Use of a physiological reflex to standardize vagal nerve stimulation intensity improves data reproducibility in a memory extinction assay

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ABSTRACT

Background: Modulating brainstem activity, via electrical vagus nerve stimulation (VNS), influences cognitive functions, including memory. However, controlling for changes in stimulus efficacy during chronic studies, and response variability between subjects, is problematic.

Objective/Hypothesis: We hypothesized that recruitment of an autonomic reflex, the Hering-Breuer reflex, would provide robust confirmation of VNS efficacy. We compared this to measurement of electrode resistance over time. We also examined whether VNS modulates contextual memory extinction.

Methods: Electrodes for VNS and diaphragm electromyography recording were implanted into anesthetized Sprague Dawley rats. When conscious, we measured the electrode resistance as well as the minimum VNS current required to evoke the Hering-Breuer reflex, before, and after, an inhibitory avoidance assay - a two chamber, dark/light model, where the dark compartment was paired with an aversive foot shock. The extinction of this contextual memory was assessed in sham and VNS treated rats, with VNS administered for 30 s at 1.5 times the Hering-Breuer reflex threshold during extinction memory formation.

Results: Assessment of VNS-evoked Hering-Breuer reflex successfully identified defective electrodes. VNS accelerated extinction memory and decreased multiple physiological metrics of fear expression. We observed an inverse relationship between memory extinction and respiratory rate during the behavioural assay. Additionally, no current - response relationship between VNS and extinction memory formation was established.

Conclusion: These data demonstrate that reliable, experimental VNS studies can be produced by verifying reflex initiation as a consequence of stimulation. Further, studies could be standardised by indexing stimulator efficacy to initiation of autonomic reflexes.

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Introduction

The vagus nerve contains the efferent axons of parasympathetic preganglionic neurons and afferent axons of visceral sensory neurons. Vagal efferent (motor) neurons form part of the parasympathetic arm of the autonomic nervous system to modulate visceral organ function. Whereas vagal afferent neurons sense changes in visceral organ function and convey these signals to the brainstem to initiate autonomic reflexes and modulate behavior [1]. Electrical stimulation of the left cervical vagus nerve (VNS) is approved for treatment of refractory epilepsy [2], obesity [3] and depression [4] and additional experimental phenomena have been reported.

The extinction of an aversive contextual memory is a phenomenon produced by learned associations dissipating, or being relearned, over time in the absence of expected reinforcement. In experimental models, auditory based extinction learning is enhanced by VNS [5–10], as is anxiety behavior [8,9,11]. While the acute cognitive effects of VNS have been reproduced, chronic outcomes are often variable for both human and rodent cohorts, with evidence for both facilitation [12–15] and no effect [16,17].

Improved understanding of the underlying mechanisms by which VNS acts is required to enable reliable experimental and ultimately clinical efficacy. Current VNS administration protocols, in both clinical and experimental settings are highly variable.

https://doi.org/10.1016/j.brs.2021.02.012
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induced with inhalation of isoflurane (5% in room air; Abbott Australasia, Sydney, Australia) followed by intramuscular (i.m.) injection of ketamine and medetomidine (0.5 mg/kg and 250 μg/kg, Pfizer, New York City, America) for ongoing anesthesia. Surgical anesthesia was assessed by the absence of both pedal withdrawal and corneal reflexes. Meloxicam (0.1 mg/100g, IP, Boehringer Ingelheim, Ingelheim am Rhein, Germany) and Polyclivc eye ointment (Alcon New Zealand Limited, Auckland, New Zealand) were administered. Supplemental ketamine (0.5 mg/kg, i.m.) was administered every 30 min as required. The rat was placed supine on a heating pad (Exoterra, China) and body temperature was maintained at 37 °C. A midline incision was made on the ventral surface of the neck and the left cervical vagus nerve exposed. A transverse incision was then made on the dorsal surface, approximately 1 cm below the rat’s shoulders blades, and a subcutaneous tunnel opened between the two incisions. The VNS electrodes were placed subcutaneously in the tunnel and the cuff placed carefully around the cervical vagus. A lateral transverse incision was then made across the most distal rib on the left side, and another subcutaneous tunnel made to the dorsal incision. The dEMG electrodes were placed through this tunnel and sutured to the left costal diaphragm, through a small sub-diaphragmatic incision. At this point the VNS electrode was connected to a stimulator (Iso-Flex with Master-8, A.M.P.I, Jerusalem, Israel) delivering electrical pulses (100 μs pulse width, 0.8 mA at 20 Hz for 5s). A stimulation-locked apnea was used to confirm recruitment of the HBR. The electrodes were then fixed to a connection plug (MS363, Plastics One, Bio Scientific Pty Ltd, Sydney, Australia) which was sutured into the dorsal incision (4.0 non-absorbable silk black braided surgical suture; Surgical Specialties, Taunton Somerset, England). The ventral incisions were sutured with absorbable suture material (4.0 absorbable polyglycolic acid surgical suture; Surgical Specialties, Taunton Somerset, England). During the recovery period, animals were provided with hydration gel (TMNectarH2O, 99% pure sterile water gel, Able Scientific, Auckland, New Zealand) and glucose energy powder (100% glucose; Glucodin, Auckland, New Zealand), in addition to standard chow. The rats were monitored for any signs of surgical complications and weighed every day for 5 days.

Recruitment of the Hering-Breuer reflex and electrode resistance measurement

Five days after surgery, the dEMG was recorded with the rat resting in its home cage. The electrodes were connected to an amplifier (NL104 Neurolog systems, Digitimer Ltd, UK; 1k gain), and the signal filtered (50–300 Hz) and sampled (5 kHz) using an A-D converter (Power1401 Mk2-CED, Cambridge Electronic Design, UK). The signal was recorded and integrated ([dEMG; Spike2 version 7; CED], and the length of each dEMG burst from the peak to the inspiratory phase to its termination was used to define the expiratory length, whereas the periods between bursts were measured to defined apnea. The rate of these bursts occurring in a minute was used to define the respiratory rate. The vagus nerve electrodes were connected to a stimulator (AMPI ISO-Flex driven by an AMPI Master-8) and electrical pulses applied (100 μs pulse duration, at 20 Hz for 5 s). The current amplitude was increased in 0.2 mA increments, with a minimum of 20 s between each test, until either the threshold current required for recruitment of the HBR was reached or a maximum of 2 mA delivered. The average expiratory length and time between bursts was calculated over 5 s before and after, the onset of VNS. These baseline measurements were then used to normalize the greatest period of apnea that occurred during the 5 s bout of VNS. The threshold for recruitment of the HBR was defined as the minimum VNS current required to
produce an apnea. To determine VNS electrode resistance, we first passed 0.2 mA across a 10 kΩ resistor to verify our system. Implanted VNS electrodes were connected to the stimulator in parallel with a 10 kΩ resistor. Current pulses (0.2 mA) were administered and the voltage drop recorded and used to calculate resistance.

Inhibitory avoidance to assay memory extinction

Instrumented animals were kept in their home cage and transported to, and habituated in, the testing room for 1 h/day over the next six days. Whilst in the testing room, each animal was handled, by the same investigator who performed all subsequent tests, for 5 min/day, for 5 consecutive days, prior to commencement of the inhibitory avoidance assay. Rats were not exposed to the inhibitory avoidance assay apparatus during this habituation procedure. The inhibitory avoidance assay occurred in an apparatus that consisted of two compartments (300 × 120 mm and height 150 mm) that were divided by a retractable door. One compartment was dark with opaque walls. The other was transparent, with a floor of both compartments, but those in the dark compartment were electrified to elicit foot shocks. The rats were filmed throughout the test, and this footage used to measure their freezing behavior. The dEMG was recorded while the rats were in the light compartment for each session. This recorded burst frequency was normalized (day 1) to account for differences in basal respiration.

On the first exposure to the apparatus (day 1) all rats were connected to the recording cables and placed in the light compartment. After 60 s the door was retracted allowing access to the dark compartment. The retractable door was shut after the rat entered the dark compartment, and foot shocks were delivered (2 × 1 s trains of 100 μs pulses at 40 Hz and 0.3 mA constant current separated by 1 s (Precision animal shocker-Coulbourne H13-15, SPW industrial, Laguna Hills, California, USA). The animals were then immediately returned to their home cage. The next day (day 0) the contextual aversive memory was assessed by placing the rat back in the light compartment. After 15 s, the retractable door was opened and the time to the first entry into the dark compartment measured. Upon entering the dark compartment each rat received a single foot shock (1 mA; 40 Hz; 1 s) to reinforce the contextual aversion. Immediately after receiving a foot shock the animals were removed from the dark compartment and returned to their home cage.

Over the next 7 days the impact of VNS (days 1–7) on memory extinction (days 2–7) was assessed. Each rat was placed into the light compartment, and 15 s later the retractable door was opened to allow access to the dark compartment. After the animal entered the dark compartment the door closed and VNS was administered (1.5 × HBR threshold; a range of 0.2–1.5 mA across the cohort; 100 μs pulse width; 20 Hz for 30 s). The sham group were treated identically but no VNS was administered, and they remained in the dark compartment for 30 s. If the rat did not enter the dark compartment within 900 s, it was guided into the dark compartment and the VNS or sham protocol administered. On the last day (day 7) the HBR threshold, and VNS electrode resistance, were re-tested.

Data analysis

To analyze the data for HBR electrode patency, animals that had a functional electrode pre- (n = 25) or post- (n = 18) inhibitory avoidance assay were included in the one data set (n = 43). Of these animals, 3 dEMG signals prevented accurate quantification of expiration length and apnea, and thus 40 animals are included for statistical analysis of these parameters (Fig. 1C). However, an unbiased, trained observer could clearly see a lack of respiratory bursts and hence these animals were still included for current and resistance measurements and comparisons (Figs. 1 and 2). In one animal it was not possible to accurately measure electrode resistance, and so this animal was removed from the analysis (Figs. 1E, 2B and 2D). The HBR failure data was derived from combining rats with a failed HBR pre- (n = 12) and post- (n = 7) the inhibitory avoidance assay. The data presented in Fig. 1D, E and 2B are pooled measurements made from both pre- and post-inhibitory avoidance assay HBR/electrode resistance testing. Unpaired t-tests were used to analyze the HBR and electrode resistance, and regression analysis was used to examine a relationship between current to induce the HBR and electrode resistance.

![Fig. 1. Vagal nerve stimulation reliably activates the Hering-Breuer reflex (HBR) in conscious rodents.](image-url)
The following parameters were measured during the inhibitory avoidance assay. Latency to enter the dark compartment—defined as the time from when the door between the chambers was retracted until all 4 paws of the rat were in the dark compartment. Freezing—a freezing episode was defined as the absence of movement for /21 s. As rats spent different amounts of time in the light compartment, the freezing time was normalized to the total time spent in the light compartment on each day. Fear recovery—analogous to fear relapse in humans [10], is defined as days when the latency to enter the dark compartment was greater than that of the previous day for a given animal [34]. Total fear recovery is the accumulated time across testing days. Across the test days the latency to enter the dark compartment and freezing were assessed with a two-way repeated measures analysis of variance followed by the Bonferroni or Dunnett’s method for multiple comparison tests. Unpaired t-tests were used to analyze total fear recovery. Regression analysis was performed to examine the relationship between different variables. All data are reported as mean ± SEM. In all instances p < 0.05 was considered statistically significant.

Exclusion criteria: Successful fear conditioning requires the formation of a robust fear memory. We defined this as an animal having a latency to enter the dark compartment that was longer than 300 s on day 1. This has been selected as a criterion for previous inhibitory avoidance studies [35]. Rats that re-entered the dark compartment in less than 300 s on day 1 were excluded from analysis (VNS, n = 3). Rats that showed substantially greater latency to enter the dark compartment after the day 1 mild foot shock (more than ± two SD - 232 s) to enter the dark compartment after the pair of 1s foot shocks at 0.3 mA) were defined as hypersensitive and removed from analysis (VNS n = 3, sham n = 1, time to enter dark compartment: 655.5 ± 69.4 s) [36]. Previous studies have described that multiple foot shock sessions or strong foot shocks can induce extinction resistance [37]. As we utilized 2 sessions of foot shock, animals that did not show a decrease in their latency to enter the dark compartment from 900s over days 1–7 were defined as extinction resistant and excluded from analysis (VNS n = 3, sham n = 1). Any animal in which the HBR could not be elicited after completion of the inhibitory avoidance assay was removed from all days of the inhibitory avoidance assay data set (n = 7). There was an overlap of n = 3 for animals lacking an HBR post inhibitory avoidance assay and the previously described exclusion criteria. In some cases, movement artifacts prevented accurate dEMG analysis during the inhibitory avoidance assay and these recordings were not included in statistical tests of respiratory function (n = 5).
Results

Utilizing the Hering-Breuer reflex as a functional correlate of vagal nerve stimulation electrode patency

Implantation of dEMG recording electrodes along with VNS electrodes enabled observation and measurement of respiratory related autonomic reflexes induced by VNS. We performed a standard protocol, increasing the amplitude of VNS current, and measuring dEMG, in all animals before and after the inhibitory avoidance assay. Using this protocol, we readily identified a pronounced apnea induced by VNS after a threshold stimulation current, that was defined as an HBR (Fig. 1A). Successful HBR recruitment was evidenced by extended expiratory periods (Fig. 1B; unpaired Student’s t-test, t = 3.04, p < 0.05, VNS: 0.2 ± 0.15 s, n = 40; sham: 0.1 ± 0.6 s, n = 19) and apnea (Fig. 1C; unpaired Student’s t-test, t = 7.06, p = 0.05, VNS: 6.3 ± 0.5 normalized units, n = 40; sham: 1.1 ± 0.16 normalized units, n = 19). The VNS current required to elicit the HBR across all tests, ranged between 0.2 and 1.5 mA (Fig. 1D; 0.82 ± 0.06 mA; n = 43). We observed no relationship between the respiration rate of each animal and the current required to recruit the HBR (Fig. 1F; linear regression, R² = 0.0053, p > 0.05). In some animals, despite increasing VNS shock currents to 2 mA, no HBR was evoked (Fig. 1A, no response) and this defined a faulty VNS electrode. The electrode resistance was also measured and varied between 2.45 and 101 kΩ (Fig. 1E; 22.1 ± 2.4 kΩ; n = 42). Thus, we find recruitment of the HBR is a readily discernible consequence of VNS in rats.

Most animals that exhibited an HBR prior to behavioral testing (n = 25) also did so post behavioral testing (n = 17). In these rats we observed negligible changes in electrode resistance between tests (Fig. 2Aii, Δ 0.75 kΩ). Interestingly, negligible changes in electrode resistance were also observed in some rats that did not exhibit the HBR after the behavioral testing, despite doing so at the first test prior to the behavioural assay (Figure 2Aii, 1.13 kΩ). The electrode resistance, from both before and after, the inhibitory avoidance assay was plotted against the corresponding HBR threshold current. We observed no correlation between the two measures (Fig. 2B; Linear regression, n = 42, R² = 0.03, p = 0.3740). Additionally, the current required to elicit the HBR both before and after the behavioral assay was not statistically different for functional electrodes (Fig. 2C; unpaired Student’s t-test; t = 0.39, p = 0.6984). Comparison of the change in electrode resistance, from pre to post behavioral assay, in the rats divided into whether or not VNS recruited the HBR at both time points, indicated there was no difference between the groups (Fig. 2D; unpaired Student’s t-test; t = 0.32, p = 0.75). We conclude that electrode resistance from the pre to post behavioral testing did not provide an accurate prediction of stimulus-induced afferent reflex recruitment and is not a reliable indicator of stimulus delivery.

Vagal nerve stimulation enhances the extinction of a contextually based fear memory

To determine if VNS hastens contextual memory extinction, an inhibitory avoidance foot-shock paradigm was utilized to condition a robust fear memory. We then examined how pairing VNS in the dark compartment of the apparatus, with the absence of contextual foot shock, affects memory extinction over the subsequent 7 days (Fig. 3A). On the first test day of extinction, all rats displayed strong avoidance of the dark compartment, where the shock was delivered, remaining in the light compartment for most of the test period. The latency to enter the dark compartment was 853.7 ± 32 s, n = 22, indicating reliable induction of the contextual fear for all animals (Fig. 3B). In sham animals (n = 10), the latency to enter the dark compartment gradually reduced over the subsequent test days, with characteristic fear recovery on day 5. Despite this gradual extinction of contextual avoidance over time, the sham group latency to enter the dark remained elevated, compared to their pre-stimulus period (day –1) for the entire test period. In contrast, pairing exposure to the dark compartment with VNS (n = 12) significantly increased the rate of memory extinction (Fig. 3B; two-way RM ANOVA, F(8,179) = 19.56, p < 0.05), such that the latency to enter the dark compartment had returned to pre-stimulus (day –1) values by day 4 (Fig. 3B and C; Dunnett’s post hoc test, p = 0.43). Freezing decreased similarly in both groups on day 2, but in the sham group, freezing remained at approximately at day 2 levels for the remaining test period. In contrast, the VNS group showed further decreases in freezing behavior, such that they were significantly different from the sham group (Fig. 3D; two-way RM ANOVA, F(6,119) = 3.193, p < 0.05), although, a difference between groups on any particular day was not detected (Fig. 3D; Bonferroni post hoc test, p > 0.05). The VNS group also showed reduced total fear recovery across testing days (Fig. 3E; unpaired Student’s t-test, t = 2.49, p < 0.05). Regression analysis confirmed a significant correlation between the latency to enter the dark compartment and freezing behavior (Fig. 3F; linear regression, R² = 0.596, p < 0.05). Thus, the daily administration of VNS, in a context previously associated as aversive, accelerates the extinction of behavioral correlates of fear.

Respiration as a correlate of contextual memory extinction

To determine if physiological responses were correlated with the extinction of aversive memory and the impact of VNS, we measured inspiratory bursts in the dEMG recordings, when the rats were in the light compartment, to index respiration rate. We first noted that respiration rate was significantly lower after the rats had experience foot shocks (Fig. 5G, p < 0.05 day 1 compared to day –1, t-test) regardless of which group they were in. Over the extinction days (1–7), a regression analysis between respiration rate and memory extinction (latency) across both cohorts combined indicates an inverse relationship between the two variables (Fig. 3G; Linear regression, R² = 0.081, p < 0.05). Where these regression data are derived from the VNS (n = 10; magenta) and sham cohorts (n = 7; green), i.e., less 5 animals in Fig. 3B, D, E & F, as noise induced artifacts preventing accurate quantification of the signal. We conclude respiratory rate is correlated to both latency and freezing, indices of fear, as such changes in respiratory rate may also be used to track changing perceptions of fear during the inhibitory avoidance assay.

Modeling the impact of utilizing vagal nerve stimulation evoked Hering-Breuer reflex as an experimental criterion.

We wanted to assess what impact HBR criteria had on behavioral outcomes. For inclusion in the analysis (Fig. 3B), rats had to exhibit a VNS-evoked HBR both before and after behavioral testing and pass the assay inclusion criteria defined in the methods. Rats that exhibit a VNS-evoked HBR during the pre-behavioral assay, but not after, were excluded (n = 4). Change in electrode resistance is a commonly used exclusion parameter for VNS studies [25,27,36]. In these animals, electrode resistance changed 4.2 ± 1.5 kΩ; less than a 25%, from pre to post behavioral assay. To demonstrate how data quality is impacted by not using a physiological metric to score electrode patency, we plotted (Fig. 4A) the latency to enter the dark compartment for the VNS (n = 12; magenta symbols) and sham (n = 10; green symbols) groups as in Fig. 3B; along with all rats exhibiting a pre-test VNS HBR response (n = 17; open symbols) as well as those excluded due to a lack of post-test VNS HBR (n = 4; grey filled symbols). Analysis of the effect of VNS on memory extinction in all rats showing a pre-test HBR remained significantly
Fig. 3. Electric VNS accelerates extinction of contextual aversive memory. A. Cartoon showing the stages of the behavioral assay. During the extinction period, VNS was delivered at 1.5 times the threshold for activation of the Hering-Breuer reflex (HBR) determined for each individual animal, at the time they entered the dark compartment on days 1–7. B. Line graph showing the latency to enter the dark compartment following the development of the strong contextual aversion. Rats receiving VNS (n = 12; magenta) entered the dark compartment more quickly than the control group (n = 10; green; * denotes p < 0.0001 compared to the control group, two-way RM ANOVA; $ denotes p < 0.05 compared to the sham group on any given day; Bonferroni post hoc test; # denotes p < 0.05 compared to day −1 of the VNS group, Dunnett’s post hoc test; ^ denotes p < 0.05 compared to day −1 of the sham group, Dunnett’s post hoc test). C. VNS accelerated the time to extinguish the contextual memory of the dark compartment in treated animals. D. Freezing behavior, when initially placed in the light compartment, was reduced in the VNS group (n = 12; magenta) compared to shams (n = 10; green; * denotes p < 0.05 compared to control, two-way RM ANOVA). E. Total fear recovery, accumulated time from days when the latency to enter the dark compartment was greater than that of the previous day, was significantly reduced in the VNS administered group (n = 12; magenta) compared to controls (n = 10; green; * denotes p < 0.05 compared to control, unpaired Student’s t-test). F. Regression analysis of latency to enter the dark compartment and the time spent freezing (%) when placed in the light compartment showed a strong correlation (R² = 0.96, p < 0.05). Each point on the graph represents the paired means for all animals (VNS and sham) on each day of the assay (n = 22). G. Rate of dEMG bursts (inspiration) while in the light compartment prior to any foot shocks upon entering the dark compartment (day −1) and after aversive conditioning (day 1; * denotes p < 0.05 compared to day −1, t-test). H. Respiratory activity, measured in the light compartment in all animals (n = 17), was significantly correlated to the latency to enter the dark compartment (linear regression on days 1–7, R² = 0.81, p < 0.05) with day −1 (prior to foot shock) in light grey. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
Vagal nerve stimulation at Hering-Breuer reflex threshold does not produce a dose response relationship with respect to increased memory extinction.

We wanted to determine whether delivery of VNS current indexed to a physiological outcome might standardize response patterns across subjects. To address this, we divided the VNS rats into those that received either low (0.3–1.2 mA) or high (1.3–2.2 mA) current stimulation (Fig. 5). We observed no difference in measures of fear extinction between these animals receiving relatively high or low VNS intensity (Fig. 5A; two-way RM ANOVA, F(2,171) = 13.10, p = 0.7983). Both groups showed a return to pre-test latency to enter the dark compartment by day 4 (Fig. 5A; Dunnett’s post hoc test, p > 0.05) with similarly derived one phase exponential decay functions for decreasing latency (low VNS: Y = 900e−0.35tx; high VNS: Y = 900e−0.248x). Combined: Y = 900e−0.275x.

This provided confidence in using the equation to calculate each individual animal’s decay constant and half-life. Interestingly, when current is correlated to each individual rat’s decay constant for fear extinction (Fig. 5B; R² = 0.2, p = 0.1269), or half-life of the fear extinction curve (Fig. 5C; linear regression, R² = 0.0001, p = 0.9671), we did not observe a dose-response relationship between current intensity and memory extinction performance. Similarly we observed no difference in freezing behavior between high and low groups (not shown).

Discussion

We have demonstrated that electrical VNS increased the extinction of a contextual aversive fear memory, as evidenced by a number of behavioral and physiological parameters. Importantly, we utilized a physiological consequence of vagal afferent activation to demonstrate VNS efficacy throughout the behavioral assay and demonstrate the ability of this approach to provide robust conclusions. We used the HBR, a well-characterised vagal afferent reflex that affects respiration, as a direct measure that VNS was stimulating a group of vagal afferent axons. This simple test, that can provide a robust measure of VNS efficacy, is superior to commonly applied methods that examine electrode resistance. Using the HBR as a physiological determinant of stimulus intensity enabled us to observe that there was not a stimulus intensity-effect response relationship in this experimental paradigm. Together the results demonstrate a novel memory-related effect of VNS, when using a physiological reflex to determine stimulus amplitude, and describe an optimized protocol for animal studies requiring longer-term, repeated stimulation of peripheral nerves.
A major concern of VNS studies is verification of electrode and VNS efficacy. Previous studies rely upon small, or no, changes in the VNS electrode resistance to imply continued electrode patency [25,32]. Large increases in electrode resistance usually indicate malfunctioning electrodes, due to lead breaks or fluid leakage into the cuff for example, and has been shown to be an effective criterion for chronic paradigms [39]. In this study we examined the electrode resistance before, and after, a behavioral assay and found that a lack of change in electrode resistance provides little confidence that VNS was being administered effectively (Fig. 2B). Most of our implanted electrodes shared a similar range of resistances (Fig. 2C), despite requiring different currents to elicit the HBR (0.2–1.5 mA). We observed instances in which electrodes passed the criterion for no change in resistance over the course of the study, and yet were unable to elicit a HBR in the post-assay period (Fig. 2Aii). Yet we did not observe a relationship between change in resistance and electrode efficacy (evoked HBR). Whilst our evidence does not imply that resistance changes cannot identify ineffective electrodes, our observations suggest exclusion/inclusion criteria based on changes in, or absolute electrode resistance may introduce unnecessary variability to experimental results. Electrode resistance will remain a measure of electrode patency as it is readily measured, yet it could be supplemented with measurement or observation of respiration to provide an additional assessment of electrode viability. Other reflexes or physiological consequences of vagal fiber activation could be used to verify electrode patency, such as ECG recordings to assess vagal efferent activation [39]. Less invasive measures may also be employed, such as pulse oximetry, to assess physiological responses to VNS.

Establishing criteria requiring expression of the HBR before (and after) the behavioral assay improved our data quality. We demonstrate this by comparing the results from animals expressing a VNS evoked HBR before and after the inhibitory avoidance assay with datasets that included all animals exhibiting a VNS evoked HBR before the assay or those that we were not able to evoke the HBR at all (Fig. 4A). Those animals with faulty electrodes, identified by a lack of HBR response, were indistinguishable from the sham animals with respect to memory extinction. Though there was no difference in latency across extinction days between animals exhibiting HBR pre-assay compared to those doing so pre and post HBR assay, there was a difference in when each cohort reached complete extinction. With successful amelioration of fear behavior taking one day longer to detect in the pre HBR group compared to the pre and post HBR group (Fig. 4B). Yet, in considering our approach, it is also important to highlight that we could not identify the day VNS electrodes failed during the inhibitory avoidance protocol in those animals where a HBR was evoked prior to, but not after, the behavioural assay. Thus, data stringency improved by removing false positives that would not have been identified on the basis of changes in electrode resistance alone.

Examination of the HBR under anesthesia, has been used in other chronic VNS studies to test electrode viability at the completion of electrode implantation [25,32] and at the end of testing [5,6]. We propose that examination of the HBR in the conscious state may be important. Although we did not compare the threshold for eliciting the HBR in the conscious and unconscious state, there is ample evidence in the literature to suggest that central responses to VNS are state dependent. Where inspiratory drive is reduced under anesthesia, leading to the threshold for eliciting the HBR being substantially reduced (25–45 μA) [40]. In the conscious state, increased modulatory drives from the forebrain and cortex interact with various brainstem nuclei to affect viscero-sensory processing [41]. In addition, the animal’s metabolic drive is greatly enhanced, and inspiratory motor neurons are more active [42]. As a consequence, the reported range of current thresholds for eliciting the HBR in conscious rats is between 0.2 and 1 mA [29]. This is very similar range to that reported in this study (0.2–1.5 mA). These observations are important in the context of this study, because the biophysical properties and activation of the vagus nerve are expected to be independent of state - the same current should drive the same afferent input in conscious or unconscious rats. Yet the efficacy of the centrally driven vagal circuitry to modulate behaviour is likely altered in different conscious states. An interesting, but unknown, question is whether central modulation of autonomic reflex circuits also impacts the ability of VNS to modulate higher order functions, such as memory.

Electrical VNS, with a set current intensity (0.4 mA), accelerates extinction memory [5–7,10] and reduces anxiety [8,9] in auditory tone fear conditioning and extinction paradigms. However, a test that ensures VNS is administered for the duration of a study, with an intensity tuned appropriately for all subjects has not been performed across studies. Delivering efficacious stimulation to each subject is critically important for overcoming biological and technical variability. Biological variability will occur because the vagus consists of a complex organisation of heterogeneous fascicles, containing sensory and motor axons whose position within the vagal trunk, and thus in relation to the stimulating electrode, is inconsistent between subjects, and this varies across species [20]. This establishes a seemingly random gradient that determines which axonal populations are stimulated, where the fibres of interest directly adjacent to the electrode may vary between subjects. Technical variability will occur due to electrode placement or damage to axonal fibres. Electrode placement is an important consideration because minor alterations in cuff dimensions can increase the current required to recruit the same proportion of fibres by over 1 mA [43]. These factors likely have therapeutic consequences. For example in the treatment of epilepsy, this biological and mechanical variability likely contributes to the need to altering stimulation duty cycles over a range of current amplitudes (0.25–3 mA) [18]. Cardiovascular responses to VNS in the clinic also vary under the same stimulatory conditions [31]. Recordings from neurons in the locus coeruleus, a central nucleus thought to be critical for anti-epileptic and depressive effects of VNS [44,45], show variability in spike frequency across animals under the same current administration [30]. To address this, we utilized a physiological reflex to determine current intensity in each rat, rather than using a nominal current, in order to be certain that at least one subset of vagal afferents was being consistently activated during the study.

Electrical VNS studies examining memory consolidation [23,24] and neural plasticity in the auditory or motor cortex [25–28] report an inverted U dose response relationship. As we controlled stimulus intensity for physiological efficacy, we used a range of current amplitudes (0.3–2.2 mA). In splitting our VNS cohort dataset into low (0.3–1.2 mA) and high (1.3–2.2 mA) stimulatory groups, we observed no difference between the groups (Fig. 5A). To determine if increased extinction memory was similar across subjects despite the varying currents administered, we examined if the decrease in latency could be modelled accurately with a one phase exponential decay model (Fig. 5C). Each animal’s decay constant and extinction half-life were then determined and correlated to the VNS current they received during the assay. We had expected to see a similar dose response relationship for VNS current and latency to enter the dark in this study, and a lack of this relationship could be interpreted as evidence for an all or nothing response with respect to this avoidance behavior. It is likely that increasing VNS current amplitudes alters the population of vagal afferents being recruited, indeed locus coeruleus activation increases accordingly [30]. Thus, standardization of stimulus intensity and administration of current...
is critical for VNS paradigms and indexing current intensity to a reflex is one way to achieve this.

In this study, VNS current amplitude, delivered at 1.5 times the threshold to initiate the HBR, accelerated the extinction of a conditioned fear memory. We chose 1.5 times threshold to ensure reliable recruitment of a cohort of vagal afferent neurons across the assay period. We standardised pulse width and frequency, which also affects vagal afferent recruitment [46], using parameters previously demonstrating memory improvements with VNS [8,9,47]. We observed decreased latency to enter the aversive context (dark compartment), decreased freezing upon being placed in the apparatus, and reduced total fear recovery. Where these measures relate to avoidance, fear and fear relapse, respectively. Our observation that VNS decreased total fear recovery concurs with previous reports [5]. This is the first time an experimental study has indexed VNS parameters based to the recruitment of a physiological reflex in conscious animals over a chronic extinction paradigm. The outcomes of our evaluation extend upon the findings of the impact of VNS on freezing and fear reinstatement reported in other studies of auditory tone fear conditioning extinction [5,6,8,9,33,47]. The HBR is mediated by pulmonary stretch receptors, mechanosensitive, myelinated, neurons that are activated with lung inflation. Here we can confidently say these neurons are activated over each day of the inhibitory avoidance assay. However, we do not know to what degree other vagus nerve fibres are recruited. We likely initiated additional unmyelinated C-fibre afferents on the basis of stimulation at 1.5 times the HBR threshold and we had no measures, such as bradycardia, to index vagal efferent activation. This begins to highlight the limitations of attempting to target specific vagal fibres with electrical activation. We and others are pursuing optogenetic [21] and novel chemogenetic strategies [48] to gain greater specificity and insight into the mechanisms underlying VNS.

We observed that respiratory rate is inversely correlated with indicators of fear, such as latency to enter dark compartment and freezing. Autonomic measures, such as respiration, may be somewhat obvious markers of fear behavior, although they are not often measured in behavioral studies. Yet the interpretation of this correlation is complex. For example, in the context of this assay with changing levels of fear-related behavior, the increase in respiration may be due to reduced freezing or increased exploratory behavior. During periods of freezing, the animals often exhibited large intervals between breaths, in contrast to exploratory periods when respiration rate was rapid. Altered respiratory rate could also be due to changes in ultrasonic vocalizations [49], although these were not measured in this study. It is important to consider that respiratory frequency alone will not provide solid conclusions about central states or processes. However, in a context where the environmental conditions and thus central state is associated to a specific behavior through robust conditioning, such as in this assay, we suggest physiological measures may be less variable across animals and thus able to aid in the interpretation of behavior. Future studies could incorporate more autonomic correlates into other behavioral assays to not only identify patent electrodes, but simultaneously provide a fuller picture of animal behavior.

Conclusion

Indexing VNS stimulation to a response that provides a direct readout of stimulus efficacy, both before, during and after the stimulation protocol is likely to increase the reliability of experiments designed to understand mechanisms underlying specific VNS outcomes. We posit that the vagal afferent mediated HBR is a relatively simple measure to incorporate into experimental vagal nerve stimulating studies that provides a robust measure of electrode viability. We demonstrate that VNS accelerates extinction of a contextual aversive memory and decreases behavioral correlates of fear perception. We observed respiratory responses that provide a novel understanding of the physiological correlates underlying fear behavior in the context of this contextual aversive assay. These data provide a robust model for future studies designed to understand neural mechanisms underlying chronic peripheral nerve activation.

CRediT authorship contribution statement

Andrew G. Butler: Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. Erin L. O’Callaghan: Methodology, Software. Allen M. Allen: Conceptualization, Writing – review & editing, Funding acquisition. Stuart J. McDougall: Conceptualization, Supervision, Visualization, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare no competing financial interests.

Supported by grants from the Australian Research Council (Discovery Project 170,104,861 and 170,104,582, SJM & AMA), the National Health and Medical Research Council of Australia (AP112000717, AMA), University of Melbourne Research Grant Support Scheme (SJM) and the Victorian Government’s Operational Infrastructure Support Program (Florey).

All authors have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

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Title:
Use of a physiological reflex to standardize vagal nerve stimulation intensity improves data reproducibility in a memory extinction assay

Date:
2021-03-05

Citation:
Butler, A. G., O'Callaghan, E. L., Allen, A. M. & McDougall, S. J. (2021). Use of a physiological reflex to standardize vagal nerve stimulation intensity improves data reproducibility in a memory extinction assay. BRAIN STIMULATION, 14 (2), pp.450-459. https://doi.org/10.1016/j.brs.2021.02.012.

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