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The Interval Between VNS-Tone Pairings Determines the Extent of Cortical Map Plasticity

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Abstract—Repeatedly pairing vagus nerve stimulation (VNS) with a tone or movement drives highly specific and long-lasting plasticity in auditory or motor cortex, respectively. Based on this robust enhancement of plasticity, VNS paired with rehabilitative training has emerged as a potential therapy to improve recovery, even when delivered long after the neurological insult. Development of VNS delivery paradigms that reduce therapy duration and maximize efficacy would facilitate clinical translation. The goal of the current study was to determine whether primary auditory cortex (A1) plasticity can be generated more quickly by shortening the interval between VNS-tone pairing events or by delivering fewer VNS-tone pairing events. While shortening the inter-stimulus interval between VNS-tone pairing events resulted in significant A1 plasticity, reducing the number of VNS-tone pairing events failed to alter A1 responses. Additionally, shortening the inter-stimulus interval between VNS-tone pairing events failed to normalize neural and behavioral responses following acoustic trauma. Extending the interval between VNS-tone pairing events yielded comparable A1 frequency map plasticity to the standard protocol, but did so without increasing neural excitability. These results indicate that the duration of the VNS-event pairing session is an important parameter that can be adjusted to optimize neural plasticity for different clinical needs.

Key words: VNS, vagal, plasticity, auditory cortex, interval between stimulations, acoustic trauma.

INTRODUCTION

Repeatedly pairing vagus nerve stimulation (VNS) with tones or movements drives highly specific and long-lasting cortical plasticity. For example, VNS-tone pairing expands the region of primary auditory cortex (A1) that responds to the paired tone frequency (Engineer et al., 2011; Borland et al., 2015). Pairing a rapid train of tones with VNS increases the number of A1 neurons that can respond to rapidly presented sounds (Shetake et al., 2012). VNS-speech pairing expands the region of A1 that responds to the paired English words (Engineer et al., 2015). Pairing movement with VNS expands the region of primary motor cortex that generates the paired movement (Porter et al., 2012; Hulsey et al., 2016). Based on this robust enhancement of plasticity, VNS has emerged as a potential adjunctive strategy to treat a range of neurological disorders (Hays, 2016).

When VNS is paired with sensory or motor therapy, the enhanced plasticity substantially improves recovery compared to therapy delivered alone, even when delivered long after the neurological insult. Pairing tones with VNS reduces tinnitus symptoms in both animal models and in patients (Engineer et al., 2011; De Ridder et al., 2014, 2015). Pairing physical therapy with VNS improves motor function in both animal models and in patients with chronic stroke (Hays et al., 2013, 2014a; Khodaparast et al., 2014; Dawson et al., 2016).

Given the potential of VNS to enhance the benefits of rehabilitation, it is important to identify parameters that maximize plasticity to boost therapeutic benefits. A recent study demonstrated that the amount of VNS current delivered during tone pairing determines the degree of cortical map plasticity. VNS-tone pairing resulted in auditory cortex map plasticity when moderate

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Abbreviations: CF, characteristic frequency; IP, intraperitoneal; PSTH, post stimulus time histogram; SC, subcutaneous; VNS, vagus nerve stimulation.

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VNS currents were used, but not when higher VNS current levels were used (Engineer et al., 2011; Borland et al., 2015). This observation indicates that the specific VNS parameters paired with particular events determine the degree of plasticity. Likewise, other stimulation parameters may also modulate VNS-tone pairing plasticity.

The extent of neural plasticity and learning are strongly influenced by the interval between individual events (Roberts, 1974; Zhou et al., 2003; Xue et al., 2011; Kornmeier and Sosic-Vasic, 2012; Kornmeier et al., 2014). In the standard VNS protocol used in the preclinical and clinical studies described above, VNS is paired with a sensory or motor event several hundred times per day (Engineer et al., 2011, 2015; Shetake et al., 2012; De Ridder et al., 2014, 2015; Borland et al., 2015). VNS-tone pairing is typically separated by 30 s, so each session requires several hours, which is longer than most clinical therapies.

The goals of the current study were to (1) determine whether it is possible to generate cortical plasticity more quickly by reducing the interval between pairings, (2) determine whether it is possible to generate greater plasticity by increasing the interval between pairings, and (3) determine whether it is possible to normalize neural and behavioral responses following acoustic trauma by decreasing the interval between pairings. The results of this study will be useful for developing the most effective VNS-event pairing parameters for different clinical subpopulations.

EXPERIMENTAL PROCEDURES

Neural activity from 2949 A1 recording sites from 70 female Sprague–Dawley rats (250–370 g) was analyzed in this experiment. Experimental rats were implanted with vagus nerve stimulators, as in our previous studies (Engineer et al., 2011, 2015; Porter et al., 2012; Shetake et al., 2012; Borland et al., 2015). After recovery, the rats were randomly assigned to one of four groups and received VNS-tone pairing for twenty days (Fig. 1). Ten rats received VNS-tone pairing for 40 min per day during which 300 pairings were separated by an average of 8 s. Ten rats received the standard VNS-tone pairing protocol for 150 min per day during which 300 pairings were separated by an average of 30 s. Eight rats received VNS-tone pairing for 600 min per day during which 300 pairings were separated by an average of 120 s. Nine rats received VNS-tone pairing for 25 min per day during which 50 pairings were separated by an average of 30 s. Ten additional rats served as naïve controls. An additional 15 rats experienced VNS-tone pairing following acoustic trauma and eight additional rats served as naïve controls. All rats were housed in a 12:12-h reversed light dark cycle. All handling, housing, stimulation, and surgical procedures were approved by The University of Texas at Dallas Institutional Animal Care and Use Committee.

Vagus nerve surgery

Rats were anesthetized using ketamine hydrochloride (80 mg/kg, intraperitoneal (IP) injection) and xylazine (10 mg/kg IP) and given supplemental doses as needed. Ringer's lactate solution was given to the rats to prevent dehydration throughout the surgery and recovery. Doses of cefotaxime sodium solution (2×10 mg, subcutaneous (SC) injection) were given to the rats before and after the surgery to prevent infection. Rats were implanted with a skull mounted connector. Rats were placed in a stereotaxic frame, and marcaine (1 mL, SC) was injected into the scalp at the incision site. An initial incision and blunt dissection of the scalp exposed the bregma and lambda landmarks on the skull. Four bone screws were manually drilled into the skull, one near the bregma suture, one near the sagittal suture, one near the lambda suture and one over the cerebellum. The connector was attached to the cranial screws with acrylic. The experimental groups of rats were implanted with a custom made cuff electrode around the left vagus nerve as used in previous studies (Engineer et al., 2011, 2015; Porter et al., 2012; Shetake et al., 2012; Borland et al., 2015). As in humans, only the left vagus nerve was stimulated because the right vagus nerve contains efferents that stimulate the sino-atrial node and can cause cardiac complications (Ben-Menachem, 2001). The cuff electrode consisted of two Teflon-coated multi stranded platinum iridium wires connected to a 4-mm section of Micro Renethane tubing. The wires were spaced 1.5 mm apart along the length of the tubing. An 8-mm region of the wires lining the inside circumference of the tube was stripped of insulation. A cut was made lengthwise along the tubing to allow the cuff to be wrapped around the nerve and then closed with silk threads. The impedance of the cuff electrodes was between 1 and 10 kΩ.

Lidocaine (0.5 mL SC) was injected in the neck at the incision site. An incision and blunt dissection of the muscles in the neck exposed the left vagus nerve. The vagus nerve was placed into the cuff electrode, and leads from the electrode were tunneled subcutaneously to the top of the head. Once the leads were connected to the skull-mounted connector, the connector was encapsulated in acrylic. Immediately after surgery, the vagus nerve was stimulated and an oxygen saturation drop was observed to ensure the cuff was working properly. A topical antibiotic cream was applied to both incision sites and the rats were given amoxicillin (5 mg) and carprofen (1 mg) for two days after surgery to prevent infection and facilitate recovery.

VNS-tone pairing

After two to seven days of recovery from surgery, the rats were placed in a 25-cm × 25-cm × 25-cm wire cage, located inside of a 50-cm × 60-cm × 70-cm chamber lined with acoustic insulating foam. Sounds were presented from a speaker hanging above the wire cage. The rats were exposed to a 9-kHz 50-dB SPL tone paired with VNS (Fig. 1). All experimental rats heard the same tone paired with VNS for twenty days. VNS was delivered as a train of fifteen 0.8 mA, 100-μs charge-balanced, biphasic pulses presented at 30 Hz (500 ms train duration). The average interval between stimulations was either 8, 30, or 120 s, depending on

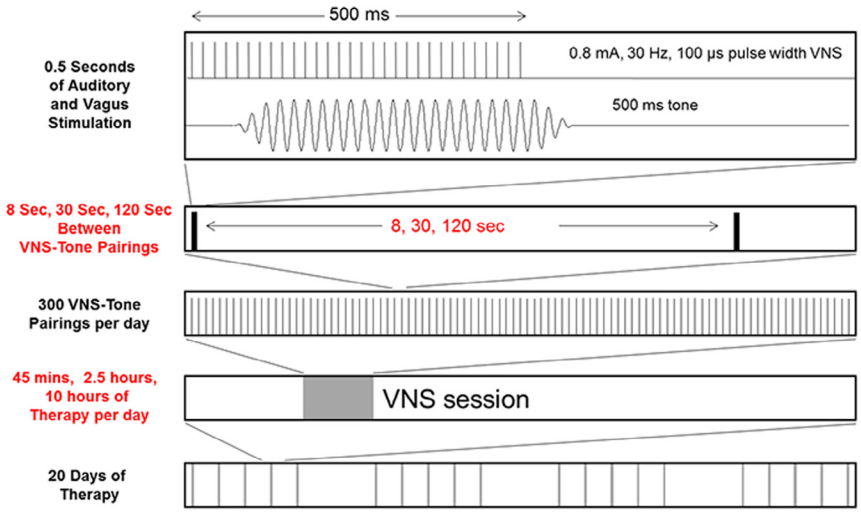

Fig. 1. Schematic diagram of the VNS-tone pairing procedure. A 0.5-s, 30-Hz train of 100-µs wide biphasic pulses was delivered to the left vagus nerve via a cuff electrode. Rats received VNS paired with a 9-kHz tone during 20 daily pairing sessions. The inter-stimulus interval between VNS-tone pairings was either 8, 30, or 120 s. Cortical recordings were made 24 h after the last pairing session.

the experimental group. To prevent rats from anticipating stimulation timing, there was a 50% chance that vagus nerve stimulation was delivered every 4, 15, or 60 s, respectively.

Daily electrode impedance testing was used to test whether each VNS implant was functional, in addition to pulse oximetry both after implantation and following auditory cortex recordings (Zaaimi et al., 2008). Seventeen rats were removed from this study because of surgical death, head cap failure, lead breakage or a lack of a drop in blood oxygen saturation in response to 20 s of VNS under anesthesia.

VNS-tone pairing following acoustic trauma

Fifteen rats were anesthetized with sodium pentobarbital (50 mg/kg) before undergoing 1 h of 16-kHz 115-dB SPL octave band noise exposure, as in previous studies (Engineer et al., 2011). Rats were bilaterally exposed to the noise from a speaker positioned 5 cm from the left ear. The Turner gap detection method was used to quantify behavioral recovery following noise exposure (Turner et al., 2006). Following noise exposure, rats underwent gap detection testing using continuous band-pass filtered sounds centered at 8, 10, and 16 kHz. A 100-dB SPL 20-ms burst of white noise was used to elicit a startle response. In half of the trials, a 50-ms gap in the continuous sound was introduced to serve as a warning of a subsequent white noise startle sound, which reduces the startle amplitude. The gap in the continuous sound does not effectively warn noise-exposed rats, who do not exhibit a reduced startle amplitude.

In noise-exposed rats, VNS was paired with tones that were distinct from the 16-kHz noise-exposed frequency (1.3, 2.2, 3.7, 17.8, and 29.9 kHz). There were 3 experimental groups ($n = 5$ rats/group): (1) VNS exposure using an 8-s inter-stimulus interval (sham therapy), (2) VNS-tone pairing using an 8-s inter-stimulus interval, and (3) VNS-tone pairing using a 30-s inter-stimulus interval. Rats underwent gap detection testing 4 weeks after noise exposure and after 10 days of VNS-tone pairing therapy.

Auditory cortex recordings

Twenty-four hours after the last VNS-tone pairing session, rats were anesthetized with sodium pentobarbital (50 mg/kg). Anesthesia depth was maintained throughout the procedure with a supplemental dose of diluted pentobarbital as needed or every 30–60 min (0.2–0.4 mL, 8 mg/mL). Dehydration was prevented by using a one-to-one ratio of dextrose (5%) and standard Ringer's lactate solution. A tracheotomy was performed to minimize breathing problems and breathing sounds. A cisternal drain was made to minimize cerebral edema. The section of the skull over the temporal ridge was removed to expose the right primary auditory cortex. The dura was removed and the cortex was maintained under a thin film of silicone oil to prevent desiccation. Four parylene-coated tungsten microelectrodes (1.5–2.5 MΩ, FHC) were lowered simultaneously to depths of approximately 600 µm to ensure that they were in layer IV/V of the primary auditory cortex. During the acute electrophysiology recordings, sounds were delivered in a foam-shielded double-walled sound-attenuated chamber via a speaker positioned directly opposite the left ear at a distance of 10 cm. Frequency and intensity calibrations were performed with an ACO Pacific microphone (PS9200-7016) and TDT SigCal software. Multiunit neural activity was captured using a software program (Brainware, TDT) and each recording site location was logged on a detailed digitized photo of the exposed auditory cortex. Auditory frequency tuning curves were determined at each site by presenting tones at 81 logarithmically spaced frequencies spanning 1–32 kHz in 0.125 octave steps at 16 intensities from 0 to 75-dB SPL in 5-dB steps. The tones were randomly interleaved and presented every 500 ms. Experimenters were blinded to the experimental conditions of each rat during electrophysiology recordings. Auditory cortex recordings were obtained in the noise-exposed rats three weeks after the last VNS-tone pairing session. At the conclusion of auditory cortex recordings, the vagus nerve was stimulated and an oxygen saturation drop was observed to ensure the cuff was functional. If VNS failed to elicit a drop in blood oxygen saturation, the recordings were excluded from analysis.

Data analysis

Control and experimental rats were analyzed using an automated MATLAB program that defined the receptive

fields and latency characteristics at each site. The lowest intensity that evoked a reliable neural response was defined as the threshold; the frequency at which the threshold occurred was defined as the characteristic frequency (CF). Four bandwidths were calculated as the range of frequencies that evoked responses at 10, 20, 30 and 40 dB above threshold. A post stimulus time histogram (PSTH) with 1-ms width bins was constructed from all of the responses to tone–intensity combinations within the receptive field. The spontaneous firing rate at each site was estimated as the spike rate in the first 5 ms recorded after tone onset (before any neural response to sounds). The peak latency for each site was calculated as the time of the maximum number of spikes in the PSTH. The onset latency was the first time point in the PSTH when the response strength reached 2 standard deviations above the spontaneous firing rate for a consecutive period of 2 ms. Onset latency was examined only in sites with thresholds above 35-dB SPL to ensure that the presented tones were sufficiently loud to evoke the shortest possible response latency.

Statistical analysis was performed using SPSS software. We tested for and found normal distributions (Shapiro–Wilk test) and equal variances (Levene's test) in our sampled distributions. A repeated measures ANOVA was conducted using tone frequency (1–2, 2–4, 4–8, 8–16, and 16–32 kHz) as the within-subjects variable and the experimental group (Naïve, 8, 30, and 120 s) as the between-subject variable for both the characteristic frequency tuning and area responding analysis. The Hotelling's trace statistic was reported and simple contrast analyses were used to determine whether there were statistically significant differences in response characteristics after VNS-tone pairing. For the receptive field and response strength analysis, mixed-effects models were used to account for the different number of recording sites obtained in each rat. The experimental group was evaluated as a fixed factor, and the individual rats were evaluated as a random factor. All post hoc comparisons were Bonferroni corrected to account for multiple comparisons. To determine whether there is a difference in the probability that an animal will recover (yes or no) based on the startle response during gap detection after VNS-tone pairing, we applied a logistic regression with group (sham therapy, VNS-tone pairing with an 8-s interval, and VNS-tone pairing with a 30-s interval) as an independent categorical variable and recovery as a binary independent variable.

RESULTS

The inter-stimulus interval between VNS-tone pairings alters A1 plasticity

This study was designed to determine whether the interval between VNS-tone pairing events has a significant effect on the extent of cortical map plasticity. Fig. 2a shows the A1 tone frequency map in a representative naive rat. Each polygon represents a single electrode penetration. A1 is tonotopically organized with high CF neurons on the anterior side and low CF neurons on the posterior side (Sally and Kelly, 1988; Kilgard and Merzenich, 1999). In a representative naïve rat, 18% of A1 recording sites (7 out of 38, Fig. 2a) were tuned to frequencies between 8 and 16 kHz. In a representative experimental rat, 35% of A1 sites

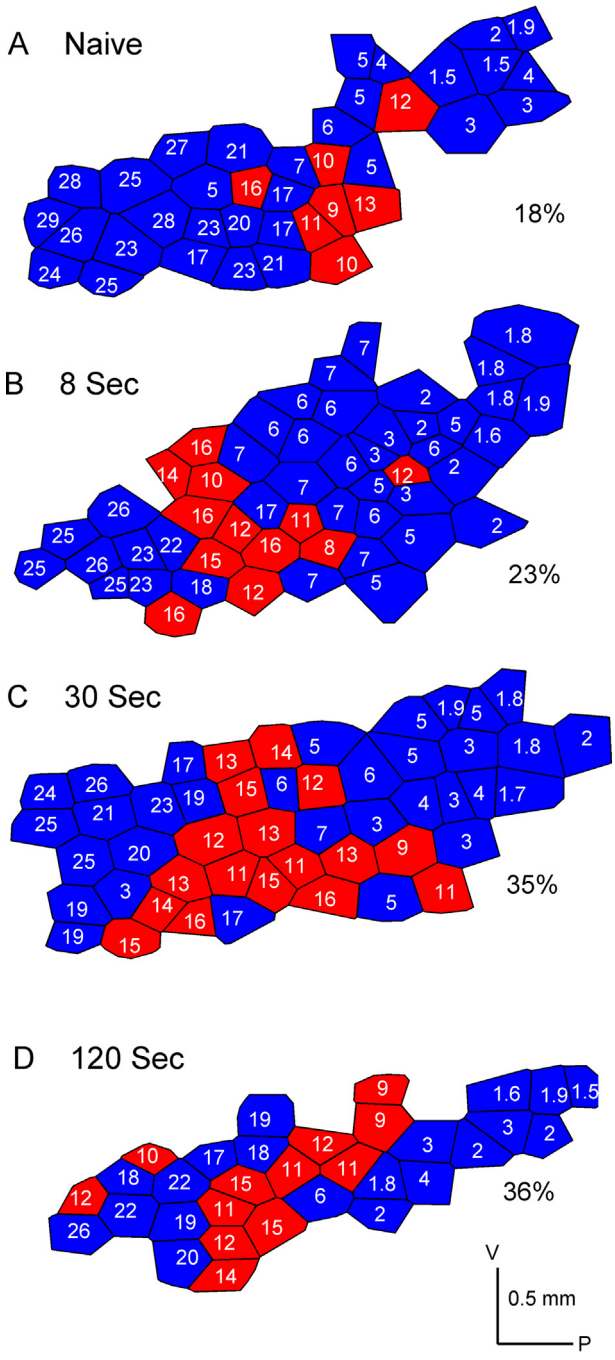

Fig. 2. Frequency map plasticity in primary auditory cortex. Each polygon represents a single electrode penetration. The characteristic frequency (CF) of each site is indicated in kHz. The red color indicates that the value of the CF is between 8 and 16 kHz. (a) Representative frequency map from a naive rat. Representative frequency maps from a rat that received VNS paired with 9-kHz tones with (b) an inter-stimulus interval of 8 s, (c) an inter-stimulus interval of 30 s, and (d) an inter-stimulus interval of 120 s. Map plasticity is greatest with longer inter-stimulus intervals. The scale bar indicates a distance of 0.5 mm. Anterior is shown to the left and dorsal is down.

(17/49) were tuned between 8 and 16 kHz after VNS-tone pairing with an inter-stimulus interval of 30 s (Fig. 2c).

VNS-tone pairing significantly altered the proportion of A1 sites tuned to tone frequencies surrounding the paired tone frequency. There was a significant interaction between experimental group and the tuning frequency ($F(12,89) = 1.85$, $p = 0.05$, Fig. 3). Previous studies have demonstrated that pairing VNS with 9-kHz tones using a 30-s inter-stimulus interval expands the 8–16-kHz region of the A1 tone frequency map (Engineer et al., 2011; Borland et al., 2015). As expected, VNS-tone pairing with an inter-stimulus interval of 30 s significantly increased the proportion of A1 sites tuned between 8 and 16 kHz from an average of 19 ± 3% in naïve animals to an average of 31 ± 3% ($F(1,34) = 8.95$, $p = 0.005$, Fig. 3). A second experimental group of rats experienced a shorter 8 s inter-stimulus interval between VNS-tone pairings. Surprisingly, the degree of map plasticity was not increased with a shorter interval between VNS-tone pairing events ($F(1,34) = 1.46$, $p = 0.24$, Fig. 3). In a representative 8-s VNS paired rat, 23% of A1 sites (12/52) were tuned to frequencies between 8 and 16 kHz (Fig. 2b). This short inter-stimulus interval group experienced VNS-tone pairing for a shorter period of time each day compared to the standard 30-s VNS pairing inter-stimulus interval used in all previous studies of VNS-tone pairing (40 min/day with a VNS-tone pairing inter-stimulus interval of 8 s compared to 150 min/day with a VNS-tone pairing inter-stimulus interval of 30 s). Rats in this short inter-stimulus interval group did not exhibit a statistically significant increase in the proportion of A1 sites tuned between 8 and 16 kHz (19 ± 3% in naïve animals to 24 ± 3% in the 8 s VNS group, Fig. 3). In contrast, rats that received VNS-tone pairing with a longer inter-stimulus interval, with an average of 120 s between pairings (600 min/day), had significantly more A1 sites tuned between 8 and 16 kHz (19 ± 3% in naïve animals to 33 ± 3% in the 120 s VNS group, $F(1,34) = 11.17$, $p = 0.002$, Figs. 2d and 3). These observations confirm the hypothesis that the interval between VNS-tone pairing events influences the degree of cortical plasticity.

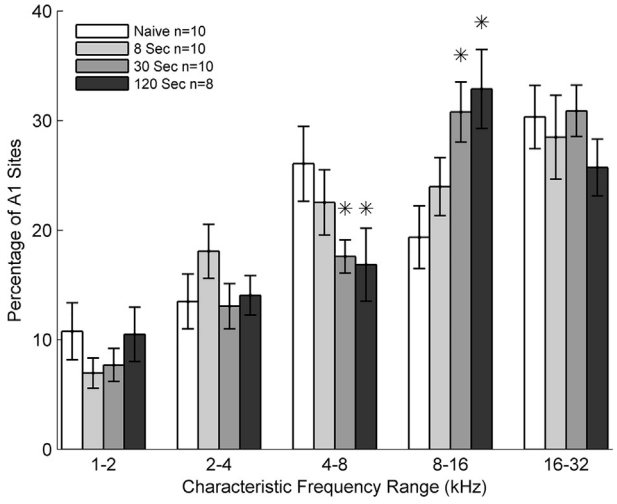

Fig. 3. The percentage of A1 recording sites tuned to each of five one-octave frequency bins. There was a significant shift in tuning between 8 and 16 kHz when VNS was paired with a 9-kHz tone using longer inter-stimulus intervals. Error bars indicate standard error of the mean across rats. Asterisks indicate experimental groups that were statistically significant from the naïve control group ($p < 0.05$).

In addition to quantifying changes in the response to tones at the response threshold, we assessed the proportion of A1 neurons that responded to suprathreshold tones. VNS-tone pairing significantly altered the proportion of A1 sites responding to tone frequencies surrounding the paired tone frequency. There was a significant interaction between experimental group and tone frequency ($F(3,34) = 3.63$, $p = 0.02$, Figs. 4 and 5). Each of the VNS-tone pairing groups of rats exhibited a significantly increased proportion of A1 sites responding to tones between 8 and 16 kHz compared to naïve animals (naïve vs. VNS 8 s group $F(1,34) = 6.31$, $p = 0.02$; naïve vs. VNS 30 s group $F(1,34) = 7.49$, $p = 0.01$; naïve vs. VNS 120 s group $F(1,34) = 4.4$, $p = 0.04$). VNS-tone pairing did not significantly alter the proportion of A1 sites responding to tones distinct from the paired tone frequency. There was no significant difference in the proportion of A1 sites responding to tones between 1 and 2 kHz in any of the experimental groups compared to the naïve group (naïve vs. VNS 8 s group $F(1,34) = 0.14$, $p = 0.71$; naïve vs. VNS 30 s group $F(1,34) = 2.03$, $p = 0.16$; naïve vs. VNS 120 s group $F(1,34) = 4.05$, $p = 0.05$). These results indicate that VNS-tone pairing at multiple inter-stimulus intervals was sufficient to expand the cortical response to the paired high-frequency tone, but was not sufficient to contract the response to low-frequency tones.

VNS-tone pairing did not alter receptive field bandwidth for neurons tuned between 8 to 16 kHz ($F(3,32.3) = 1.17$, $p = 0.34$, Table 1). However, VNS-tone pairing did result in significant alterations to the response onset latency ($F(3,30.18) = 5.39$, $p = 0.004$, Table 1). While the response latency in the 8-s VNS-tone pairing group significantly decreased compared to the naïve group ($p = 0.05$), there was no difference in response latency in the 30-s VNS-tone pairing group ($p = 0.09$) or the 120-s VNS-tone pairing group ($p = 0.99$) compared to the naïve group (Table 1). In addition, VNS-tone pairing did not alter the level of spontaneous A1 firing ($F(3,31.92) = 1.34$, $p = 0.28$, Table 1). Finally, VNS-tone pairing did not alter the driven A1 response rate ($F(3,33.95) = 1.76$, $p = 0.17$, Table 1).

VNS-tone pairing altered the number of action potentials generated by high- and low-frequency tones. There was a significant interaction between experimental group and tone frequency ($F(7,80.49) = 8.12$, $p < 0.0001$, Figs. 6 and 7). Compared to naïve rats, the cortical response strength to 50-dB 8–16-kHz tones was unaltered in the 8-s VNS-tone pairing group ($t(44.08) = -1.03$, $p = 0.31$), the 30 s VNS-tone pairing group ($t(46.64) = -1.8$, $p = 0.08$), and the 120-s VNS-tone pairing group ($t(47.91) = -0.28$, $p = 0.79$, Fig. 7). However, VNS-tone pairing altered the A1 spike firing rate in response to unpaired low-frequency tones

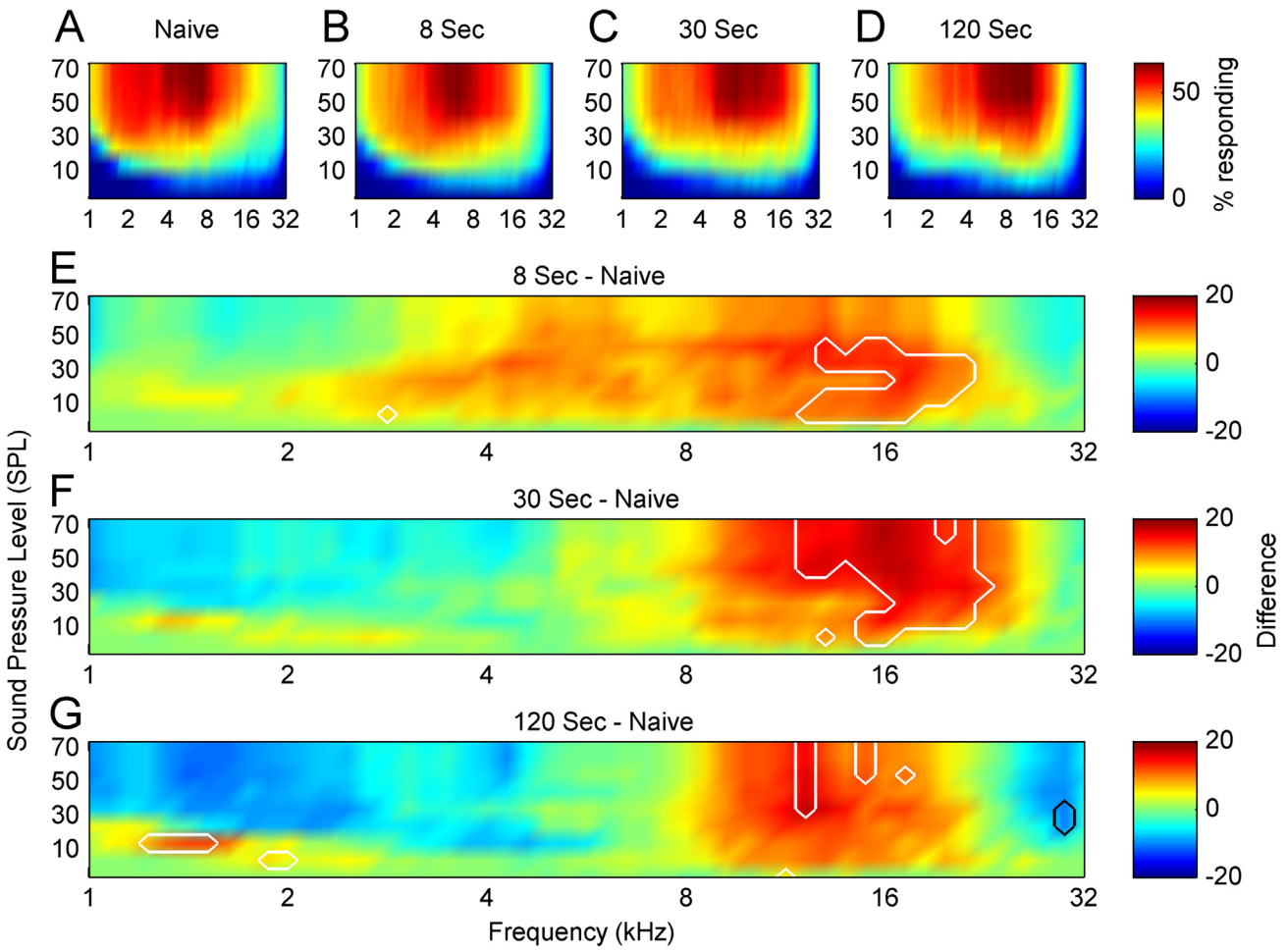

Fig. 4. Percent of A1 neurons responding to each tone frequency intensity combination for (a) naive control rats and for rats that received VNS-tone pairing (b) every 8 s, (c) 30 s, and (d) 120 s. (e-g) The difference in the percent of A1 neurons that respond to tones between VNS-tone paired rats and control rats. Red indicates a greater percent of A1 neurons that respond in VNS-tone paired rats compared to control rats, while blue indicates a decrease in the percent of A1 neurons that respond in VNS-tone paired rats. White lines delineate the frequency intensity combinations which activate significantly more neurons after VNS-tone pairing ($p < 0.01$). Black lines delineate significantly decreased responses ($p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Figs. 6 and 7). Compared to naïve rats, the cortical response strength to 1–2-kHz tones was unaltered in the 8-s VNS-tone pairing group ($t(44.08) = 0.91$, $p = 0.37$) and the 30-s VNS-tone pairing group ($t(46.64) = 0.89$, $p = 0.38$), but was significantly weaker in the 120-s VNS-tone pairing group ($t(47.91) = 3.32$, $p = 0.002$, Fig. 7). Collectively, these results demonstrate that the interval between VNS-tone pairing events can significantly influence map plasticity and the excitability of primary auditory cortex (Fig. 8, Table 2).

Reducing the number of VNS-tone pairings does not enhance A1 responses

In addition to altering the inter-stimulus interval to shorten the therapy protocol, it is also possible to reduce the number of VNS-tone pairings experienced each day. An additional experimental group was used to determine whether A1 plasticity can be generated when delivering fewer VNS-tone pairing events than the standard protocol. This group experienced 50 VNS-tone pairings per day with an inter-stimulus interval of 30 s (25 min/day) compared to the standard 300 VNS-tone pairings per day with an inter-stimulus interval of 30 s (150 min/day). There was a significant interaction between experimental group and tone frequency ($F(8,44) = 2.37$, $p = 0.03$, Fig. 9), although rats who experienced 50 VNS-tone pairings per day did not exhibit a statistically significant increase in the proportion of A1 sites tuned between 8 and 16 kHz compared to naïve rats ($F(1,26) = 0.04$, $p = 0.85$, Fig. 9).

The difference between the proportion of A1 responding to tones between 8 and 16 kHz and tones between 1 and 2 kHz was significantly altered following VNS-tone pairing ($F(4,42) = 3.12$, $p = 0.03$, Fig. 8a). However, the 50 VNS-tone pairing group did not exhibit an alteration in the difference between the proportion of A1 responding to tones between 8 and 16 kHz and tones between 1 and 2 kHz ($p = 0.99$, Fig. 8b).

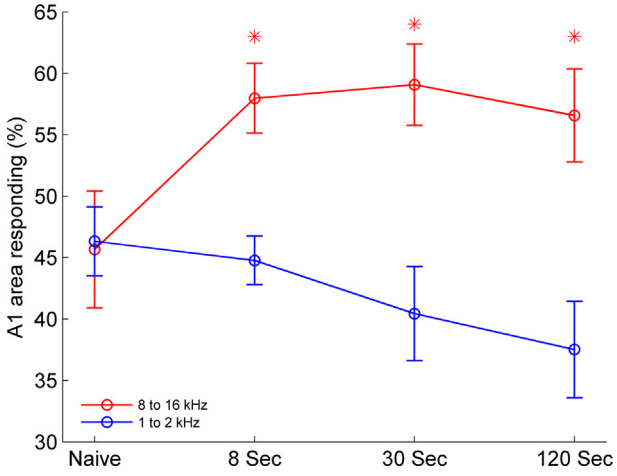

Fig. 5. VNS-tone pairing reorganizes the auditory cortex frequency map. Red indicates the proportion of A1 neurons that respond to 50-dB SPL tones between 8 and 16 kHz. Blue indicates the proportion of A1 neurons that respond to 50-dB SPL tones between 1 and 2 kHz. Error bars indicate standard error of the mean across rats. Asterisks indicate experimental groups that were statistically significant from the naïve control group ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Similarly, the difference in the number of spikes responding to high versus low tones was significantly altered following VNS-tone pairing ($F(4,42.22) = 3.18$, $p = 0.02$, Fig. 8c). The 50 VNS-tone pairing group also did not demonstrate a difference in the number of spikes responding to high versus low tones compared to the naïve group ($p = 0.99$, Fig. 8c). These findings demonstrate that delivering fewer VNS-tone pairings per day does not generate auditory cortex plasticity and suggests that delivering fewer VNS-tone pairings is not an effective method to use to shorten therapy sessions.

The inter-stimulus interval between VNS-tone pairings affects recovery from acoustic trauma

To further examine how the inter-stimulus interval of VNS-tone pairings affects auditory cortex plasticity, we examined both neural and behavioral recovery following acoustic trauma. Following 1 h of 16-kHz 115-dB SPL octave-band noise, it is known that a significantly greater percentage of A1 neurons are tuned to low-frequency tones compared to naïve rats (Engineer et al., 2011). It has previously been demonstrated that pairing VNS with tones using a 30-s inter-stimulus interval reverses auditory cortex damage and restores A1 tonotopy following acoustic noise exposure trauma (Engineer et al., 2011). In the current study, there was a significant difference in the percent of A1 that responds to low-frequency tones across groups ($F(3,16) = 3.34$, $p = 0.046$, Fig. 10a). While there was no significant difference in the percent of A1 that responds to low-frequency tones in naïve rats compared to the 30-s VNS-tone pairing group ($p = 0.48$, Fig. 10a), both the sham therapy group (VNS alone delivered every 8 s, $p = 0.03$) and the 8-s VNS-tone pairing group ($p = 0.04$) had a significantly greater percent of A1 tuned to low frequencies compared to the 30-s VNS-tone pairing group.

Additionally, recovery can be quantified behaviorally using the Turner gap detection method (Turner et al., 2006). The inter-stimulus interval between VNS-tone pairings also affected the recovery of the ability of rats to detect 50-ms gaps embedded in continuous background noise ($\chi^2(2) = 8.46$, $p = 0.015$, Fig. 10b). Only 20% of noise-exposed rats that experienced sham therapy with an 8-s inter-stimulus interval recovered gap detection ability. In contrast, 60% of noise-exposed rats that experienced VNS-tone pairings with an 8-s inter-stimulus interval recovered gap detection ability. 100% of noise-exposed rats that experienced VNS-tone pairings with a 30 s inter-stimulus interval recovered gap detection ability. These findings suggest that longer VNS pairing inter-stimulus intervals may be necessary in order to reliably normalize neural and behavioral responses following acoustic trauma.

DISCUSSION

This study confirms that VNS-tone pairing can generate neural plasticity in auditory cortex. Moreover, a stimulation paradigm with shorter intervals between VNS-tone pairings can drive plasticity in less time than the standard protocol used in previous studies (Engineer et al., 2011; Borland et al., 2015). However, the extent of plasticity was reduced when the VNS-tone pairings were delivered closer in time. Shortening the inter-stimulus interval between VNS-tone pairing events also failed to normalize neural and behavioral responses following acoustic trauma. No A1 plasticity was observed when fewer VNS-tone pairings were delivered. Extending the interval between VNS-tone pairings yielded comparable plasticity to the standard protocol, but did so without increasing neural excitability. The observation that both the number of VNS-pairing events and the interval between them determines the extent of cortical plasticity suggests that careful consideration is needed to develop effective VNS-based targeted plasticity therapies (Hays et al., 2014b).

Table 1. VNS-tone pairing induced receptive field plasticity. Asterisks indicate experimental groups that are significantly different than the naïve group ($p = 0.05$).

	Naive	VNS-tone 8 s	VNS-tone 30 s	VNS-tone 120 s
Bandwidth 30 (octaves)	2.2 ± 0.2	2.4 ± 0.2	2.5 ± 0.2	2.2 ± 0.2
Onset latency (ms)	12.3 ± 0.3	**11.3 ± 0.3***	11.4 ± 0.3	12.7 ± 0.3
Spontaneous (Hz)	21 ± 2.9	19.1 ± 2.8	15.6 ± 2.8	13.3 ± 3.1
Driven rate (spikes/tone)	2.8 ± 0.2	2.9 ± 0.2	2.9 ± 0.2	2.2 ± 0.2

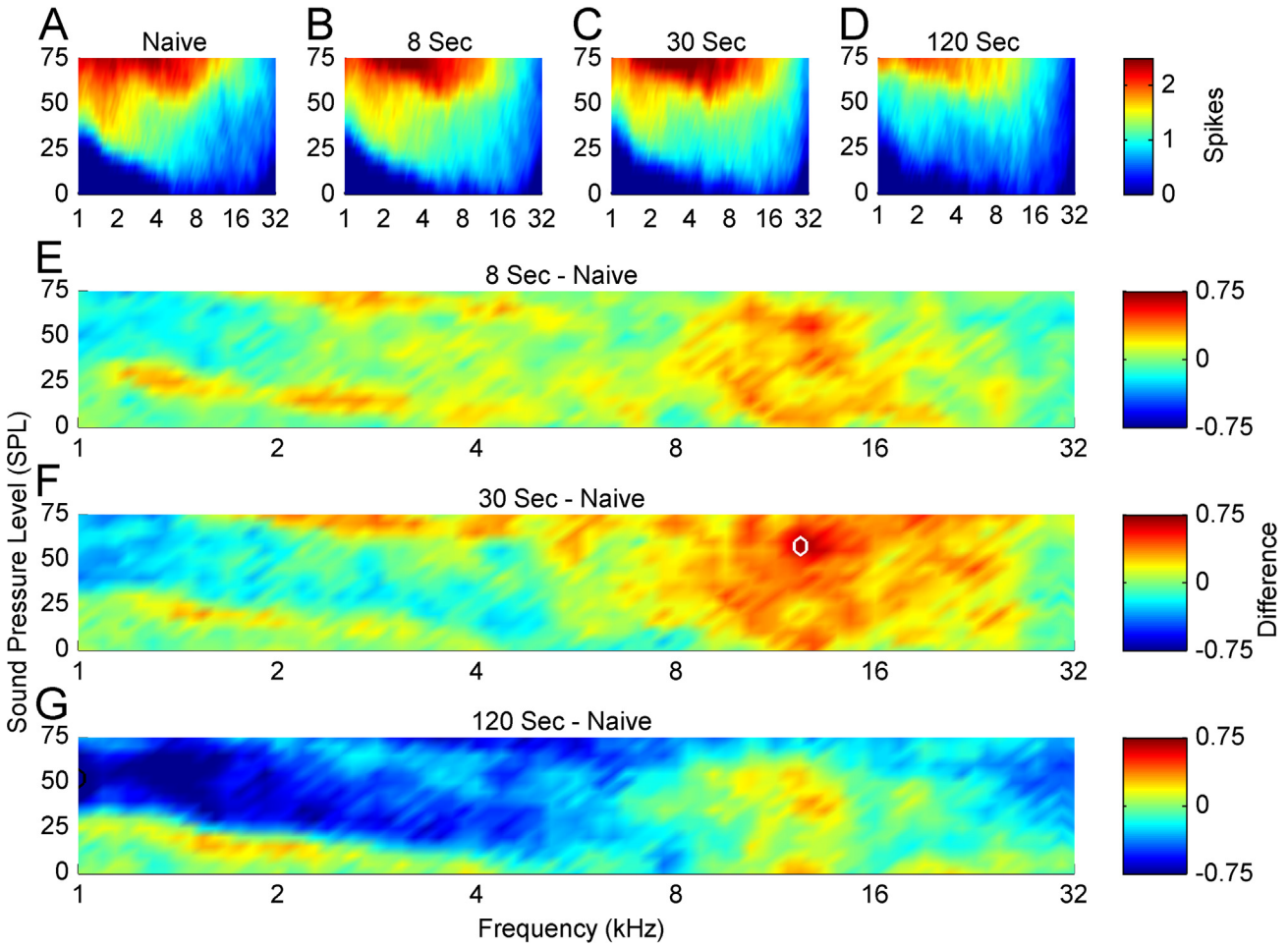

Fig. 6. The average number of A1 action potentials (spikes) evoked by each tone frequency intensity combination for (a) naive control rats and for rats that received VNS-tone pairing (b) every 8 s, (c) 30 s, and (d) 120 s. (e-g) The difference between the number of spikes evoked in experimental rats and control rats reveals the range of tones that evoked a stronger (red) or weaker (blue) response in each of the experimental groups. White lines delineate the frequency intensity combinations which generate significantly more spikes after VNS-tone pairing, while black lines delineate significantly fewer spikes ($p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Long intervals between VNS-tone pairing events may be particularly useful for conditions where reducing excitability is desirable, such as chronic tinnitus (Engineer et al., 2013). Current VNS-based tinnitus therapy requires patients to listen to tones delivered via headphones connected to a computer, which restricts mobility (De Ridder et al., 2014, 2015). Thus, it is not practical for daily therapy sessions to be longer than a few hours. The equivalent number of VNS-tone pairings could be separated by long intervals if VNS is triggered from a mobile device and tones are wirelessly transmitted to an ear piece or hearing aid. Such a strategy would be more convenient and help improve compliance because it would interfere less with daily activities and might be more effective since longer intervals between VNS pairings increase frequency selectivity and decrease excitability, which are associated with reduced tinnitus severity (Engineer et al., 2011).

Longer intervals may not be needed for conditions like recovery from deafness or stroke, where greater neural excitability is likely to be therapeutic (Nudo et al., 1996; Moore and Shannon, 2009; Murphy and Corbett, 2009). Shorter intervals between VNS-movement pairing events may also be desirable to minimize the time commitment and expense of physical therapy. Previous studies of learning and synaptic plasticity show an optimal range of interstimulus intervals to achieve the best behavior or synaptic plasticity outcome (Zhou et al., 2003; Kornmeier et al., 2014). Five-minute intervals generate maximal long-term synaptic plasticity in the developing retinotectal system, while very little long-term synaptic plasticity was observed when very short (seconds) or very long (10 min) intervals were used (Zhou et al., 2003). As with many other aspects of neurorehabilitation, there is likely to be an optimal range of inter-stimulus intervals between VNS-event pairings to maximally enhance plasticity.

The timing of neural activity is a key mediator of plasticity. A number of studies indicate that longer intervals between training events (referred to as spaced training) typically yield faster performance gains than short intervals (massed training) (Cepeda et al., 2006).

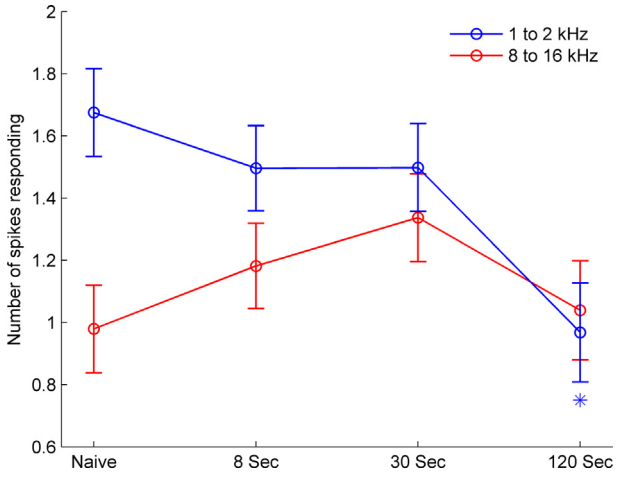

Fig. 7. VNS-tone pairing reduces the number of spikes evoked by low-frequency tones (1–2 kHz, blue), and does not significantly alter the number of spikes evoked by high-frequency tones (8–16 kHz, red). Error bars indicate standard error of the mean across rats. Asterisks indicate experimental groups that were statistically significant from the naïve control group ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

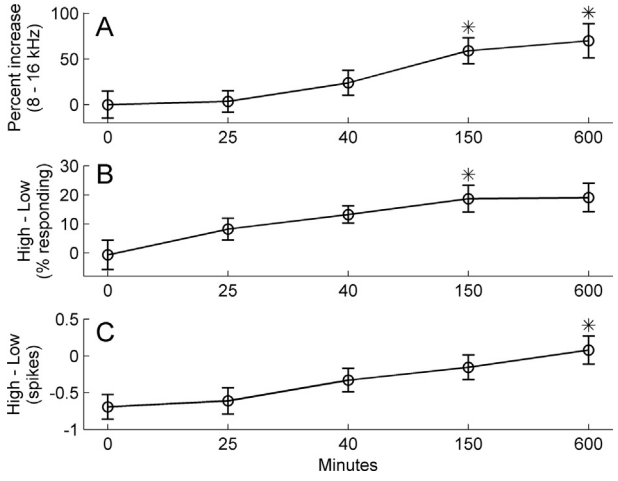

Fig. 8. Summary of the effects of VNS-tone pairing on A1 responses. The degree of cortical plasticity was a monotonic function that increased with longer daily pairing session durations.

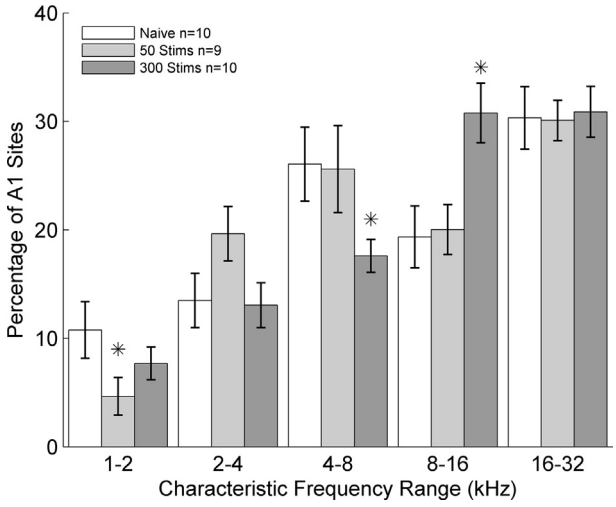

Fig. 9. The percentage of A1 recording sites tuned to each of five one-octave frequency bins. There was a significant shift in tuning between 8 and 16 kHz when VNS was paired with tones 300 times per day, but not when VNS was paired with tones 50 times per day. Error bars indicate standard error of the mean across rats. Asterisks indicate experimental groups that were statistically significant from the naïve control group ($p < 0.05$).

Aligning with these behavioral changes, spaced training produces synaptic modifications more rapidly than massed training (Aziz et al., 2014). Several neuronal mechanisms likely contribute to the influence of timing on plasticity, including engagement of signaling pathways, modulation of protein synthesis, and timing of neuromodulator release (Farah et al., 2009; Philips and Carew, 2009; Naqib et al., 2011, 2012; He et al., 2015). VNS is known to trigger release of acetylcholine, norepinephrine, serotonin, fibroblast growth factor (FGF), and brain-derived neurotrophic factor (BDNF) (Roosevelt et al., 2006; Follesa et al., 2007; Manta et al., 2009; Nichols et al., 2011). These modulators lead to post-synaptic changes in phosphorylation of tropomyosin receptor kinase B (TrkB) and calcium- and calmodulin-dependent protein kinase II (CaMKII) and increased expression of NMDA receptor subunits, cadherin, neurexin, and voltage-dependent calcium channels (Zhang and Zhang, 2002; Furmaga et al., 2012; Alvarez-Dieppa et al., 2016; Alexander et al., 2017). In the present study, we observed that longer intervals between VNS-tone pairings yield greater plasticity than shorter intervals. One plausible explanation for a greater degree of plasticity with longer intervals between VNS-tone pairings is that the longer spacing between events yields more rapid plasticity. In general, our findings are consistent with the notion that timing between events is a critical mediator of the degree of plasticity and point to the need for optimization of VNS-event timing to maximize clinical benefits of

Table 2. Summary of experimental results

	Daily session duration			
	25 min	40 min	150 min	600 min
# of VNS-tone pairings/day	50	300	300	300
Interval between pairings (sec)	30	8	30	120
Map plasticity		+ +	+ + +	+ + +
Excitability			+	+ +

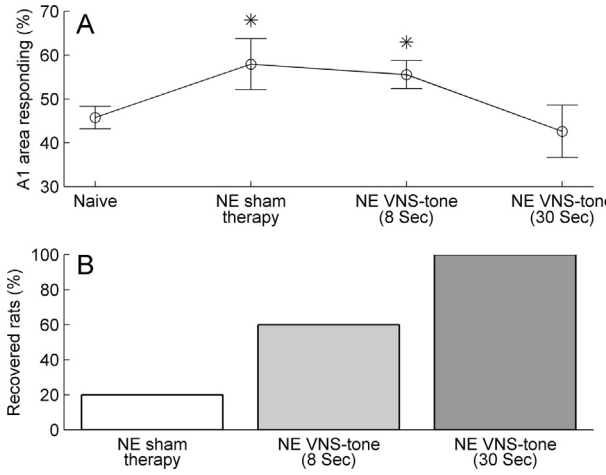

Fig. 10. The short VNS-tone pairing inter-stimulus interval was not sufficient to reverse the neural or behavioral deficits observed following acoustic trauma. (a) Sham therapy and VNS-tone pairing therapy delivered every 8 s were not sufficient to reduce the percent of A1 responding to low-frequency tones. Error bars indicate standard error of the mean across rats. Asterisks indicate experimental noise-exposed (NE) groups that were statistically significant from the 30-s VNS-tone paired group ($p < 0.05$). (b) The inter-stimulus interval between VNS-tone pairings affected the ability of noise-exposed rats to detect 50 ms gaps embedded in continuous background noise.

VNS-based plasticity therapy. The most likely reason that longer intervals between VNS-tone pairing events generate more plasticity and better recovery is that the structural changes that underlie these improvements require many seconds to minutes to develop. Future studies are needed to clarify the cost-benefit of increasing the interval between VNS-tone pairings given that this necessarily results in fewer total pairing events.

The results of the current study suggest that a better mechanistic understanding of VNS-induced plasticity may lead to neural plasticity that is more specific and more rapidly produced. Optimizing the speed, specificity, and extent of VNS-induced plasticity is likely to improve clinical outcomes of VNS-enhanced rehabilitation of chronic tinnitus, stroke, and other serious conditions.

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