The timing and amount of vagus nerve stimulation during rehabilitative training affect poststroke recovery of forelimb strength

Seth A. Hays\textsuperscript{a,b}, Navid Khodaparast\textsuperscript{a,b}, Andrea Ruiz\textsuperscript{a,b}, Andrew M. Sloan\textsuperscript{a,b}, Daniel R. Hulsey\textsuperscript{a,b}, Robert L. Rennaker II\textsuperscript{a,b,c} and Michael P. Kilgard\textsuperscript{a,b}

Loss of upper arm strength after stroke is a leading cause of disability. Strategies that can enhance the benefits of rehabilitative training could improve motor function after stroke. Recent studies in a rat model of ischemic stroke have demonstrated that vagus nerve stimulation (VNS) paired with rehabilitative training substantially improves recovery of forelimb strength compared with extensive rehabilitative training without VNS. Here we report that the timing and amount of stimulation affect the degree of forelimb strength recovery. Similar amounts of Delayed VNS delivered 2 h after daily rehabilitative training sessions resulted in significantly less improvement compared with that on delivery of VNS that is paired with identical rehabilitative training. Significantly less recovery also occurred when several-fold more VNS was delivered during rehabilitative training. Both delayed and additional VNS confer moderately improved recovery compared with extensive rehabilitative training without VNS, but fail to enhance recovery to the same degree as VNS that is timed to occur with successful movements. These findings confirm that VNS paired with rehabilitative training holds promise for restoring forelimb strength poststroke and indicate that both the timing and the amount of VNS should be optimized to maximize therapeutic benefits. \textit{NeuroReport} 2014, 25:676–682 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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\*Department of Neuroscience, School of Behavioral Brain Sciences, \textsuperscript{b}Texas Biomedical Device Center and \textsuperscript{c}Department of Bioengineering, Erik Jonsson School of Engineering and Computer Science, The University of Texas at Dallas, Richardson, Texas, USA

Correspondence to Seth A. Hays, PhD, School of Behavioral Brain Sciences, The University of Texas at Dallas, 800 West Campbell Road, GR41, Richardson, TX 75080-3021, USA
Tel: +1 972 883 2376; fax: +1 972 883 2491; e-mail: sxh129730@utdallas.edu

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Introduction

As many as 795 000 Americans suffer a stroke each year. As a result, stroke is a leading cause of disability, with many patients showing chronic impairment of the upper limbs. Loss of strength is believed to be the major contributing factor to poststroke disability [1]. Physical rehabilitation is insufficient to restore function in the majority of patients; therefore, strategies that enhance the recovery of upper arm strength are needed.

Adjuvants to physical therapy that increase neural plasticity would be expected to improve stroke recovery [2]. Recent studies in rats have demonstrated that stimulation of the vagus nerve paired with forelimb movement causes substantial reorganization of the primary motor cortex [3]. Stimulation of the vagus nerve paired with forelimb movement during rehabilitative training can substantially improve recovery of motor function after stroke. In one study, vagus nerve stimulation (VNS) paired with rehabilitative training normalized forelimb strength, whereas extensive rehabilitation without VNS provided only modest gains [4]. A second study demonstrated that VNS paired with rehabilitative training improves recovery of forelimb speed after stroke compared with rehabilitative training alone [5]. In addition to preclinical evidence of efficacy, VNS represents a potential therapy for stroke because it is Food and Drug Administration approved for the treatment of epilepsy and is well tolerated by patients [6]. Previous stimulation paradigms and those described in this study deliver much less total charge than that used for epilepsy control [4,7], suggesting that VNS paired with physical rehabilitation for stroke recovery can be safely delivered with potentially even fewer adverse effects. Delivery of VNS timed to coincide with tones is effective in treating chronic tinnitus in animal models and patients [7,8].

To further evaluate VNS combined with physical therapy for poststroke rehabilitation, we tested the effectiveness of different stimulation paradigms in restoring forelimb strength after ischemic lesion of the motor cortex in rats. If VNS acts to reinforce the effects of rehabilitative training to improve recovery of motor function, a delay between rehabilitative training and VNS delivery would be expected to reduce the beneficial effect of VNS. However, several studies have shown that activation of the vagus nerve can promote memory retention when delivered up to hours after training [9]. In this study we evaluated whether VNS delivered hours after rehabilitative training was effective in improving stroke recovery.
Our previous findings showed that delivery of short durations of VNS during rehabilitative training results in significant recovery of forelimb strength. It is not known whether additional stimulation enhances stroke recovery. Memory enhancement driven by VNS shows an inverted-U-shaped response for stimulation intensity, suggesting that greater stimulation might not increase VNS efficacy. In this study, we also evaluated whether additional VNS delivered during rehabilitative training is more or less effective compared with the amount of VNS delivered in our earlier studies.

Methods

Subjects

Forty-six adult female Sprague–Dawley rats, ~4 months old and weighing ~250 g when the experiment began, were used. The rats were housed in a 12:12 h reversed light cycle environment and behavioral testing took place during the dark cycle to increase daytime activity levels. Rats were food deprived to no less than 85% of their normal body weight during training. Data from some rats in the paired VNS (N = 6) and rehabilitative training alone (N = 9) groups were published in a previous study [4]. We include these data along with data from additional interleaved rats in the same treatment groups to allow comparison with previous data and to reduce the number of rats used in this study. Eleven rats were removed from the study because of lack of impairment (defined as a postlesion reduction in hit rate of less than 20%) during the postlesion assessment. Three rats were removed from the study because of device failure within the first 4 weeks. If device failure occurred after more than 4 weeks of therapy, data from these rats were included (n = 4). All handling, housing, and surgical procedures, as well as behavioral training of the rats, were approved by the University of Texas at Dallas Institutional Animal Care and Use Committee.

Isometric force task

The isometric force task was conducted as previously described [4,10]. Rats were trained to reach out through a narrow slot in the cage and pull a handle attached to a force transducer (Motor Pull Device and Motor Controller; Vulintus LLC, Sachse, Texas, USA). Force measurements were sampled at 20 or 100 Hz and measured with ±1 g accuracy. Custom software was used to control the task and collect data. If pull force exceeded 120 g within 2 s of initial contact with the handle, the trial was recorded as a success and the software triggered an automated pellet dispenser (Vulintus LLC) to deliver a sucrose pellet (45 mg dustless precision pellet; BioServ, Frenchtown, New Jersey, USA) to a receptacle located in the front left corner of the cage. If the force did not exceed 120 g within 2 s, the trial was recorded as a failure and no reward was given.

Training sessions lasted 30 min and were conducted twice daily, 5 days a week, with sessions on the same day separated by at least 2 h. Shaping was conducted in stages as previously described [4,10]. Once proficient, rats were held in the prelesion stage until they had 10 successive sessions with an average hit rate of 85%. The prelesion data reported in this study are the average of these 10 sessions. After this point, the rats were administered an ischemic lesion followed by 7 days of recovery, after which they were returned for testing until they had undergone four sessions with greater than 10 trials each during the postlesion assessment. Rats were then subjected to the therapy, during which stage VNS was delivered as appropriate for 25 days. A subset of rats underwent 2–5 days of additional testing without VNS (Week 6) to assess persistent effects of VNS.

Unilateral motor cortex ischemic lesion

Unilateral ischemic lesions in the primary motor cortex were administered as previously described [4,5,10,11]. Rats were anesthetized with ketamine hydrochloride (80 mg/kg, intraperitonely) and xylazine (10 mg/kg, intraperitonely) and administered supplemental doses as needed. A craniotomy exposed the primary motor cortex contralateral to the trained forelimb. Injections of 1.0 µl endothelin-1 (6.66 nM/s; Bachem, Torrance, California, USA; 1 mg/ml in saline) were administered at eight different locations within the forelimb area of the motor cortex: anteroposterior 2.5, 1.5, 0.5, and −0.5 mm and mediolateral 2.5 and 3.5 mm from the bregma and 1.8 mm below the cortical surface. The craniotomy was covered with KwikCast (World Precision Instruments, Sarasota, Florida, USA), sealed with acrylic, and the skin was sutured.

Vagus nerve cuff implantation

All subjects underwent headcap and VNS cuff implantations, as previously described [3–5,7,12]. Immediately after lesion surgery, a two-channel connector was attached with acrylic to four skull screws. An incision and blunt dissection of the neck exposed the left cervical vagus nerve. Stimulation of the left branch of the vagus avoids cardiac complications [4,5,7]. The nerve was placed inside the cuff (5–6 kΩ impedance), and cuff leads were tunneled subcutaneously and attached to the two-channel connector atop the skull. Rats were administered amoxicillin (5 mg) and carprofen (1 mg) for 3 days after surgery.

Application of vagus nerve stimulation

Behavioral testing sessions were identical for all rats. When appropriate, VNS was delivered as a 500-ms train of 15 biphasic, 0.8-mA, 100-µs phase duration pulses at 30 Hz, the same parameters as those used in previous studies [4,5,7]. The headcaps of all subjects were connected with a tether wire during postimplantation sessions. The Rehab group freely performed the task...
without VNS delivery (Fig. 1). The Paired VNS group received VNS during rehabilitative training sessions on successful trials for \( \sim 6500 \) total stimulations over the 25-day therapy period. At least 2 h after the completion of daily training sessions, the Delayed VNS group received VNS every 10 s for 1 h in a dummy cage without a manipulandum each day, for 9000 stimulations over the 25-day period. The delayed stimulation protocol was designed to ensure sufficient stimulation and was based on previous data according to which the subject with the most paired VNS received 8024 stimulations over 25 days. The Extra VNS group received VNS during rehabilitative training sessions at 1- and 3-s pseudorandom interstimulus intervals (averaging stimulation every 2 s). This resulted in an approximately six-fold increase in amount of VNS compared with that in the Paired VNS group, with 45 000 total stimulations over the 25-day therapy period.

**Statistics**

All data are reported as the mean±SEM. Significant effects of treatment were determined using one-way or two-way analysis of variance (ANOVA) and post-hoc unpaired \( t \)-tests where appropriate. Paired \( t \)-tests were used to compare prelesion and post-therapy (Week 6) performances of the same subjects. Statistical tests for each comparison are provided in the text. The \( \alpha \)-level was set at 0.05 for all comparisons.

**Results**

Rats were trained to perform the isometric force task and reached stable proficiency in 30±3 days. Before lesion, the maximal pull force was 148.8±1.9 g, with most trials exceeding the 120-g hit threshold. No significant difference was observed in prelesion maximal force between groups [Fig. 2a; pre, one-way ANOVA, \( F(3,27) = 1.55, P = 0.22 \)]. The hit rate was 86.4±0.6%, with no differences observed between groups [Fig. 2b; pre, one-way ANOVA, \( F(3,27) = 0.95, P = 0.43 \)]. As expected, ischemic lesions reduced multiple measures of forelimb function. Maximal pull force was significantly reduced compared with prelesion levels (Fig. 2a; post: 100.8±2.5 g; paired \( t \)-test, \( P = 1.12 \times 10^{-15} \) compared with prelesion). No differences were observed between groups [one-way ANOVA, \( F(3,27) = 1.58, P = 0.22 \)]. Similarly, hit rate was significantly reduced after lesion (Fig. 2b; post: 36.9±2.6%; paired \( t \)-test, \( P = 5.74 \times 10^{-19} \) compared with prelesion) with no differences observed between groups [one-way ANOVA, \( P(3,27) = 0.98, P = 0.42 \)].

Vagus nerve stimulation paired with forelimb training enhances recovery of forelimb strength after stroke

As previously reported, VNS paired with successful trials during rehabilitative training (Paired VNS, \( n = 8 \)) significantly improved recovery of maximal force compared with rehabilitative training without VNS (Rehab, \( n = 10 \)), indicative of restoration of forelimb strength [Paired VNS vs. Rehab, two-way ANOVA, \( F(1,95) = 53.86, P = 7.25 \times 10^{-11} \)]. The Paired VNS group also showed a significantly improved hit rate compared with the Rehab group [Paired VNS vs. Rehab, two-way ANOVA, \( F(1,95) = 39.88, P = 8.63 \times 10^{-9} \)]. VNS paired with rehabilitative training resulted in increased maximal pull force (Paired VNS vs. Rehab, unpaired \( t \)-test, all \( P \)-’s < 0.05 for Weeks 2–6) and hit rate (Paired VNS vs. Rehab, unpaired \( t \)-test,
all \( P < 0.05 \) for Weeks 2–6) beginning at the second week of therapy compared with rehabilitative training without VNS. Paired VNS resulted in 95.0 ± 5.1% recovery of forelimb strength at the end of therapy, and forelimb strength was not significantly different from prelesion levels (Paired VNS, prelesion vs. Week 6, paired \( t \)-test, \( P = 0.13 \), \( n = 7 \)), indicative of full recovery. In the Rehab group 40.7 ± 18.8% of forelimb strength was restored and there was a significant deficit compared with prelesion levels (Rehab, Prelesion vs. Week 6, paired \( t \)-test, \( P = 0.011 \), \( n = 10 \)), suggesting a long-lasting impairment in strength. These findings indicate the VNS paired with rehabilitative training substantially improves recovery of forelimb strength after stroke.

### Vagus nerve stimulation delivered after forelimb training is less effective than vagus nerve stimulation paired with forelimb training

We hypothesized that VNS delivered after the daily therapy session would be less effective at improving recovery than VNS paired with training. To test this, a group of rats underwent identical rehabilitative training but received VNS 2 h after daily training sessions (Fig. 1; Delayed VNS, \( n = 7 \)). Delayed VNS is less effective at improving forelimb strength [Paired VNS vs. Delayed VNS, two-way ANOVA, \( F(1,76) = 22.48, P = 9.71 \times 10^{-6} \)] and hit rate [Paired VNS vs. Delayed VNS, two-way ANOVA, \( F(1,76) = 14.24, P = 3.0 \times 10^{-4} \)] compared with paired VNS. Delayed VNS results in significantly lower maximal pull force during the last 2 weeks of therapy compared with paired VNS (Paired VNS vs. Delayed VNS, unpaired \( t \)-test, all \( P < 0.01 \) for Weeks 5 and 6). Similar results are observed for hit rate (Paired VNS vs. Delayed VNS, unpaired \( t \)-test, all \( P < 0.01 \) for Weeks 5 and 6). These findings suggest that delayed delivery of VNS after rehabilitative training is less effective than VNS paired with forelimb movement during rehabilitative training.

Delayed VNS resulted in an improvement in recovery of forelimb strength [Rehab vs. Delayed VNS, two-way ANOVA, \( F(1,89) = 9.28, P = 0.003 \)] and hit rate [Rehab vs. Delayed VNS, two-way ANOVA, \( F(1,89) = 6.34, P = 0.014 \)] compared with rehabilitative training without VNS. However, at most weeks, no significant differences were observed in maximal pull force (Rehab vs. Delayed VNS, unpaired \( t \)-test, \( P = 0.027 \) for Week 5, all other weeks \( P > 0.05 \)) or hit rate performance (Rehab vs. Delayed VNS, unpaired \( t \)-test, \( P = 0.039 \) for Week 5, all other weeks \( P > 0.05 \)). Although the group mean at each time point shows a modest increase, most time points fail to reach statistical significance, suggesting that Delayed VNS is not consistently better than rehabilitative training without VNS. Delayed VNS resulted in 65.7 ± 18.6% recovery of forelimb strength after therapy. Maximal pull force was still significantly impaired compared with prelesion levels (Delayed VNS, Prelesion vs. Week 6, paired \( t \)-test, \( P = 0.042 \), \( n = 6 \)), showing incomplete recovery of strength. These findings establish that Delayed VNS is less effective at improving forelimb strength after stroke compared with VNS paired with rehabilitative training.
Additional vagus nerve stimulation is less effective than vagus nerve stimulation paired with forelimb training

Additional VNS delivered during rehabilitative training may promote enhanced recovery or could desensitize the response and subsequently reduce poststroke recovery. To examine the effects of additional VNS, a group of rats underwent identical rehabilitative training but received VNS on average every 2 s during the training session (Fig. 1; Extra VNS, n = 6), resulting in an approximately six-fold increase in the number of stimulations. Extra VNS did not improve recovery compared with paired VNS. Rather, Extra VNS results in a trend toward a decrease in recovery of forelimb strength [Paired VNS vs. Extra VNS, two-way ANOVA, F(1,69) = 3.80, P = 0.055]. Extra VNS results in a statistically significant reduction in hit rate compared with paired VNS [Paired VNS vs. Extra VNS, two-way ANOVA, F(1,69) = 4.57 P = 0.036]. These findings suggest that additional VNS that is not precisely paired with movement is less effective at enhancing recovery compared with paired VNS.

Extra VNS results in only moderately improved recovery of maximal pull force compared with rehabilitative training without VNS [Rehab vs. Extra VNS, F(1,82) = 14.23, P = 3.0 × 10^{-4}], which reaches significance at Week 2 (Rehab vs. Extra VNS, unpaired t-test, P = 0.01 for Week 2). No significant differences were observed at other time points, suggesting a transient improvement that is absent by the end of therapy (Rehab vs. Extra VNS, unpaired t-test, all P’s > 0.05 for Weeks 1, 3–6). Similar results were obtained for hit rate performance [Rehab vs. Extra VNS, F(1,82) = 9.82, P = 0.002; unpaired t-test, P = 0.014 for Week 2, all P’s > 0.05 for Weeks 1, 3–6]. Extra VNS resulted in 83.6±20.1% recovery of forelimb strength [Paired VNS vs. Extra VNS, unpaired t-test, all P’s > 0.05 for Weeks 1, 3–6]. However, no differences were observed in the intensity of training for any delivery paradigms of VNS, as evidenced by a similar number of pulls over the course of the 5-week therapy period [Rehab: 34787±3356 pulls, Paired VNS: 25366±1871 pulls, Delayed VNS: 40972±6313 pulls, Extra VNS: 34214±2674 pulls; one-way ANOVA, F(3,27) = 2.73, P = 0.063]. This suggests that the differences in recovery observed between groups cannot be accounted for by differences in the intensity of training.

Intensity of training was not different between groups

The intensity of rehabilitative training can affect the degree of functional recovery [13]. Therefore, we evaluated whether VNS could improve recovery by increasing the intensity of training. Consistent with previous studies [4,5], no differences were observed in the intensity of training for any delivery paradigms of VNS, as evidenced by a similar number of pulls over the course of the 5-week therapy period [Rehab: 34787±3356 pulls, Paired VNS: 25366±1871 pulls, Delayed VNS: 40972±6313 pulls, Extra VNS: 34214±2674 pulls; one-way ANOVA, F(3,27) = 2.73, P = 0.063]. This suggests that the differences in recovery observed between groups cannot be accounted for by differences in the intensity of training.

Discussion

This study evaluated different VNS delivery paradigms to improve recovery of forelimb strength after ischemic lesion of the motor cortex. First, we evaluated the importance of temporally precise delivery of VNS during rehabilitative training. We find that delayed delivery of VNS at least 2 h after training is less effective at improving recovery compared with VNS paired with rehabilitative training. Second, we evaluated whether additional VNS distributed in time during rehabilitative training would improve recovery. We find that rather than improving recovery, additional VNS is less effective at restoring forelimb strength than VNS paired with successful forelimb movements. The findings from these experiments suggest that temporally precise delivery of VNS paired with rehabilitative training drives the most recovery of function after stroke.

Reorganization of motor representations in the motor cortex is associated with recovery after stroke [14]. In unlesioned rats, VNS paired with forelimb training drives training-specific map reorganization in the motor cortex [3]. This robust, specific enhancement of plasticity driven by VNS is believed to underlie the improvement in functional recovery observed when VNS is paired with rehabilitative training [4,5]. The majority of vagus nerve fibers are ascending and project into the nucleus tractus solitarius [15]. Stimulation of the vagus nerve activates neurons in the noradrenergic locus coeruleus and the cholinergic basal forebrain, resulting in release of neuromodulators throughout the central nervous system [16]. Release of neuromodulators during external events enhances event-specific cortical plasticity [17–19]. Therefore, VNS that is delivered outside of the time of motor training would not be predicted to enhance plasticity or improve recovery of function. However, previous studies have documented enhancements in memory retention when VNS is delivered after training, likely by enhancing consolidation [20]. As such, VNS may still confer beneficial effects when delayed after training. Our results indicate that Delayed VNS is significantly less effective at improving recovery of forelimb function after stroke compared with VNS paired with rehabilitative training and is only modestly more effective than rehabilitative training without VNS. This corroborates preliminary findings from a previous study indicating that VNS must be paired with training to improve poststroke recovery [5]. The importance of temporal precision between VNS and rehabilitative training supports a plasticity-dependent mechanism of recovery rather than alternative mechanisms that would not be predicted to require temporal precision, such as neuroprotection or modulation of the immune system. Consolidation and poststroke recovery share common molecular mechanisms [21]; therefore, the modest improvement in recovery caused by Delayed VNS compared with rehabilitative training without VNS may be due to enhancement of consolidation. Our findings are consistent with those
of previous reports in that precisely timed stimulation methods drive more effective recovery after brain injury than techniques that do not allow precise timing [22], and they suggest that clinical implementations of VNS and rehabilitative training use precisely timed delivery of stimulation to maximize benefits.

Additional VNS delivered during rehabilitative training may further increase recovery or desensitize the effectiveness of VNS and occlude recovery. Our findings indicate that delivery of six-fold more stimulation results in reduced recovery compared with less VNS paired with rehabilitative training and only moderately improved recovery compared with rehabilitative training without VNS. One limitation of the current study design is that it is not possible to precisely pair the timing of VNS during forelimb movement and also deliver several-fold more stimulation. Rats in the Paired VNS group already received stimulation on more than half of the trials during the first week of therapy, and therefore stimulation was delivered repeatedly at random intervals during training rather than precisely paired with forelimb movement to achieve substantially more stimulation.

Two mechanisms could explain why more VNS results in less stroke recovery. Additional VNS may cause desensitization that occludes the reinforcing effects of VNS paired with forelimb movement. Previous studies using VNS to enhance neural plasticity and memory observed an inverted-U-shaped response. Moderate VNS intensity enhanced plasticity and memory, but greater and lesser stimulation intensities did not [20]. Desensitization of the G-protein-coupled receptors that respond to the acetylcholine and norepinephrine released on VNS could account for these results, although many other cellular mechanisms are possible [16,23]. A second possible explanation is that the additional VNS reinforces non-task-specific movements. Paired VNS and Extra VNS groups received the same number of stimulations within 290 ms of forelimb movement (~135 per session). The 765 additional stimulations in the Extra VNS group are delivered during non-task-specific movements, including movement of the unimpaired forelimb, jaw, vibrissae, and hind limb, as well as during periods of inactivity. Stimulation during movements other than that of the impaired forelimb may competitively interfere with the reinforcement of impaired forelimb movements, thus leading to reduced recovery [3,24,25]. Our findings indicate that the amount and timing of stimulation can impact the benefits of VNS during rehabilitative training.

Insufficient intensity of rehabilitative training after a stroke can limit functional recovery [13]. Previous studies have indicated that VNS does not affect the intensity of rehabilitative training [4,5]. The findings from the present study confirm this, as no differences in intensity of training were observed between groups. Therefore, training intensity cannot account for the differences in recovery observed between groups. With stimulation beginning at least 9 days after lesion administration, VNS would not be predicted to confer any neuroprotective effects. Using a similar stimulation schedule, previous studies do not report a reduction in lesion size [4,5]. Therefore, it is unlikely that reduced lesion size accounts for the differences in recovery observed between groups in this study. The lack of changes in training intensity and lesion size suggest that VNS acts through an alternative mechanism, likely by enhancing neural plasticity, to promote recovery.

Here we report that VNS paired with rehabilitative training improves recovery of forelimb function in a rat model of ischemic stroke. Delaying VNS by 2 h after daily rehabilitative training results in less recovery of forelimb function. Additional stimulation of the vagus nerve also results in less recovery. These findings indicate that VNS paired with rehabilitative training holds potential as a poststroke therapy to improve recovery of motor function, and suggest that future implementations of the therapy should accordingly optimize the timing and amount of stimulation to maximize beneficial effects.

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Conflicts of interest

M.P.K. is a consultant and has a financial interest in MicroTransponder Inc. A.M.S. is an employee of, and R.L.R. owns, Vulintus LLC. For the remaining authors there are no conflicts of interest.

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