Vagus Nerve Stimulation During Rehabilitative Training Improves Functional Recovery After Intracerebral Hemorrhage

Seth A. Hays, PhD; Navid Khodaparast, PhD; Daniel R. Hulsey, MS; Andrea Ruiz, MS; Andrew M. Sloan, PhD; Robert L. Rennaker II, PhD; Michael P. Kilgard, PhD

Background and Purpose—Vagus nerve stimulation (VNS) delivered during rehabilitative training enhances neuroplasticity and improves recovery in models of cortical ischemic stroke. However, VNS therapy has not been applied in a model of subcortical intracerebral hemorrhage (ICH). We hypothesized that VNS paired with rehabilitative training after ICH would enhance recovery of forelimb motor function beyond rehabilitative training alone.

Methods—Rats were trained to perform an automated, quantitative measure of forelimb function. Once proficient, rats received an intrastriatal injection of bacterial collagenase to induce ICH. Rats then underwent VNS paired with rehabilitative training (VNS+Rehab; n=14) or rehabilitative training without VNS (Rehab; n=12). Rehabilitative training began ≥9 days after ICH and continued for 6 weeks.

Results—VNS paired with rehabilitative training significantly improved recovery of forelimb function when compared with rehabilitative training without VNS. The VNS+Rehab group displayed a 77% recovery of function, whereas the Rehab group only exhibited 29% recovery. Recovery was sustained after cessation of stimulation. Both groups performed similar amounts of trials during rehabilitative, and lesion size was not different between groups.

Conclusions—VNS paired with rehabilitative training confers significantly improved forelimb recovery after ICH compared to rehabilitative training without VNS. (Stroke. 2014;45:00-00.)

Key Words: cerebral hemorrhage ■ rehabilitation ■ vagal nerve stimulation ■ vagus nerve

Spontaneous intracerebral hemorrhage (ICH) is a devastating subtype of stroke and often leaves survivors with significant disability.1 There is no consistently effective poststroke rehabilitative intervention; therefore, methods to improve recovery of motor function represent a significant clinical need.

Neuroplasticity is thought to support recovery of function after stroke, so methods that enhance plasticity may promote greater recovery after ICH. Stimulation of the vagus nerve releases neuromodulators associated with plasticity.2–4 Consequently, vagus nerve stimulation (VNS) paired with forelimb training drives robust neuroplasticity.3 On the basis of this enhancement of plasticity, we found that VNS paired with rehabilitative training represents a potential method to improve recovery after stroke. Studies in models of ischemic stroke demonstrate that VNS paired with rehabilitation results in significantly greater recovery of forelimb strength and movement speed than extensive rehabilitative training without VNS.6–9 VNS paired with rehabilitation is currently being investigated in patients with ischemic stroke.3

Despite the efficacy of VNS paired with rehabilitation after cortical ischemic stroke, ICH bears different pathologic features that may interfere with the beneficial effects of VNS. In this study, we evaluate whether VNS paired with rehabilitative training can improve recovery of motor function beyond rehabilitative training without VNS in a rat model of ICH.

Methods

Subjects

All procedures were approved by the University of Texas Institutional Animal Care and Use Committee. Fifty-eight female Sprague–Dawley rats (Charles River), weighing ≈250 g at the beginning of the experiment, were used. The rats were individually housed in a 12:12 hours reversed light cycle environment and were food deprived to no <85% of their normal body weight during training.

Behavioral Training

The bradykinesia assessment task (Vulintus Inc, Dallas, TX) was performed as previously described10 (online-only Data Supplement).

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Once proficient at the task, rats received a lesion and VNS implant. After 7 days of recovery, rats were returned for postlesion testing and were then assigned to groups (online-only Data Supplement). Rehabilitative training continued for the following 6 weeks.

**ICH and VNS Implant Surgery**

ICH was performed similar to previous descriptions.\(^5\)\(^–\)\(^8\),\(^12\) Rats were anesthetized with ketamine hydrochloride (80 mg/kg, IP) and xylazine (10 mg/kg, IP). Body temperature was maintained at 37°C throughout the surgery. Bacterial collagenase type IV-S (Sigma-Aldrich Corp, St Louis, MO) of 0.18 U in 1.0-µL saline was injected into the left hemisphere at 3.0 mm lateral and 6.0 mm ventral relative to bregma using a 26-gauge Hamilton syringe. Injections took place over a 2-minute period, and the syringe remained in place for additional 3 minutes. A 2-channel connector was then affixed to the skull, and a bipolar stimulating cuff with platinum-iridium leads (5 kΩ impedance) was implanted around the left cervical vagus nerve, as previously described.\(^5\)\(^–\)\(^8\),\(^12\) Amoxicillin (5 mg) and carprofen (1 mg) tablets were provided for 3 days after surgery.

**Group Assignment and Exclusion Criteria**

The Rehab group (n=12) underwent rehabilitative training for 6 weeks, which consisted of freely performing the task during training sessions. The VNS+Rehab group (n=14) underwent identical rehabilitative training but received VNS during training based on 1 of 2 paradigms (online-only Data Supplement). One group (n=8) received VNS on successful trials, similar to previous studies.\(^6\)\(^–\)\(^8\) Another group (n=6) received VNS on all trials. VNS was delivered using identical parameters to previous studies: 500 ms train, 15 biphasic 0.8 mA pulses, 100 µs each, 30 Hz.\(^5\)\(^–\)\(^8\) No stimulation was delivered on the sixth week in any group to allow assessment of effects persisting after VNS cessation. Estrous phase was not monitored during the study because behavioral testing and stimulation occur over multiple cycles. Experimenters were blind to treatment group during testing, and automated data analysis eliminated any bias.\(^10\) Thirty-two rats were excluded from the main text because of (1) death, (2) failure to demonstrate a postlesion impairment, (3) impairment too severe to perform task, or (4) stimulation device failure (online-only Data Supplement). Data for all subjects are included in the online-only Data Supplement. Exclusion had minimal effects on statistical comparisons.

**Histological Processing**

After behavioral testing, subjects were perfused with 4% paraformaldehyde. Cresyl violet staining and analysis were performed as previously described.\(^6\)\(^–\)\(^7\) Histology could not be performed on 3 of the 26 included subjects because of technical difficulties.

**Statistics**

All data are expressed as mean±SEM. Significant differences between groups were determined using 2-way ANOVA or 2-tailed t tests where appropriate. α level was set at 0.05 for all comparisons.

**Results**

Before ICH, all rats were highly proficient at the task (Movie I in the online-only Data Supplement). No significant difference in hit rate, second press latency, or number of trials was observed between groups (Figure 1, PRE; Rehab versus VNS+Rehab, unpaired t test, all P>0.05). ICH significantly worsened multiple measures of forelimb performance in both groups (Movie II in the online-only Data Supplement). No differences were observed in postlesion performance metrics between groups (Figure 1, POST; unpaired t test, all P>0.05).

VNS paired with rehabilitative training (VNS+Rehab; Movie III in the online-only Data Supplement) significantly enhances recovery when compared with rehabilitative training without VNS (Rehab; Movie IV in the online-only Data Supplement). ANOVA comparing hit rate for Rehab and VNS+Rehab groups during the course of therapy (weeks 1–6) revealed a significant effect of treatment (Figure 1A, 2-way ANOVA, F\(1,144\)=39.59; \(P=3.54\times10^{-9}\)). Enhanced recovery is maintained on week 6 after the cessation of VNS (Movie V in the online-only Data Supplement). ANOV A comparing hit rate for Rehab and VNS+Rehab groups at each time point.

**Figure 1.** Vagus nerve stimulation (VNS) paired with rehabilitative training improves forelimb recovery after intracerebral hemorrhage. A, VNS paired with rehabilitative training (n=14) improves hit rate by the second week of therapy when compared with rehabilitative training without VNS (n=12). Recovery is maintained after the cessation of VNS on week 6. B, VNS paired with rehabilitative training improves second press latency when compared with rehabilitative training without VNS. C, The number of trials performed by the Rehab and VNS+Rehab groups is not different at any time point during testing. *P<0.05 between Rehab and VNS+Rehab group at each time point.
weeks after the initial injection. In the present study, VNS+Rehab, 11.09±1.58 mm³; unpaired t test, \( P=0.38 \). No lesion metrics were correlated with impairment in individual subjects (online-only Data Supplement).

**Discussion**

Previous studies show that VNS paired with rehabilitative training improves recovery of forelimb speed and strength after cortical ischemic lesion. The results from the present study extend the efficacy of VNS to a model of ICH that includes subcortical damage to both white and gray matter. VNS therapy, therefore, may be useful in patients with stroke bearing similar pathology and could potentially generalize to other mechanisms of brain injury.

The collagenase injection model of ICH results in prolonged neuronal death, with lesion size evolving as long as 4 weeks after the initial injection. In the present study, VNS did not begin until ≥9 days after collagenase injection, after which the lesion is predicted to have reached ≥75% of its final size. As expected, we did not observe a difference in tissue loss between groups; therefore, the improved functional outcomes resulting from VNS cannot be attributed to reduced lesion size. The absence of neuroprotective effects when VNS is delivered on this timescale after lesion onset is consistent with previous studies. The lack of a difference in lesion size between groups suggests that VNS is not enhancing forelimb recovery through neuroprotection but rather acting through a different mechanism, such as enhancing neuroplasticity. The degree of forelimb impairment after ICH was not correlated with any of the anatomic measures in this study. This suggests that a feature not observed with gross anatomy, such as partial damage to projections or pathological plasticity, may underlie at least part of the functional impairment after ICH. VNS has been successfully used to reverse pathological plasticity and confer benefits in chronic tinnitus patients. Similarly, VNS paired with rehabilitative training may promote beneficial plasticity to drive functional recovery after ICH.

Neuroplasticity is thought to be a substrate for recovery after brain damage. Similar to ischemic stroke and traumatic brain injury, rehabilitative training after ICH likely supports recovery by promoting reorganization within motor circuitry. Previous studies correlate increased dendritic complexity, a morphological feature associated with plasticity, with improved motor recovery in subjects that receive rehabilitative training after ICH. Brain-derived neurotrophic factor is known to promote increased dendritic complexity, and VNS provides a potential direct link to plasticity by driving increased expression of brain-derived neurotrophic factor and activation of TrkB signaling. Despite links to plasticity, the mechanism by which VNS improves recovery after ICH remains unclear and should be addressed in future studies.

Unlike studies in models of ischemic stroke, VNS paired with rehabilitative training results in an incomplete recovery of forelimb function after ICH, which is likely accounted for by the differences in the lesion characteristics described above. To attempt to improve recovery, 2 different stimulation paradigms were used (online-only Data Supplement). One group received stimulation on successful trials similar to the design used in previous studies, and the second group received stimulation on all trials, resulting in 40% more stimulations during the course of the therapy. Consistent with previous reports, additional VNS does not result in greater recovery. No difference in recovery was observed between either VNS paradigm. Parameters, such as current intensity and timing of stimulation, modulate the effects of VNS. Therefore, optimizing these parameters is of key importance for clinical implementation.

VNS paired with physical rehabilitation represents a potentially attractive method to improve recovery after stroke and is currently under evaluation in patients with ischemic stroke. VNS is Food and Drug Administration approved to treat epilepsy and depression, and >60,000 patients are implanted with VNS devices. VNS is safe and well tolerated. The implementation of VNS in the present study uses 100-fold less daily stimulation than is approved for epilepsy, which may further reduce any occurrence of adverse effects. Along with the evidence of safety and preclinical efficacy of VNS paired with rehabilitation in models of ischemic and hemorrhagic stroke, this report strengthens the viability of VNS as a poststroke therapy.

**Figure 2.** Lesion size is not affected by vagus nerve stimulation (VNS). Representative images showing intracerebral hemorrhage lesions from a subject in the Rehab group (A) and the VNS+Rehab group (B). C. No difference was observed in tissue loss between groups.
Conclusions and Future Directions
This study demonstrates that VNS paired with rehabilitative training improves recovery of forelimb function after ICH compared with rehabilitative training without VNS. This extends the efficacy of VNS to models that include subcortical and white matter damage. Furthermore, the beneficial effects last after the cessation of VNS, suggesting that functional improvements may be lasting. Clinical investigation in patients may be warranted. Further preclinical studies should evaluate the cellular and molecular mechanisms underlying VNS-dependent enhancement of recovery.

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Disclosures
Dr Kilgard is a consultant and has a financial interest in MicroTransponder, Inc. Dr Sloan is an employee of, and Dr Rennaker owns Vulintus, Inc. The other authors report no conflicts.

References
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SUPPLEMENTAL MATERIAL

Vagus Nerve Stimulation Delivered during Rehabilitative Training Improves Recovery of Forelimb Function After Intracerebral Hemorrhage

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II. Supplementary videos

III. Supplementary references

\textbf{I.1. Behavioral Training}

The bradykinesia assessment task was performed as previously described\textsuperscript{1}. The apparatus consists of a lever (Lever Device, Vulintus Inc., Dallas, TX) located 1.25 cm outside of a behavioral chamber. A control board and custom software (Motor Controller, Vulintus Inc., Dallas, TX) sample the switch at 20 Hz and measure lever press times with an accuracy of ± 1 msec. Custom MATLAB software analyzed, displayed, and stored the data. If a trial was successful, the software triggered an automated pellet dispenser (Vulintus Inc., Dallas, TX) to deliver a sucrose pellet (45mg dustless precision pellet, BioServ, Frenchtown, NJ) to a receptacle in the chamber. Behavioral sessions were conducted for 30 min twice daily, five days a week, with daily sessions separated by at least 2 hours. For each trial, a timer was initiated on the first press of the lever. If the lever was depressed a second time within 500 msec, the trial was recorded as a success and a reward pellet was delivered. If the lever was not pressed again or the second press occurred more than 500 msec later, the trial was recorded as a failure and no reward was given. Rats were held at the pre-lesion stage until they had 10 successive sessions averaging over 75% success rate. Upon reaching this performance level, rats received a lesion and VNS implant. After 7 days of recovery, rats returned for post-lesion testing and were held at this stage until they had 4 sessions averaging at least 15 trials each. After the post-lesion stage, rats were assigned to groups (see below) and testing continued for 6 weeks.

\textbf{I.2. Exclusion of subjects}

We employed three primary exclusion criteria for the study. 1) Rats did not survive ICH. Nine rats were excluded based on this criterion. All deaths occurred before group assignment, and therefore could not bias outcomes. 2) Rats were excluded if they failed to display at least a 20% decrease in hit rate compared to pre-lesion following stroke. Six rats were excluded based on this criterion. 3) Rats were excluded if there were too impaired to perform 4 sessions averaging at least 15 trials during within the first 4 weeks of post-lesion assessment. Ten rats were excluded based on this criterion. Exclusion based on either the first, second, or third criterion took place before assignment to either the Rehab or VNS+Rehab group, and therefore did not impact the interpretation of the effects of therapy. 4) Rats were excluded if their headcap connector or vagus nerve cuff failed due to mechanical breakage or high impedance (>30 kΩ). Three rats were removed due to a high impedance measurement and four rats were removed due to headcap malfunction. These exclusion occurred after assignment to treatment groups and therefore could potentially impact the interpretation of the results.
However, addition of these excluded subjects up to the point of device failure had little effect on the significance of any comparison (Fig. I). The only statistical effect of inclusion of the additional subjects is loss of significance in the across group comparison of hit rate at week 5 of treatment and the emergence of a significant difference at the post-lesion time point for inter-press interval.

Supplementary Figure I. Forelimb performance data including all subjects for (A) hit rate, (B) second press latency, and (C) number of trials.

I.3. Group Assignment

Following post-lesion baseline assessment of performance, rats were sorted into balanced groups based on hit rate. The first four rats were randomly assigned to a treatment group. For all subsequent rats, the post-lesion hit rate of each rat was compared to the average for each group and added to the group that minimized the between-group difference. This ensured evenly balanced performance between groups after lesion, allowing accurate comparison of the effects of treatment.

I.4. Raw statistical values for comparisons

The table below contains the statistical comparison for all t-test comparisons between pre-lesion, post-lesion, and during therapy time points and across groups for subjects included in the main text.
Supplementary Table I

<table>
<thead>
<tr>
<th>Measure</th>
<th>Comparison</th>
<th>Group</th>
<th>Wk 1</th>
<th>Wk 2</th>
<th>Wk 3</th>
<th>Wk 4</th>
<th>Wk 5</th>
<th>Wk 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hit rate</td>
<td>Pre v. week</td>
<td>Rehab</td>
<td>2.98 x 10⁴</td>
<td>0.0021</td>
<td>0.0016</td>
<td>0.0018</td>
<td>0.0054</td>
<td>0.0052</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VNS+Rehab</td>
<td>0.0030</td>
<td>0.0433</td>
<td>0.0144</td>
<td>0.0306</td>
<td>0.1145</td>
<td>0.1874</td>
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<tr>
<td></td>
<td>Post v. week</td>
<td>Rehab</td>
<td>8.85 x 10⁴</td>
<td>0.0307</td>
<td>0.1907</td>
<td>0.1068</td>
<td>0.0260</td>
<td>0.0308</td>
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<tr>
<td></td>
<td></td>
<td>VNS+Rehab</td>
<td>4.64 x 10⁴</td>
<td>3.05 x 10⁵</td>
<td>1.85 x 10⁵</td>
<td>6.46 x 10⁵</td>
<td>1.55 x 10⁵</td>
<td>6.85 x 10⁵</td>
</tr>
<tr>
<td></td>
<td>Rehab v. VNS+Rehab</td>
<td>n/a</td>
<td>0.1402</td>
<td>0.0087</td>
<td>0.0046</td>
<td>0.0112</td>
<td>0.0260</td>
<td>0.0143</td>
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<tr>
<td>Second press latency</td>
<td>Pre v. week</td>
<td>Rehab</td>
<td>0.0062</td>
<td>0.0049</td>
<td>0.0026</td>
<td>0.0030</td>
<td>0.0070</td>
<td>0.0103</td>
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<td></td>
<td></td>
<td>VNS+Rehab</td>
<td>0.1565</td>
<td>0.7188</td>
<td>0.3212</td>
<td>0.4191</td>
<td>0.4624</td>
<td>0.5949</td>
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<tr>
<td></td>
<td>Post v. week</td>
<td>Rehab</td>
<td>0.0020</td>
<td>0.0437</td>
<td>0.1553</td>
<td>0.1722</td>
<td>0.0780</td>
<td>0.0844</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VNS+Rehab</td>
<td>0.0011</td>
<td>0.0010</td>
<td>0.0057</td>
<td>0.0107</td>
<td>0.0143</td>
<td>0.0075</td>
</tr>
<tr>
<td></td>
<td>Rehab v. VNS+Rehab</td>
<td>n/a</td>
<td>0.0722</td>
<td>6.23 x 10⁴</td>
<td>0.0020</td>
<td>0.0027</td>
<td>0.0120</td>
<td>0.0102</td>
</tr>
<tr>
<td>Trials</td>
<td>Pre v. week</td>
<td>Rehab</td>
<td>0.9173</td>
<td>0.4460</td>
<td>0.2332</td>
<td>0.3640</td>
<td>0.8684</td>
<td>0.7745</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VNS+Rehab</td>
<td>0.5197</td>
<td>0.6119</td>
<td>0.5324</td>
<td>0.9469</td>
<td>0.9396</td>
<td>0.4546</td>
</tr>
<tr>
<td></td>
<td>Post v. week</td>
<td>Rehab</td>
<td>0.0055</td>
<td>0.0027</td>
<td>0.0036</td>
<td>0.0037</td>
<td>0.0074</td>
<td>0.0125</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VNS+Rehab</td>
<td>0.0077</td>
<td>0.0109</td>
<td>0.0023</td>
<td>0.0082</td>
<td>0.0066</td>
<td>7.52 x 10⁴</td>
</tr>
<tr>
<td></td>
<td>Rehab v. VNS+Rehab</td>
<td>n/a</td>
<td>0.8028</td>
<td>0.6743</td>
<td>0.7706</td>
<td>0.6035</td>
<td>0.9589</td>
<td>0.7752</td>
</tr>
</tbody>
</table>

1.5. Correlation of lesion size and behavioral parameters

We analyzed several measures of lesion size in cresyl violet stained sections and attempted to correlate post-stroke performance in individual subjects. Pearson correlation was calculated comparing post-lesion hit rate to each of the measures of lesion size listed in the table below. We found that none of these measurements yielded a significant correlation.

Supplementary Table II

<table>
<thead>
<tr>
<th>Histological Measure</th>
<th>Pearson’s r</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lesion volume: [(Volume of unlesioned hemisphere) – (Volume of ventricle on unlesioned side)] – [(Volume of lesioned hemisphere) – (Volume of ventricle on lesioned side) – (Cellular debris)]</td>
<td>-0.0044</td>
<td>0.9841</td>
</tr>
<tr>
<td>Volume of lesioned tissue: total damaged tissue, including gray and white matter</td>
<td>0.0779</td>
<td>0.7239</td>
</tr>
<tr>
<td>Ventricle size ratio: (Volume of ventricle in lesioned hemisphere) / (Volume of ventricle in unlesioned hemisphere)</td>
<td>0.0407</td>
<td>0.8537</td>
</tr>
<tr>
<td>Total volume of white matter damage: total damaged white matter throughout the corpus callosum and external capsule</td>
<td>-0.2563</td>
<td>0.2379</td>
</tr>
<tr>
<td>Area of white matter damage: total surface area of white matter damage in the corpus callosum and external capsule parallel to the cortical surface (independent of white matter thickness)</td>
<td>0.0349</td>
<td>0.8745</td>
</tr>
<tr>
<td>Maximal width of white matter damage: in the coronal plane, length of the widest area of white matter damage in the corpus callosum and external capsule parallel to the cortical surface</td>
<td>-0.0711</td>
<td>0.7471</td>
</tr>
<tr>
<td>Anterior extent of white matter damage: most anterior site of white matter damage relative to bregma</td>
<td>0.0278</td>
<td>0.8998</td>
</tr>
<tr>
<td>Posterior extent of white matter damage: most posterior site of white matter damage relative to bregma</td>
<td>0.0313</td>
<td>0.8873</td>
</tr>
<tr>
<td>Total length of white matter damage: distance between most anterior and posterior sites of white matter damage</td>
<td>-0.0050</td>
<td>0.9818</td>
</tr>
<tr>
<td>Anterior extent of striatal damage: most anterior site of striatal damage relative to bregma</td>
<td>-0.0694</td>
<td>0.7528</td>
</tr>
<tr>
<td>Posterior extent of striatal damage: most posterior site of striatal damage relative to bregma</td>
<td>-0.0078</td>
<td>0.9720</td>
</tr>
<tr>
<td>Total length of striatal damage: distance between most anterior and posterior sites of striatal damage</td>
<td>-0.0478</td>
<td>0.8286</td>
</tr>
</tbody>
</table>
I.6. Comparison of single and double press VNS groups

VNS was delivered according to one of two paradigms. In the first paradigm (N = 8), VNS was delivered coincident with trials in which the second press occurred within 500 msec of the first press. This is similar to previous studies\textsuperscript{6-8}. In the second paradigm (N = 6), VNS was delivered on the first press regardless of whether a second press occurred. This resulted in ~40% more stimulations during the therapy period (Wks 1 – 6; Paradigm 1: 6187 ± 439 stimulations, Paradigm 2: 10037 ± 1427 stimulations). In accordance with a previous study\textsuperscript{8}, additional VNS did not further improve recovery. Number of stimulations over the course of therapy was not correlated to recovery in individual subjects (r = 0.33, \( P = 0.24 \)). Minimal differences in performance were observed for any parameters between either paradigm during the therapy period. Both groups display similar recovery (VNS on successful trials: 77 ± 22% recovery, VNS on all trials: 76 ± 14% recovery; unpaired t-test, \( P = 0.969 \)). Based on similar performance in each paradigm, subjects were combined and regarded as the VNS+Rehab group.

II. Supplementary videos

\textit{Video 1. Performance of the bradykinesia assessment task before ICH}
Prior to surgery (Pre-lesion) all rats were highly proficient on the bradykinesia assessment task. This movie illustrates a rat reaching out of the cage with the forelimb to press the lever rapidly.

\textit{Video 2. Performance after ICH}
One week after ICH, rats returned for post-lesion assessment. This movie shows the typical degree of impairment. Note the difficulty reaching and lack of forelimb control.

\textit{Video 3. Performance after the completion of VNS delivered during rehabilitative training (VNS+Rehab)}
This movie illustrates recovery after five weeks of VNS paired with rehabilitative training. Note the marked improvement in forelimb reaching, control, and movement speed.

\textit{Video 4. Performance after the completion of rehabilitative training with VNS (Rehab)}
This movie shows performance after five weeks of rehabilitative training without VNS. Note the sustained lack of forelimb control despite the extensive rehabilitative training.

III. Supplementary references


SUPPLEMENTAL MATERIAL

Vagus Nerve Stimulation Delivered during Rehabilitative Training Improves Recovery of Forelimb Function After Intracerebral Hemorrhage

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Contents

I. Supplemental Methods
   1. Behavioral training
   2. Exclusion of subjects
   3. Group assignment
   4. Raw statistical values for comparisons
   5. Correlation of lesion size and forelimb performance
   6. Comparison of single and double press VNS groups

II. Supplementary videos

III. Supplementary references

I.1. Behavioral Training

The bradykinesia assessment task was performed as previously described1. The apparatus consists of a lever (Lever Device, Vulintus Inc., Dallas, TX) located 1.25 cm outside of a behavioral chamber. A control board and custom software (Motor Controller, Vulintus Inc., Dallas, TX) sample the switch at 20 Hz and measure lever press times with an accuracy of ± 1 msec. Custom MATLAB software analyzed, displayed, and stored the data. If a trial was successful, the software triggered an automated pellet dispenser (Vulintus Inc., Dallas, TX) to deliver a sucrose pellet (45mg dustless precision pellet, BioServ, Frenchtown, NJ) to a receptacle in the chamber. Behavioral sessions were conducted for 30 min twice daily, five days a week, with daily sessions separated by at least 2 hours. For each trial, a timer was initiated on the first press of the lever. If the lever was depressed a second time within 500 msec, the trial was recorded as a success and a reward pellet was delivered. If the lever was not pressed again or the second press occurred more than 500 msec later, the trial was recorded as a failure and no reward was given. Rats were held at the pre-lesion stage until they had 10 successive sessions averaging over 75% success rate. Upon reaching this performance level, rats received a lesion and VNS implant. After 7 days of recovery, rats returned for post-lesion testing and were held at this stage until they had 4 sessions averaging at least 15 trials each. After the post-lesion stage, rats were assigned to groups (see below) and testing continued for 6 weeks.

I.2. Exclusion of subjects

We employed three primary exclusion criteria for the study. 1) Rats did not survive ICH. Nine rats were excluded based on this criterion. All deaths occurred before group assignment, and therefore could not bias outcomes. 2) Rats were excluded if they failed to display at least a 20% decrease in hit rate compared to pre-lesion following stroke. Six rats were excluded based on this criterion. 3) Rats were excluded if there were too impaired to perform 4 sessions averaging at least 15 trials during within the first 4 weeks of post-lesion assessment. Ten rats were excluded based on this criterion. Exclusion based on either the first, second, or third criterion took place before assignment to either the Rehab or VNS+Rehab group, and therefore did not impact the interpretation of the effects of therapy. 4) Rats were excluded if their headcap connector or vagus nerve cuff failed due to mechanical breakage or high impedance (>30 kΩ). Three rats were removed due to a high impedance measurement and four rats were removed due to headcap malfunction. These exclusion occurred after assignment to treatment groups and therefore could potentially impact the interpretation of the results.
However, addition of these excluded subjects up to the point of device failure had little effect on the significance of any comparison (Fig. I). The only statistical effect of inclusion of the additional subjects is loss of significance in the across group comparison of hit rate at week 5 of treatment and the emergence of a significant difference at the post-lesion time point for inter-press interval.

**Supplementary Figure I.** Forelimb performance data including all subjects for (A) hit rate, (B) second press latency, and (C) number of trials.

### I.3. Group Assignment

Following post-lesion baseline assessment of performance, rats were sorted into balanced groups based on hit rate. The first four rats were randomly assigned to a treatment group. For all subsequent rats, the post-lesion hit rate of each rat was compared to the average for each group and added to the group that minimized the between-group difference. This ensured evenly balanced performance between groups after lesion, allowing accurate comparison of the effects of treatment.

### I.4. Raw statistical values for comparisons

The table below contains the statistical comparison for all t-test comparisons between pre-lesion, post-lesion, and during therapy time points and across groups for subjects included in the main text.
### Supplementary Table I

<table>
<thead>
<tr>
<th>Measure</th>
<th>Comparison</th>
<th>Group</th>
<th>Wk 1</th>
<th>Wk 2</th>
<th>Wk 3</th>
<th>Wk 4</th>
<th>Wk 5</th>
<th>Wk 6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hit rate</strong></td>
<td>Pre v. week</td>
<td>Rehab</td>
<td>2.98 x 10⁻⁴</td>
<td>0.0021</td>
<td>0.0016</td>
<td>0.0018</td>
<td>0.0054</td>
<td>0.0052</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VNS+Rehab</td>
<td>0.0030</td>
<td>0.0433</td>
<td>0.0144</td>
<td>0.0306</td>
<td>0.1145</td>
<td>0.1874</td>
</tr>
<tr>
<td></td>
<td>Post v. week</td>
<td>Rehab</td>
<td>8.85 x 10⁻⁴</td>
<td>0.0307</td>
<td>0.1907</td>
<td>0.1068</td>
<td>0.0260</td>
<td>0.0308</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VNS+Rehab</td>
<td>4.64 x 10⁻⁴</td>
<td>3.05 x 10⁻⁴</td>
<td>1.85 x 10⁻⁴</td>
<td>6.46 x 10⁻⁵</td>
<td>1.55 x 10⁻⁵</td>
<td>6.85 x 10⁻⁶</td>
</tr>
<tr>
<td></td>
<td>Rehab v. VNS+Rehab</td>
<td>n/a</td>
<td>0.1402</td>
<td>0.0087</td>
<td>0.0046</td>
<td>0.0112</td>
<td>0.0260</td>
<td>0.0143</td>
</tr>
<tr>
<td><strong>Second press latency</strong></td>
<td>Pre v. week</td>
<td>Rehab</td>
<td>0.0062</td>
<td>0.0049</td>
<td>0.0026</td>
<td>0.0030</td>
<td>0.0070</td>
<td>0.0103</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VNS+Rehab</td>
<td>0.1565</td>
<td>0.7188</td>
<td>0.3212</td>
<td>0.4191</td>
<td>0.4624</td>
<td>0.5949</td>
</tr>
<tr>
<td></td>
<td>Post v. week</td>
<td>Rehab</td>
<td>0.0020</td>
<td>0.0437</td>
<td>0.1553</td>
<td>0.1722</td>
<td>0.0780</td>
<td>0.0844</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VNS+Rehab</td>
<td>0.0011</td>
<td>0.0010</td>
<td>0.0057</td>
<td>0.0107</td>
<td>0.0143</td>
<td>0.0075</td>
</tr>
<tr>
<td></td>
<td>Rehab v. VNS+Rehab</td>
<td>n/a</td>
<td>0.0722</td>
<td>6.23 x 10⁻⁴</td>
<td>0.0020</td>
<td>0.0027</td>
<td>0.0120</td>
<td>0.0102</td>
</tr>
<tr>
<td><strong>Trials</strong></td>
<td>Pre v. week</td>
<td>Rehab</td>
<td>0.9173</td>
<td>0.4460</td>
<td>0.2332</td>
<td>0.3640</td>
<td>0.8684</td>
<td>0.7745</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VNS+Rehab</td>
<td>0.5197</td>
<td>0.6119</td>
<td>0.5324</td>
<td>0.9469</td>
<td>0.9396</td>
<td>0.4546</td>
</tr>
<tr>
<td></td>
<td>Post v. week</td>
<td>Rehab</td>
<td>0.0055</td>
<td>0.0027</td>
<td>0.0036</td>
<td>0.0037</td>
<td>0.0074</td>
<td>0.0125</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VNS+Rehab</td>
<td>0.0077</td>
<td>0.0109</td>
<td>0.0023</td>
<td>0.0082</td>
<td>0.0066</td>
<td>7.52 x 10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>Rehab v. VNS+Rehab</td>
<td>n/a</td>
<td>0.8028</td>
<td>0.6743</td>
<td>0.7706</td>
<td>0.6035</td>
<td>0.9589</td>
<td>0.7752</td>
</tr>
</tbody>
</table>

### I.5. Correlation of lesion size and behavioral parameters

We analyzed several measures of lesion size in cresyl violet stained sections and attempted to correlate post-stroke performance in individual subjects. Pearson correlation was calculated comparing post-lesion hit rate to each of the measures of lesion size listed in the table below. We found that none of these measurements yielded a significant correlation.

### Supplementary Table II

<table>
<thead>
<tr>
<th>Histological Measure</th>
<th>Pearson’s r</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lesion volume&lt;sup&gt;+&lt;/sup&gt;:  [(Volume of unlesioned hemisphere) – (Volume of ventricle on unlesioned side)] – [(Volume of lesioned hemisphere) – (Volume of ventricle on lesioned side) – (Cellular debris)]</td>
<td>-0.0044</td>
<td>0.9841</td>
</tr>
<tr>
<td>Volume of lesioned tissue: total volume of damaged tissue, including gray and white matter</td>
<td>0.0779</td>
<td>0.7239</td>
</tr>
<tr>
<td>Ventricle size ratio&lt;sup&gt;+&lt;/sup&gt;: (Volume of ventricle in lesioned hemisphere) / (Volume of ventricle in unlesioned hemisphere)</td>
<td>0.0407</td>
<td>0.8537</td>
</tr>
<tr>
<td>Total volume of white matter damage: total damaged white matter throughout the corpus callosum and external capsule</td>
<td>-0.2563</td>
<td>0.2379</td>
</tr>
<tr>
<td>Area of white matter damage: total surface area of white matter damage in the corpus callosum and external capsule parallel to the cortical surface (independent of white matter thickness)</td>
<td>0.0349</td>
<td>0.8745</td>
</tr>
<tr>
<td>Maximal width of white matter damage: in the coronal plane, length of the widest area of white matter damage in the corpus callosum and external capsule parallel to the cortical surface</td>
<td>-0.0711</td>
<td>0.7471</td>
</tr>
<tr>
<td>Anterior extent of white matter damage: most anterior site of white matter damage relative to bregma</td>
<td>0.0278</td>
<td>0.8998</td>
</tr>
<tr>
<td>Posterior extent of white matter damage: most posterior site of white matter damage relative to bregma</td>
<td>0.0313</td>
<td>0.8873</td>
</tr>
<tr>
<td>Total length of white matter damage: distance between most anterior and posterior sites of white matter damage</td>
<td>-0.0050</td>
<td>0.9818</td>
</tr>
<tr>
<td>Anterior extent of striatal damage: most anterior site of striatal damage relative to bregma</td>
<td>-0.0694</td>
<td>0.7528</td>
</tr>
<tr>
<td>Posterior extent of striatal damage: most posterior site of striatal damage relative to bregma</td>
<td>-0.0078</td>
<td>0.9720</td>
</tr>
<tr>
<td>Total length of striatal damage: distance between most anterior and posterior sites of striatal damage</td>
<td>-0.0478</td>
<td>0.8286</td>
</tr>
</tbody>
</table>
I.6. Comparison of single and double press VNS groups

VNS was delivered according to one of two paradigms. In the first paradigm (N = 8), VNS was delivered coincident with trials in which the second press occurred within 500 msec of the first press. This is similar to previous studies. In the second paradigm (N = 6), VNS was delivered on the first press regardless of whether a second press occurred. This resulted in ~40\% more stimulations during the therapy period (Wks 1 – 6; Paradigm 1: 6187 ± 439 stimulations, Paradigm 2: 10037 ± 1427 stimulations). In accordance with a previous study, additional VNS did not further improve recovery. Number of stimulations over the course of therapy was not correlated to recovery in individual subjects (r = 0.33, P = 0.24). Minimal differences in performance were observed for any parameters between either paradigm during the therapy period. Both groups display similar recovery (VNS on successful trials: 77 ± 22\% recovery, VNS on all trials: 76 ± 14\% recovery; unpaired t-test, P = 0.969). Based on similar performance in each paradigm, subjects were combined and regarded as the VNS+Rehab group.

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