in the other compartment, rats still showed no preference to either side of the apparatus (Figure 4.3a).

Separate rats underwent a control test with an appetitive stimulus. Following the administration of food in one compartment, a paired sample t-test indicated a significant place preference for the compartment where the food reward was given (Food Side vs. Empty Side, $t(5)=5.34$ $p=1.7x10^{-2}$) (Figure 4.3b). Taken together, these results indicate that unlike FrootLoops, VNS is not rewarding.

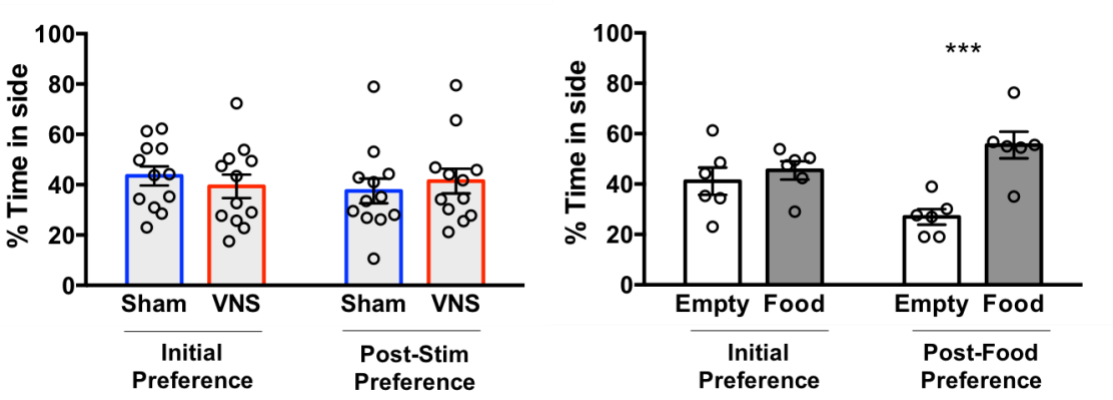

Figure 4.3. Rats show no preference for a place where VNS was administered.

A. VNS does not alter CPP. Following habituation, rats showed no difference in initial preference for either compartment of the CPP apparatus ($p=0.62$). The next day rats received 5 consecutive days of where VNS was given in one compartment of the CPP apparatus and sham stimulation in the other compartment of the CPP apparatus. At the Post-Stimulation Preference Test there was still no difference in preference for the VNS compartment versus the sham compartment of the apparatus ($p=0.55$). B. Food reward induces preference. Rats underwent the same CPP protocol, however VNS or sham stimulation were replaced by food reward (FrootLoop) or an unreinforced chamber. There was no difference in preference at the initial

preference test (p=0.60). However, following food administration in one of the chambers, rats spent significantly more time in the compartment where food was administered versus the unreinforced side ($p=1.7x10^{-2}$).

Experiment 3: Peripheral vagal blockade reverses VNS-induced anxiety reduction

VNS is anxiolytic, thus VNS may promote generalization of fear extinction by reducing anxiety during extinction training. To determine whether VNS reduces freezing in the presence of either CS by interfering with the anxiety experienced during extinction sessions, we administered VNS prior to testing on the elevated plus maze (EPM). A one-way ANOVA indicated a significant effect across groups (Figure 4.4; $F(3, 32)=16.26$ $p=1.3x10^{-6}$). Administration of VNS increased time spent in the open arms of the EPM versus sham stimulation in rats given saline (Saline with VNS vs. Saline with sham, $p=8.2x10^{-3}$). Consistent with the notion that descending parasympathetic activation via VNS promotes anxiolysis, administration of the peripherally-acting muscarinic acetylcholine receptor antagonist methyl-scop blocked VNS-dependent increase in time spent in the open arms (Methyl-scop with VNS vs. Saline with sham, p=0.27). VNS-treated rats given methyl-scop showed a significant reduction in time spent in the open arms of the EPM versus VNS-treated rats given saline (Methyl-scop with VNS vs. Methyl-scop with sham, $p=1.2x10^{-3}$). These findings indicate that VNS-dependent reduction in anxiety requires engagement of peripheral cholinergic receptors.

Experiment 4: VNS-induced enhancement of extinction does not depend on anxiety reduction

Building on this demonstration of the peripheral anxiolytic action of VNS, we tested whether the peripheral actions of VNS were required for VNS-dependent enhancement of fear extinction.

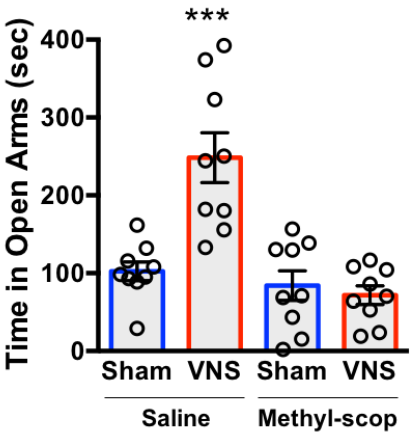

Figure 4.4. Peripheral vagal blockade attenuates the reduction in anxiety from non-contingent VNS.

Following 3 days of habituation to stimulation (1 train of stimulation in the home cage each day, or equivalent amounts of sham stimulation), rats were injected with either saline or the peripherally acting muscarinic acetylcholine receptor antagonist methyl-scop. Fifteen minutes after injection, rats were given a single non-contingent train of VNS or sham stimulation in the home cage and then subjected to EPM testing 10 minutes later. Increased time spent in the open arms indicated a reduction in anxiety. In rats treated with saline, non-contingent VNS lead to a reduction in anxiety versus sham stimulated rats ($p=8.2x10^{-3}$). However, in rats treated with methyl-scop this effect was attenuated. Methyl-scop treated rats given VNS showed no difference in anxiety versus methyl-scop treated rats given sham stimulation ($p=0.27$).

Methyl-scop was administered prior to VNS or sham-paired extinction to block VNS effects on anxiety. A one-way repeated measures ANOVA indicated a significant effect across groups (Figure 4.5; $F(4, 60)=54.62$ $p=4.2x10^{-3}$). Consistent with previous studies[29-30], administration of VNS was sufficient to enhance extinction versus sham stimulated rats that were treated with saline (Saline with VNS vs. Saline with sham, $p=7.1x10^{-3}$). Administration of VNS in rats treated with methyl-scop was also sufficient to enhance extinction versus sham stimulated rats treated with saline (Methyl-scop with VNS vs. Methyl-scop with sham, $p=4.1x10^{-4}$). Rats treated with methyl-scop and given VNS during extinction showed no difference in level of conditioned fear versus rats treated with saline and given VNS during extinction (Methyl-scop with VNS vs.

Methyl-scop with sham, p=0.54). These findings indicate VNS-dependent enhancement of fear extinction does not require reduction of anxiety.

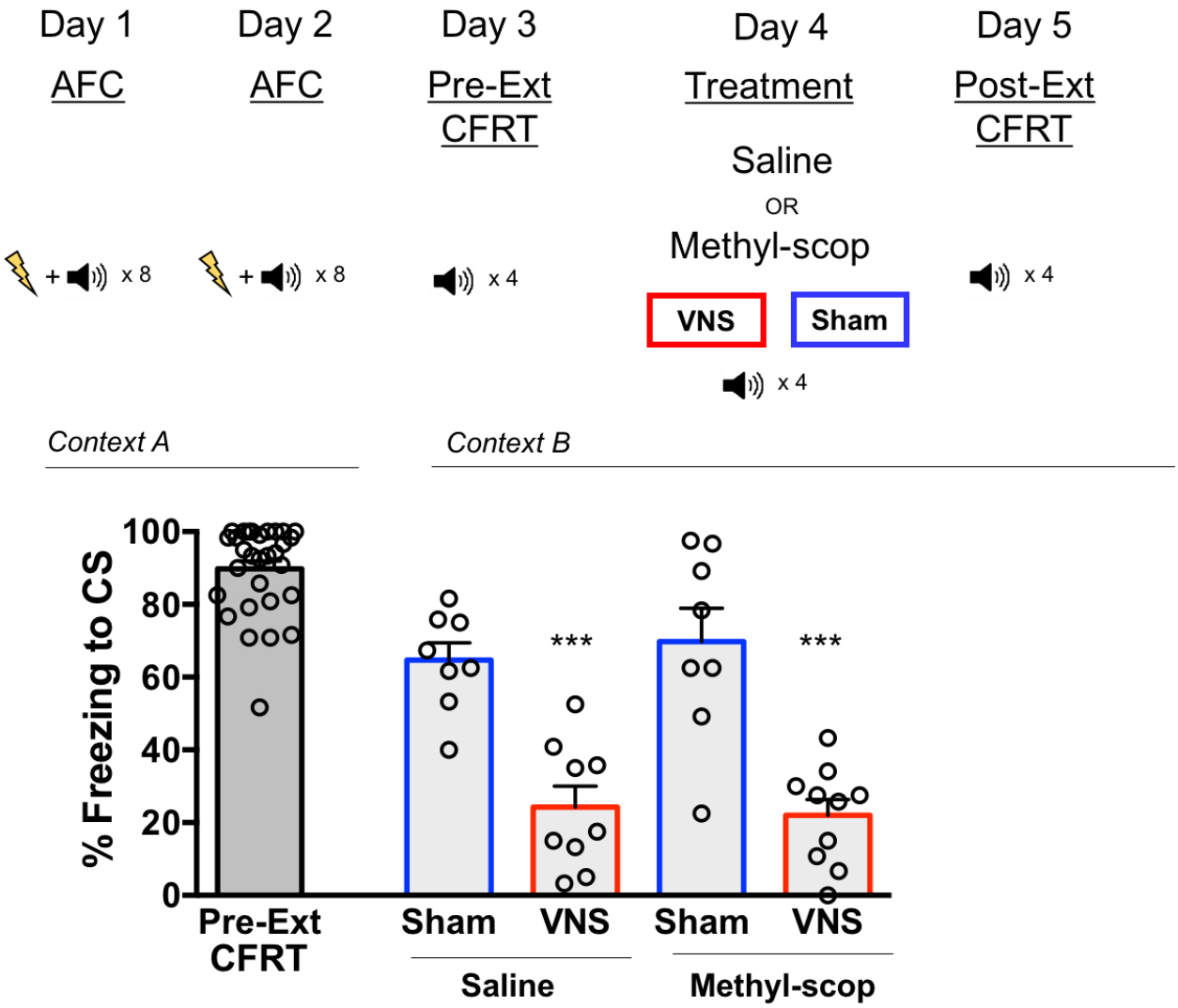

Figure 4.5. Peripheral vagal blockade does not alter VNS effects on extinction of conditioned fear.

A. Timeline for AFC, CFRT, and extinction. Rats were subjected to two days of AFC with CS3 (9kHz). Twenty-four hours later, rats underwent a CFRT to assess fear to CS 3. B. Methyl-scop does not alter VNS-enhanced extinction. There was no difference between groups at the Pre-Extinction CFRT. The next day, rats underwent extinction. On extinction day, rats were injected with either saline or methyl-scop and 15 minutes later underwent extinction where four

presentations of CS3 were paired with either VNS or sham stimulation. Twenty-four hours later, rats underwent a Post-Extinction CFRT to assess levels of conditioned fear following extinction. In rats treated with saline, VNS administration during extinction enhanced extinction versus sham stimulated rats ($p=7.1x10^{-3}$). Additionally, in rats treated with methyl-scop, VNS administration during extinction also enhanced extinction learning versus sham stimulated rats ($p=4.1x10^{-4}$). Though methyl-scop was sufficient to attenuate the VNS reduction in anxiety, there was no effect on extinction indicating that anxiolysis is not required for extinction enhancement by VNS.

Experiment 5: VNS-induced generalization of extinction does not extend to stimuli conditioned on separate days

To determine if fear memories that were acquired at different times were susceptible to VNS-induced generalization of extinction, we paired CS1 alone with a footshock during the first AFC session and then paired CS2 alone with a footshock twenty-four hours 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 ($F(2, 37)=26.65$ $p=6.8x10^{-7}$). 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, following 20 presentations of either CS1 or CS2 paired with sham stimulation, Extended Extinction rats showed a reduction in conditioned fear to the Extinguished CS versus Pre-Extinction (Extended Extinction vs. Pre-Extinction, $p=7.1x10^{-3}$). Four presentations of CS1 or CS2 paired with VNS (VNS + Ext) was also sufficient to reduce freezing to the Extinguished CS versus Pre-Extinction (VNS + Ext vs. Pre-Extinction, $p=4.4x10^{-3}$) and comparable to that observed with Extended Extinction (Figure 4.6b).

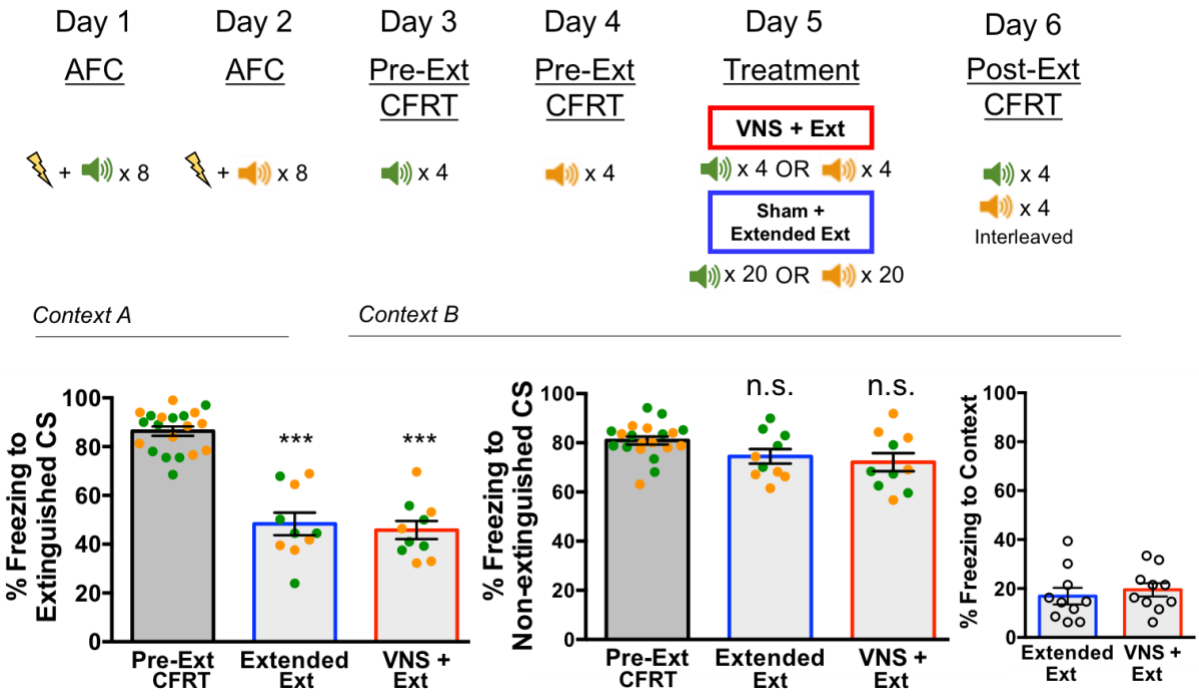

Figure 4.6. Separating AFC by twenty-four hours attenuates the VNS + Extinction effect on generalization.

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 on the other day, twenty-four hours apart. A CFRT to CS1 was administered the next day 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 with sham stimulation (Extended Extinction), or 4 extinction trials of CS1 or CS2 paired with VNS (VNS + Ext). 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 Extinguished CS. The Pre-Extinction CFRT shows rats from both groups, as levels of conditioned fear were similar between groups. The Extinguished CS was counterbalanced, there was also no difference in fear acquisition or expression to CS1 (green dots) or CS2 (orange dots). Following 20 presentations of the Extinguished CS, Extended Extinction rats show reduced freezing to the CS versus the Pre-Extinction CFRT ($p=7.1x10^{-3}$). VNS + Extinction rats also show reduced freezing to the Extinguished CS versus the Pre-Extinction CFRT ($p=4.4x10^{-3}$). C. Neither Extended Extinction rats or VNS + Extinction rats show reduced freezing to the Non-Extinguished CS. In contrast to Experiment 1, VNS + Extinction rats do not show reduced freezing to the Non-Extinguished CS versus the Pre-Extinction CFRT ($p=0.29$) after AFC has been separated by twenty-four hours.

Separating AFC attenuated the VNS + Extinction effect on generalization. Extended Extinction rats also do not show reduced conditioned fear to the Non-Extinguished CS versus the Pre-Extinction CFRT (p=0.12). D. Baseline freezing to the context is not different between groups. Extended Extinction and VNS + Extinction rats spend equivalent amounts of time freezing to the extinction context during the five minutes prior to CS presentation at the Post-Extinction CFRT (p=0.55).

Extended Extinction rats did not show a reduction in conditioned fear to Non-Extinguished CS versus Pre-Extinction (Extended Extinction vs. Pre-Extinction, 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-Extinguished CS versus Pre-Extinction (VNS + Ext vs. Pre-Extinction, p=0.29) (Figure 4.6c). An unpaired t-test indicated no significant difference in freezing to the context between Extended Extinction and VNS + Extinction (VNS + Ext vs. Extended Extinction, $t(9)=0.59$ p=0.56) (Figure 4.6d). This demonstrates that VNS does not promote generalized extinction of fear when fear-associated stimuli are not co-conditioned and thus does not broadly diminish fear.

Experiment 6: VNS-induced generalization of extinction does not extend to stimuli conditioned in separate contexts

Fear conditioning in the same context at different times prevents VNS-dependent extinction. We next tested whether fear conditioning at the same time (i.e., in a single session) in two contexts would generate separate fear memories and thus prevent VNS-dependent generalization of extinction. We administered AFC to CS1 in context A and then, on the same day, administered AFC to CS2 in context C. Extinction was given only to CS1 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 ($F(2, 29)=19.16$ $p=5.0\times10^{-6}$). 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 5 using separated AFC, following 20 presentations of either CS1 paired with sham stimulation, Extended Extinction rats showed a reduction in conditioned fear to the Extinguished CS versus Pre-Extinction (Extended Extinction vs. Pre-Extinction, $p=2.3x10^{-3}$). Four presentations of CS1 paired with VNS was also sufficient to reduce freezing to the Extinguished CS in VNS + Extinction rats versus Pre-Extinction (VNS + Ext vs. Pre-Extinction, $p=1.2x10^{-3}$) (Figure 4.7b).

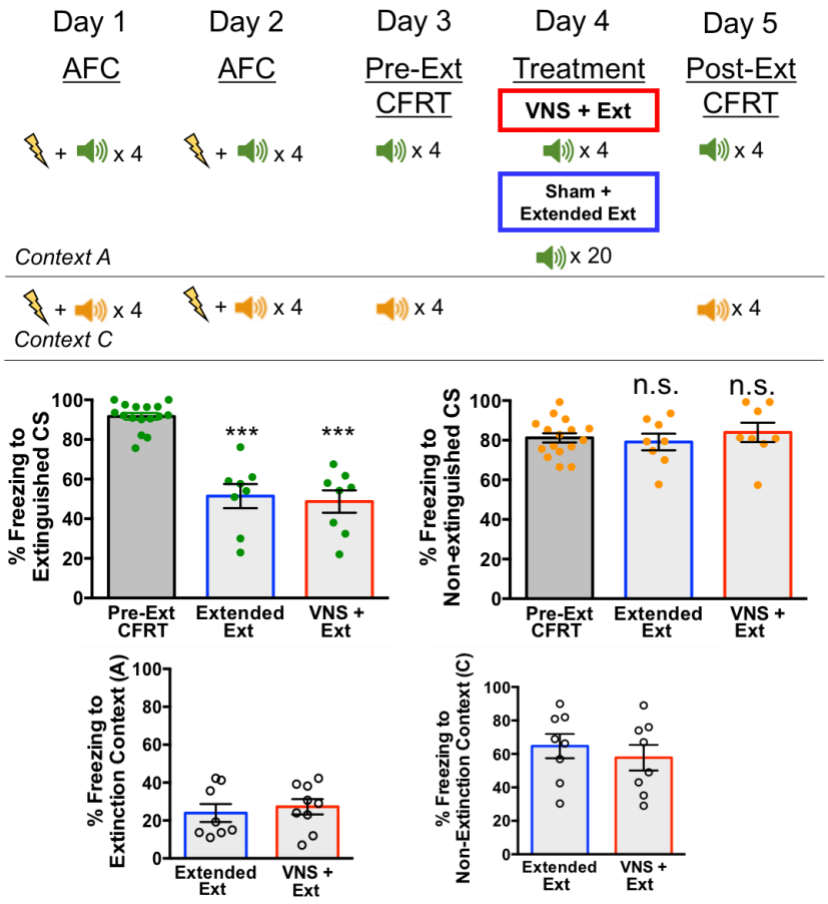

Figure 4.7. Separating AFC by context attenuates the VNS + Extinction effect on generalization.

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 on the same day. Following 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 to CS2 in context C. B. Extended Extinction and VNS + Extinction rats show equal reduction in conditioned fear to the Extinguished CS. Following extinction of CS1 in context A, Extended Extinction rats show reduced conditioned fear versus the Pre-Extinction CFRT ($p=2.3x10^{-3}$) VNS + Extinction rats also show a reduction in conditioned fear to the Extinguished CS ($p=1.2x10^{-3}$). C. Neither Extended Extinction rats or VNS + Extinction rats show reduced freezing to the Non-Extinguished CS. In contrast to Experiment 1, VNS + Extinction rats do not show reduced freezing to the Non-Extinguished CS versus the Pre-Extinction CFRT (p=0.38) after AFC has been separated by context. Extended Extinction rats also do not show reduced freezing to the Non-Extinguished CS versus the Pre-Extinction CFRT (p=0.40). D. Baseline freezing to the extinction context is not different between groups. Extended Extinction and VNS + Extinction rats spend an equal amount of time freezing to the extinction context during the five minutes 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 spend an equal amount of time freezing to the non-extinction context during the five minutes prior to CS2 presentation during the Post-Extinction CFRT (p=0.62). Freezing to the non-extinction context is high likely because the rats were not exposed to extinction in this context.

After AFC in two distinct contexts, Extended Extinction rats did not show a reduction in conditioned fear versus Pre-Extinction for the Non-Extinguished CS (Extended Extinction vs. Pre-Extinction, p=0.38). Similar to Experiment 5 when AFC was separated by time, VNS + Ext rats showed no reduction in conditioned fear versus Pre-Extinction for the Non-Extinguished CS (VNS + Ext vs. Pre-Extinction, p=0.40) (Figure 4.7c).

Unpaired t-tests indicated no significant difference in freezing to the Extinction Context between Extended Extinction and VNS + Extinction (VNS + Ext vs. Extended Extinction, t(7)=0.53 p=0.60) (Figure 4.7d) or in freezing to the Non-Extinction context (VNS + Ext vs. Extended Extinction, t(7)=0.67 p=0.52) (Figure 4.7e).

Administration of AFC in distinct contexts attenuated the VNS effect on extinction generalization indicating that the original fear memories need to occur in the same context to be susceptible to VNS-induced generalization of extinction.

Discussion

Exposure-based therapies are founded on the premise that unreinforced exposure to conditioned cues leads to extinction of learned associations[12]. Exposure therapy is used to treat disorders such as PTSD, phobia, obsessive-compulsive disorder, and addiction. Unfortunately, these therapies show high incidence of non-response, dropout, and relapse[13]. Our findings provide evidence that VNS paired with extinction training can enhance extinction of conditioned fear, not only for the CS that is presented during extinction, but also for another CS associated with the same fear experience. This generalization effect is not due to a general VNS-related reduction in freezing or anxiety, or a counter-conditioning effect. Instead, VNS-enhanced extinction generalizes to conditioned cues that were acquired during the same fear conditioning session, indicating that VNS-paired extinction training specifically enhances extinction of learned associations related to a specific trauma.

A general reduction of freezing in VNS-treated rats is not indicated by the results of this study as administration of VNS Alone did not lead to a reduction in the conditioned fear response. VNS effects on extinction generalization cannot be explained by enhanced learning of the extinction context, as VNS + Extinction and Extended Extinction rats showed equivalent levels of freezing to the extinction context. Additionally, a counter-conditioning effect where VNS replaced a negative association with a positive association is not a likely explanation for these results. Rats showed no preference for an environment where they received VNS, suggesting that VNS did

not produce hedonic effects. Taken together, these results indicate that the VNS effects on extinction generalization are specific to the fear memory.

Administration of VNS produced general anxiolytic effects consistent with previous reports [38]. This anxiolytic effect was attenuated by peripheral vagal blockade. The attenuation of the peripheral vagal response did not alter the VNS effects on extinction, indicating that the anxiety reduction VNS provides is not necessary for extinction enhancement. These findings suggest that VNS reduces anxiety, which may make exposure-based therapies more tolerable, however, the anxiety reduction is not necessary for VNS effects on extinction of conditioned fear.

Importantly, the effect of VNS on generalization of extinction was attenuated by altering fear conditioning, such that when presentations of each stimulus did not co-occur on the same day, or in the same context, VNS did not produce generalized extinction. Attenuation of the generalization effect provides evidence that VNS does not act to reduce all conditioned fear. Fear can generally be considered an adaptive biological process meant to enhance chances of survival[39]. Adjuvant therapies with the potential to generalize extinction should aim to enhance extinction for stimuli that are associated with maladaptive fears, rather than all conditioned fears. Since VNS enhances extinction generalization, but the effect is attenuated when presentations of CSs do not co-occur, there is likely an important effect on the way the fear memory is consolidated which influences the opportunity for extinction generalization. Studies regarding neuronal ensembles representing a fear memory could explain why only co-occurring stimuli are susceptible to generalization of extinction[40-43]. Optogenetic stimulation of small neuronal ensembles lead to conditioned fear expression, indicating that these neurons were responsible for the fear memory[44]. Interestingly, 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[44-46]. These results, however, cannot 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 Extinguished CS compared to VNS + Extinction. However, Extended Extinction rats do not show extinction to the Non-Extinguished CS.

Taken together, these findings indicate that there is a qualitative difference in VNS-influenced extinction and extinction achieved following extended exposure, despite the comparable levels of freezing to the Extinguished CS seen twenty-four hours after the extinction session. The degree of memory displayed at this time point does not reveal all of the facets of extinction memory, such as duration and generalization. In addition to facilitating extinction of conditioned fear, VNS-enhanced extinction is also 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. The generalization of learning is seen with other memory enhancing treatments such as administration of histone deacetylase inhibitors[47]. Similarly, the generalization of extinction is seen with other methods that enhance memory consolidation, such as mild stress and administration of D-cycloserine[23-24]. It is well-established that VNS can enhance memory consolidation in rats and humans and it also facilitates experience-dependent plasticity[48-59]. Recent work suggests that VNS enhances plasticity in the pathway from the infralimbic area of

the prefrontal cortex (IL) to the basolateral complex of the amygdala (BLA), brain regions that are involved in fear extinction. VNS-induced changes in this pathway differ from Extended Extinction alone. Although both groups reached similar levels of conditioned fear responses following extinction, administration of VNS paired with extinction predisposed synapses in the pathway from the IL to the BLA to the formation of LTP following high-frequency stimulation (HFS). In contrast, HFS did not lead to LTP in this pathway in rats subjected to Extended Extinction[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 including a reduction in expression of the activity-regulated cytoskeleton-associated protein (ARC) in the BLA[28]. These VNS-induced changes indicate that there can be lasting changes that are specific to rats given VNS despite comparable levels of conditioned fear responding across groups.

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 [53] and increases levels of norepinephrine in the amygdala[51], and intra-BLA infusions of norepinephrine enhance the consolidation of extinction memory in rats[60]. Similarly, VNS increases levels of brain-derived neurotrophic factor (BDNF) in the prefrontal cortex[61]. BDNF is important for memory consolidation and fear extinction[62-63]. BDNF engages downstream effectors and pathways related to plasticity, including Arc expression[64], which is altered following VNS-paired extinction[28]. VNS also increases phosphorylation of several sites on the BDNF receptor, tyrosine receptor kinase B (TrkB)[65]. Furthermore, multiple studies have demonstrated that infusion of BDNF into the prefrontal cortex is sufficient to generate extinction of fear, even without training[66-67]. Given that BDNF is sufficient to generate extinction without

training, and administration of VNS leads to increases in BDNF and TrkB expression, it is possible that BDNF contributes to generalization of extinction seen in response to VNS + Extinction, but not Extended Extinction. However, because VNS alone did not promote extinction, it is likely that a combination of VNS-induced effects relating to synaptic plasticity are responsible for the observed generalization of extinction.

Here, we found that VNS paired with extinction led to generalization of extinction for stimuli that co-occurred during AFC, whereas Extended Extinction training did not. VNS effects were not explained by enhanced contextual extinction, counter-conditioning, or a reduction in general anxiety. Co-occurrence of stimuli during AFC was necessary for extinction generalization, indicating that VNS influenced the extinction of conditioned cues that were associated with each other in time and context. VNS has been used in tens of thousands of patients with drug-resistant epilepsy[68]. VNS produces a more rapid and robust extinction of conditioned fear in rats[28-31]. Here, we report that VNS can lead to generalization of extinction, which may make exposure-based therapies more efficient and protect patients from cue-induced relapses elicited by reminders of a trauma not addressed during exposure-based therapy. Taken together, these results indicate that VNS has promise as an adjunct treatment during exposure-therapy to enhance extinction and increase treatment success.

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CHAPTER 5

CONCLUSIONS

Vagus nerve stimulation (VNS) enhances extinction of conditioned fear. Following VNS-paired extinction training, extinction memories are more rapid, more permanent, and more broadly tuned. VNS still expedites extinction of conditioned fear in a rat model of pathological fear that is resistant to extinction, and reduces PTSD-like symptoms one week later. In addition to VNS-induced changes in learning and memory, administration of VNS led to a reduction in anxiety. This unique combination of effects is could provide benefits over currently available pharmaceutical adjuncts. VNS-induced enhancements in memory consolidation could improve treatment success while the anxiolytic effects could improve patient tolerability during treatment. These preclinical data provide evidence that VNS could be considered as an adjunctive treatment during exposure-based therapies for anxiety-related disorders.

Chapter 3 of this dissertation examined the ability of VNS to enhance extinction even in a rat model of pathology. Using the single prolonged stress (SPS) rat model of posttraumatic stress disorder (PTSD), we found that though SPS-treated rats were resistant to extinction, VNS was sufficient to enhance extinction to levels of healthy controls. We also found that VNS administration during extinction led to an extinction memory that was resistant to reinstatement of conditioned fear, a form of relapse. Furthermore, following VNS-enhanced extinction of conditioned fear, SPS-treated rats showed a reversal of PTSD-like symptoms (outlined in Chapter 2).

VNS administration during exposure to the conditioned stimulus enhanced extinction and reduced reinstatement of conditioned fear

Rats subjected to the SPS protocol followed by auditory fear conditioning (PTSD Model rats) show impaired extinction of conditioned fear versus rats subjected to auditory fear conditioning alone (Adaptive Fear rats). Administration of VNS paired with the extinction tones enhanced extinction of conditioned fear in both PTSD Model rats and in Adaptive Fear rats. At the completion of extinction training, PTSD Model rats who received VNS reached remission of fear whereas PTSD Model rats who received sham stimulation during extinction never reached remission of fear. Additionally, following a single presentation of the footshock in the absence of any tone, VNS-treated rats were protected against reinstatement of conditioned fear. PTSD model rats who received VNS showed equivalent levels of reinstatement to those demonstrated by Adaptive Fear rats. In contrast, PTSD model rats given sham stimulation during extinction showed complete reinstatement, where levels of conditioned fear returned to the original levels prior to any extinction training.

VNS administration during extinction sessions reduced PTSD-like symptoms

One week after VNS- or sham-paired extinction, a subset of rats from each group were subjected to a battery of behavioral tests to assess PTSD-like symptoms seen in the SPS rat model of PTSD. We found that PTSD Model rats given sham stimulation showed elevated anxiety on the elevated plus maze. Administration of VNS during extinction reversed the PTSD-like symptom of heightened anxiety even one week following the completion of treatment. Interestingly, administration of VNS during extinction also reduced anxiety in Adaptive Fear rats. VNS administration during extinction also reduced startle amplitudes during an acoustic startle

response test in both Adaptive Fear rats and in PTSD Model rats. PTSD Model rats given sham stimulation during extinction showed heightened novel object avoidance during a marble burying task versus Adaptive Fear rats. VNS administration during extinction reversed the abnormal object avoidance, PTSD Model rats given VNS during extinction performed similarly to Adaptive Fear rats. Finally, PTSD Model rats given sham stimulation show social withdrawal during a social interaction task, such that the PTSD Model prefer to spend time in an empty compartment rather than with a novel rat. Administration of VNS during extinction reversed this negative social preference, and PTSD Model rats given VNS performed similarly to Adaptive Fear rats on the social interaction task.

Insights and Implications

The PTSD Model rats studied in Chapter 3 provide more complete insights into PTSD-like symptoms seen in patients. Though there are numerous animal models for PTSD[1-5], it is common for these models to focus on one specific PTSD-like symptom. According to the DSM-V, PTSD patients need to exhibit a variety of symptoms prior to diagnosis, including: intrusion symptoms, alterations in arousal or reactivity, avoidance, and alterations in cognition or mood[6]. The PTSD Model rats show PTSD-like symptoms from each of the criteria clusters required for PTSD diagnosis in patients. Though it is not clear if rats can experience PTSD as humans can, more complete animal models can provide better insights into the pathophysiology of PTSD and can hopefully lead to developments of new therapeutics to treat the disorder.

The data presented in Chapter 3 expand what is known about VNS paired with extinction, indicating that VNS can be a useful tool even in models of pathology. Even in rats with impaired extinction learning, VNS accelerated extinction to levels of healthy controls. Interestingly, these

data also provide evidence that enhancing extinction learning can alleviate additional PTSD-like symptoms without additional treatment. Even one week following VNS-paired extinction, PTSD Model rats show improvements in PTSD-like symptoms. These data support the hypothesis of many clinicians that extinction impairments are the underlying source of PTSD symptoms in patients[7-12]. If extinction impairments are the basis for PTSD symptoms, then enhancing extinction should treat the source of the disorder, leading to remission of other PTSD symptoms without treatment. Given this hypothesis, currently pharmacological options that enhance memory consolidation are used in an attempt to treat the source of PTSD by enhancing extinction of conditioned fear[13-20]. However, current pharmacological options cannot be delivered with temporal precision. Since drugs like DCS cannot be temporally controlled following administration, VNS has an advantage in that it can be turned off if needed. Furthermore, VNS has also been reported to reduce anxiety[21]. A reduction in anxiety during exposure-based therapies could make therapy more tolerable for patients. VNS appears to have a unique combination of effects where it can both enhance memory consolidation, while also reducing anxiety. This combination could be useful in the treatment of anxiety-related disorders, including PTSD.

Chapter 4 of this dissertation examined the ability of VNS to make extinction more broadly tuned by determining if VNS could enhance extinction of multiple stimuli related to a single fear event. Rats were subjected to auditory fear conditioning where two discriminable stimuli were paired with footshocks. Extinction training where only one of the conditioned stimuli was presented was given paired with VNS or sham stimulation. We found that stimuli that were presented during the same fear conditioning session were susceptible to VNS-induced

generalization of extinction. VNS could promote generalization of extinction where both stimuli were extinguished, whereas sham stimulation could only promote extinction of the single stimulus presented. Additionally, we found that the original fear memory for each stimulus needed to take place during the same conditioning session to be susceptible to VNS-induced generalization of extinction. When fear conditioning took place on different days, or in different contexts, VNS did not promote generalization of extinction.

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

Following AFC where both conditioned stimuli were interleaved, rats showed equivalent levels of conditioned fear to both stimuli. During extinction, rats were only exposed to one of the conditioned stimuli (Extinguished CS). Rats given 20 presentations of the Extinguished CS with sham stimulation (Extended Ext rats) showed reduced levels of conditioned fear for the Extinguished CS, but not for the stimulus that was not given during extinction (Non-Extinguished CS). Four presentations of the Extinguished CS paired with VNS (VNS + Ext) was sufficient to generate similar levels of freezing to the Extinguished CS compared to Extended Ext rats, as VNS enhances extinction. Interestingly, VNS + Ext rats not only showed reduced freezing to the Extinguished CS, but they also showed reduced freezing to the Non-Extinguished CS, indicating that extinction generalized to the other stimulus that was presented during the fear conditioning session.

VNS effects on generalization of extinction are specific

To exclude the possibility of confounding effects of VNS on performance, we performed additional tests to determine the specificity of the generalization effect. We determined that the effects of VNS on generalization of extinction were not due to a general reduction in freezing

from VNS, as rats given VNS in the home cage in lieu of extinction did not show reduced freezing to either conditioned stimulus. Generalization of extinction also could not be explained by enhanced extinction to the context, as VNS + Ext rats and Extended Ext rats spent equivalent amounts of time freezing to the extinction context prior to tone presentation. We also excluded the anxiolytic effects of VNS as the mechanism for generalization of extinction. VNS led to a reduction in anxiety on the elevated plus maze. VNS-induced reduction in anxiety could be blocked by systemic administration of methyl-scopolamine (methyl-scop). When methyl-scop was given prior to VNS- or sham-paired extinction, such that there was no longer an anxiolytic effect of VNS, rats still showed enhanced extinction even when methyl-scop was on board. This result indicates that VNS-induced reduction in anxiety does not contribute to the VNS-induced extinction enhancement.

VNS-induced generalization of extinction does not extend to stimuli conditioned on separate days or in separate contexts

To determine the effects of how the original fear memory was conditioned on the susceptibility for generalization, we performed two additional experiments separating fear conditioning first by time, and subsequently by context. When fear conditioning of two distinct auditory stimuli was separated by time (24 hours), VNS during extinction no longer promoted generalization of extinction. VNS + Ext rats showed only a reduction in conditioned fear to the Extinguished CS, similar to Extended Ext rats. Similarly, when fear conditioning was separated by context, such that fear conditioning to one stimulus took place in context A and then, within the same hour, fear conditioning to the other stimulus took place in context C, VNS did not promote

generalization of extinction. VNS + Ext rats only showed a reduction of conditioned fear for the Extinguished CS, similar to Extended Ext rats.

Insights and Implications

Since VNS promotes extinction generalization, but the effect is attenuated when presentations of each CS are not co-occurring, there is likely an important effect on the way the fear memory is consolidated which influences the susceptibility for extinction generalization. The concept of and search for a memory engram, a specific change in the brain where a memory exists, have been publicized and debated since the term was first used by Richard Semon in 1921[22]. Interest in the search for the engram continued to grow following early work by Karl Lashley involving lesions in entire brain regions meant to disrupt memories[23]. Since the early 1950's advancements in technology available for spatial, temporal, and cell-type precision have advanced the search for the engram from entire brain regions to specific cells; recent evidence indicates that groups of neurons, neuronal ensembles, could be the physical memory trace. Using new methods, these ensembles thought to underlie a memory can be tagged and manipulated. Activation or inactivation of the specific ensemble cells can be used to determine if those specific cells are necessary and/or sufficient to activate the memory[24-27]. Of specific interest to this paper, recent work using optogenetic stimulation of small neuronal ensembles lead to conditioned fear expression, indicating that these neurons were responsible for the fear memory[28]. Interestingly, neuronal ensembles for similar experiences that happen closely in time can overlap. However, following the passage of time or the changing of context, the memories are represented by separate neuronal ensembles, indicating that memory engram cells are timing and context-specific[28-30]. When fear conditioning co-occurs, neuronal ensembles that represent fear memory

overlap; separation of conditioning leads to separation of these neuronal ensembles. This change in fear memory representation could explain why co-occurring stimuli are susceptible to extinction generalization whereas stimuli separated by time or context are not. Although overlapping neural ensembles can explain why only certain memories are susceptible to generalization of extinction, they fail to explain the effect of VNS on extinction generalization. VNS alters levels of neuromodulators in brain regions important for extinction of conditioned fear. Notably, VNS alters levels of BDNF expression in the prefrontal cortex[31], as well as phosphorylation of sites on the BDNF receptor, TrkB[32]. BDNF is of particular interest as it is implicated in memory consolidation and fear extinction[33-34]. Administration of BDNF directly into the prefrontal cortex was found to generate extinction, even without extinction training[35-36]. It is possible that, when memories are linked, VNS is sufficient to increase BDNF concentrations in the prefrontal cortex to drive extinction of the Non-Extinguished CS, even without extinction training. Evidence that VNS Alone administered in the home cage in lieu of extinction is not sufficient to generate extinction seems to contradict this hypothesis. However, it is possible that extinction training increases levels of BDNF, and that VNS paired with extinction causes an additional increase in BDNF, such that BDNF levels would be sufficient to generate extinction without training.

The results in Chapter 4 could seem to contradict what is known about the temporal- and stimulus-specificity of VNS. However, if the neural ensembles are overlapping, VNS could still be having stimulus-specific effects which carry-over to the Non-Extinguished CS. Evidence that VNS promotes generalization of extinction for multiple stimuli relating to a single fear experience is exciting, as patients undergoing exposure-based therapies could benefit from

complete extinction of all cues related to their trauma. It is also exciting that the VNS effect on extinction generalization does not carry over to conditioned fears that are not associated with the same experience, as treatment should aim to target conditioned fears associated with a traumatic experience rather than all learned fears. Taken together, these findings indicate that VNS could enhance extinction in exposure-based therapies, but leave adaptive fears intact.

Overall conclusion

The contents of this dissertation provide evidence that VNS could be a useful adjunct for exposure-based therapies, as VNS can make extinction more rapid, more permanent, and more broadly tuned. This combination of effects on extinction could enhance treatment success during exposure-based therapies, reduce the incidence of relapse, as well as potentially shorten the duration of therapy, which could reduce dropout and non-compliance. VNS can also reduce anxiety, which could make exposure-based therapies more tolerable and reduce patient dropout. It is my hope that preclinical studies like those listed here will allow the transition of VNS into the clinic for use in patients with anxiety-related disorders.

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BIOGRAPHICAL SKETCH

Lindsey Noble grew up in Verona, New York, a small town in Upstate New York, born to Stacey Tiller and David Noble. She did her undergraduate work at The College of Saint Rose in Albany, New York and graduated with a Bachelor's degree in psychology with a concentration in neuroscience. During her freshman year of college, she became involved in the neuroscience lab of Dr. Robert Flint studying learning and memory, specifically memory reconsolidation. After graduation from Saint Rose in May 2013, Lindsey moved to Dallas and started working towards her PhD in Dr. Christa McIntyre's lab in June 2013. Her PhD work is still in learning and memory, studying the use of vagus nerve stimulation as a novel therapeutic treatment to enhance extinction learning for utility in disorders such as posttraumatic stress disorder.

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The College of Saint Rose Summer Research Grant – *Effects of MK-801 on Memory Reconsolidation in Olfactory Discrimination in Rats*. Summer, 2012.

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Peer Reviewed Publications (Chronological Order)

Chown AE, Noble LJ, & Flint RW Jr. (2010). Traumatic brain injury and memory loss: A review of non-human animal models. *Journal of Behavioral and Neuroscience Research, 8*(1), 17-28.

Flint RW Jr, Foti J, Noble LJ, & Ulmen A. (2011). Effects of the β- adrenergic antagonist propranolol or the protein synthesis inhibitor cycloheximide on reconsolidation of

auditory fear conditioning or spatial memory, respectively, in adolescent rats. *Journal of Articles in Support of the Null Hypothesis, 7*(2), 43-56.

Flint RW Jr, Noble LJ, & Ulmen A. (2013). NMDA receptor antagonism with MK-801 impairs consolidation and reconsolidation of passive avoidance conditioning in adolescent rats: Evidence for a state-dependent reconsolidation effect. *Neurobiology of Learning and Memory.*

Ketcherside A, Noble LJ, McIntyre CK, Filbey FM. (2017). Cannabinoid receptor 1 gene by cannabis use interaction on CB1 receptor density. *Cannabis and Cannabinoid Research.*

Noble LJ, Gonzalez IJ, Meruva VB, Callahan KA, Belfort BD, Ramanathan KR, Meyers E, Kilard MP, Rennaker RL, McInyre CK. (2017). Effects of vagus nerve stimulation on extinction of conditioned fear and posttraumatic stress disorder symptoms in rats. *Translational Psychiatry.*

Souza RR, Noble LJ, McIntyre CK. (2017). Using the single prolonged stress model to examine the pathophysiology of PTSD. *Frontiers in Pharmacology.*

Noble LJ, Meruva VB, Chuah A, Hays SA, Kilgard MP, McIntyre CK. (Under Review). Vagus nerve stimulation generalizes extinction to multiple conditioned stimuli presented during Pavlovian fear conditioning. *Journal of Neuroscience*

Presentations (Chronological Order)

Noble LJ & Flint RW Jr. (March, 2010). *Effects of NMDA antagonism on consolidation and reconsolidation of a 1-trial inhibitory avoidance task in juvenile Sprague-Dawley rats*. Poster presented at the 81st annual Eastern Psychological Association meeting, Manhattan, NY.

Foti J, Ulmen A, Noble LJ, & Flint RW Jr. (March, 2011). *Systemic administration of propranolol fails to disrupt reconsolidation of auditory fear conditioning in adolescent rats*. Poster presented at the 82nd annual Eastern Psychological Association meeting, Cambridge, MA.

Ulmen A, Noble LJ, & Flint RW Jr, (March, 2011). *MK-801-induced reconsolidation impairment for inhibitory avoidance conditioning in adolescent rats is state-dependent.* Poster presented at the 82nd annual Eastern Psychological Association meeting, Cambridge, MA.

Ulmen A, Noble LJ, & Flint RW Jr. (March, 2011). *NMDA antagonism with MK-801 disrupts immediate post-training and post-reactivation processing: Evidence for*

state-dependent memory reconsolidation. Poster presented at the National Conference on Undergraduate Research, Ithaca, NY.

Noble LJ, Foti J, & Flint RW Jr. (April, 2011). *Effects of systemic administration of the protein synthesis inhibitor cycloheximide on acquisition, consolidation, and retrieval of habituation of the acoustic startle response in adult male Sprague-Dawley rats*. Poster presented at the 2nd annual College of Saint Rose Undergraduate Research Symposium, Albany, NY.

Staulo T, Foti J, Noble LJ, & Flint RW Jr. (April, 2011). *Effects of systemic administration of D-glucose on consolidation and reconsolidation of habituation of the acoustic startle response in adult male Sprague- Dawley rats*. Poster presented at the 2nd annual College of Saint Rose Undergraduate Research Symposium, Albany, NY.

Noble LJ & Flint RW Jr. (March, 2012). *Effects of ketamine and PCP on acquisition, consolidation and retrieval of habituation of the acoustic startle response in Long-Evans rats*. Poster Presented at the 83rd annual Eastern Psychological Association meeting, Pittsburgh, PA.

Karow M, Noble LJ, & Flint RW Jr. (March, 2013). *Scopolamine and consolidation of inhibitory avoidance conditioning in rats.* Poster presented at the 84th annual Eastern Psychological Association meeting, NY.

Noble LJ & Flint RW Jr. (March, 2013). *Effects of MK-801 on consolidation/reconsolidation of an olfactory discrimination memory in rats.* Poster presented at the 84th annual Eastern Psychological Association meeting, NY.

Flint RW Jr, Staulo T, Foti J, & Noble LJ. (March, 2013). *Effects of systemic administration of D-glucose on consolidation and reconsolidation of habituation of the acoustic startle response in adult male Sprague-Dawley rats*. Poster presented at the 84th annual Eastern Psychological Association meeting, NY.

Noble LJ & McIntyre CK. (February, 2014). *Effects of acute vagus nerve stimulation on anxiety in rats.* Poster presented at the 18th annual Institution for Neuroscience Symposium at The University of Texas at Austin, Austin, TX.

Noble LJ, Ramananthan KR, Gonzalez IJ, Belfort BD, McIntyre CK. (June, 2014). *Effects of vagus nerve stimulation on anxiety in rats*. Poster presented at the UT Dallas Emotional Learning and Memory Conference, Richardson, TX.

Noble LJ, Ramananthan KR, Gonzalez IJ, Belfort BD, McIntyre CK. (November, 2014). *Effects of vagus nerve stimulation on anxiety in rats.* Poster presented at the annual Society for Neuroscience meeting, Washington D.C.

Noble LJ, Ramananthan KR, Gonzalez IJ, Belfort BD, McIntyre CK. (March, 2015). *Effects of vagus nerve stimulation on anxiety in rats.* Poster presented at the 7th Annual Institute for Neuroscience Symposium, College Station, TX.

Noble LJ, Gonzalez IJ, Ramanananthan KR, Meyers E, Belfort BD, Malhotra S, Rennaker RL II, & McIntyre CK (April, 2015). *Vagus nerve stimulation enhances extinction of conditioned fear in an animal model of PTSD.* Poster presented at the UT Austin Learning and Memory Conference, Austin, TX. *Poster awarded best poster presentation

Noble LJ, Gonzalez IJ, Ramanananthan KR, Meyers E, Belfort BD, Malhotra S, Rennaker RL II, & McIntyre CK (August, 2015). *Vagus nerve stimulation enhances extinction of conditioned fear in an animal model of PTSD.* Poster presented at the UT Dallas Pain Symposium, Richardson, TX.

Noble LJ, Gonzalez IJ, Ramanananthan KR, Meyers E, Belfort BD, Malhotra S, Rennaker RL II, & McIntyre CK (September, 2015). *Vagus nerve stimulation enhances extinction of conditioned fear in an animal model of PTSD.* Poster presented at the Annual Pavlovian Society Conference, Portland, OR.

Noble LJ, Gonzalez IJ, Ramanananthan KR, Meyers E, Belfort, BD, Rennaker RL II, McIntyre CK. (October, 2015). *Vagus nerve stimulation enhances extinction of conditioned fear in an animal model of PTSD.* Poster presented at the Annual Society for Neuroscience Meeting, Chicago, IL.

Noble LJ, Gonzalez IJ, Ramanananthan KR, Meyers E, Belfort, BD, Rennaker RL II, McIntyre CK. (April, 2016). *The effects of vagus nerve stimulation on a rat model of PTSD.* Poster presented at the annual Reprogramming the Brain to Health Meeting, Dallas, TX.

Noble LJ, Meruva VB, Kilgard MP, McIntyre CK. (August, 2016). *Vagus nerve stimulation reverses extinction impairments and alters PTSD symptoms in the SPS animal model.* Poster presented at the Texas FreshAIR Neuroscience Conference, Austin, TX.

Noble LJ, Meruva VB, Kilgard MP, McIntyre CK. (September, 2016). *Vagus nerve stimulation reverses extinction impairments and alters PTSD symptoms in the SPS animal model.* Poster presented at the annual Mikiten Graduate Research Symposium, San Antonio, TX.

Noble LJ, Meruva VB, Kilgard MP, McIntyre CK. (September, 2016). *Vagus nerve stimulation reverses extinction impairments and alters PTSD symptoms in the SPS animal model.* Poster presented at the annual Pavlovian Society Meeting, Jersey City, NJ.

Noble LJ, Meruva VB, Kilgard MP, McIntyre CK. (November, 2016). *Vagus nerve stimulation reverses extinction impairments and alters PTSD symptoms in the SPS animal model.* Poster presented at the Annual Society for Neuroscience Meeting, San Diego, CA.

Noble LJ, Meruva VB, Chuah AV, Callahan KA, McIntyre CK. (April, 2017). *Effects of vagus nerve stimulation on a rat model of PTSD*. Poster presented at the Austin Learning and Memory Conference, Austin, TX.

Noble LJ, Childs JE, Chuah AV, Meruva VB, Kroener S, McIntyre CK. (November, 2017). *Mechanisms of VNS-induced extinction enhancement and PTSD symptom reduction in rats*. Poster presented at the Annual Society for Neuroscience Meeting, Washington, D.C.

Professional Association Memberships

Society for Neuroscience
Pavlovian Society
Psi Chi – The International Honor Society in Psychology
Eastern Psychological Association
Council for Undergraduate Research

Professional Awards and Achievements

Best presentation award, 2015 – Poster selected as best presentation at the UT Austin Learning and Memory conference

"Hot Topic" in neuroscience, 2015 – Poster selected as part of the top 1% of newsworthy abstracts out of 31,000 attendees at the annual Society for Neuroscience meeting

"Hot Topic" in neuroscience, 2016 – Poster selected as part of the top 1% of newsworthy abstracts out of 30,300 attendees at the annual Society for Neuroscience meeting

3 Minute Thesis winner, 2016 – 3MT presented at The University of Texas at Dallas and selected as a winner