SUPPLEMENTAL MATERIAL

Vagus nerve stimulation enhances stable plasticity and generalization of stroke recovery

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I. Supplemental Methods

All behavioral testing was performed on adult female Sprague Dawley rats because females are underrepresented in preclinical stroke studies[1] and stroke is more prevalent in females[2].

I.1. Supination assessment task behavioral testing

Behavioral testing was conducted in a clear acrylic cage (30 cm x 13 cm x 25 cm) with a 1.3 cm wide slot on the right edge of the front wall (MotoTrak Base Cage Rat Model, Vulintus, Inc., Dallas, TX). The slot restricts use to the right forelimb while allowing full range of movement during interaction with the device (Fig. 1D). A textured spherical manipulandum 1 cm in diameter is centered in the slot and coupled about the center axis to an optical rotary encoder, providing turn angle measurements with a 0.25 degree resolution. The encoder is mounted on a metal slide allowing the device to be placed at various fixed distances relative to the inside wall of the cage. A pulley provides 6-grams of counterweight to the manipulandum, limiting animals to clockwise rotation (supination) while providing a constant torque (0.29mN*m) and returning the manipulandum to the original location once released (Knob Behavior Module, Vulintus, Inc., Dallas, TX). A microcontroller sampled the encoder position at a frequency of 100Hz and the signal was passed to the computer for displaying data, controlling behavioral sessions, and saving data to a file for analysis.

Custom MATLAB software was used to control the task (MotoTrak Software, Vulintus, Inc., Dallas, TX). The GUI displays real-time turn angle of the device in degrees performance over the course of the behavioral session. Data was collected and stored on a trial-by-trial basis for each animal. Trial initiation occurred when the animal rotated the device a minimum of 5 degrees. If the animal rotated the knob past the pre-determined turn angle threshold within two seconds of trial initiation, the trial was recorded as a success and a reward pellet was delivered (45 mg dustless chocolate precision pellet, BioServ, Frenchtown, NJ). If the turn angle did not exceed the threshold within the two seconds, the trial was recorded as a failure and no reward pellet was given. All trials were followed by a two-second timeout window in which no pellet rewards were delivered. All activity one second prior and four seconds following trial initiation was recorded for analysis (Fig. 1B).

A training algorithm was used as previously described to adaptively scale the success thresholds and accurately track individual performance[3]. The algorithm uses the median of the peak turn angle of the previous 10 trials to calculate the current trial success threshold, with a 15-degree minimum and 60-degree maximum adaptive threshold bounds. Success rate was defined as the percentage of trials greater than the maximum threshold. Animals underwent two 30-minute behavioral training sessions daily, five days per week, with at least a 2-hour interval between training periods (Fig. 1A).

I.2. Isometric pull task behavioral testing

Identical behavioral chambers were used as in the supination task described above, but the slot in the cage allowed access to a manipulandum that emphasizes application of volitional forelimb pull force. Instead of a spherical manipulandum attached to a rotary encoder as in the supination task, an aluminum handle affixed to a force transducer was centered on the slot (Fig. 1E). The force transducer measured the pull force applied with a resolution of 0.1 grams. The manipulandum was placed 0.75" outside relative to the inner cage wall. A microcontroller sampled the force transducer at a frequency of 100Hz, and the MATLAB software described above was used to control the task and for collection and displaying the data.

I.3. Lesion quantification and analysis

Within one week of the conclusion of behavioral testing, animals were transcardially perfused with 4% paraformaldehyde. Brains were removed and fixed in 4% paraformaldehyde overnight, and then cryoprotected in a 30% sucrose solution. Tissue was sectioned in 35-μm slices and processed with Nissl and myelin stains for lesion identification. A rat brain atlas (Paxinos and Watson, 2007) was used to help determine lesion size and location.

II. Supplemental Tables

The table below contains numerical group data, confidence intervals, t-test comparisons across groups, and effect sizes for all time points and all animals included in the main text.

Table I

	Rehab Mean	VNS Mean	Rehab CI	VNS CI	*p*-value	Effect size (Cohen's d)
Pre	67.34°	68.46°	±1.17°	±3.41°	0.466	0.37
Post	27.13°	32.24°	±6.61°	±6.02°	0.213	0.59
Week 1	30.63°	51.06°	±9.77°	±8.80°	0.003	1.63
Week 2	33.60°	55.35°	±12.54°	±9.06°	0.006	1.48
Week 3	35.25°	56.15°	±12.94°	±8.62°	0.008	1.43
Week 4	38.03°	59.11°	±12.16°	±9.90°	0.007	1.41
Week 5	36.88°	59.44°	±12.68°	±7.86°	0.004	1.61
Week 6	39.53°	58.94°	±11.71°	±7.44°	0.006	1.49
Week 7	111.35g	142.58g	±19.40g	±18.99g	0.018	1.21
Week 8	114.45g	149.56g	±18.42g	±19.91g	0.008	1.36
Week 9	125.67g	147.75g	±13.84g	±17.73g	0.036	1.04
Week 10	120.44g	149.24g	±15.61g	±14.73g	0.007	1.41
Week 11	38.54°	57.19°	±12.52°	±8.81°	0.015	1.28
Week 12	42.51°	57.67°	±13.59°	±9.90°	0.060	0.95

III. Supplemental Figures and Figure Legends

Animals were excluded from this study based on three exclusion criteria: (1) Did not survive the ischemic lesion and VNS implant (N=4); (2) Did not display at least a 50% reduction in success rate (N=4); (3) Headcap or stimulation cuff failure (N=4). Of these, 4 exclusions were done following group assignment due to headcap or stimulating cuff failure and could potentially impact the interpretation of results. Three of the four rats were removed due to headcap malfunction, and one was removed due to high impedance measurements. However, addition of these excluded subjects up to the point of device failure in an intent-to-treat analysis had little effect on the significance of any comparison (Fig. 1). The only statistical effect of inclusion of the additional subjects is loss of significance of the across group comparison at week 7 and 9 and the emergence of a significant difference at week 12.
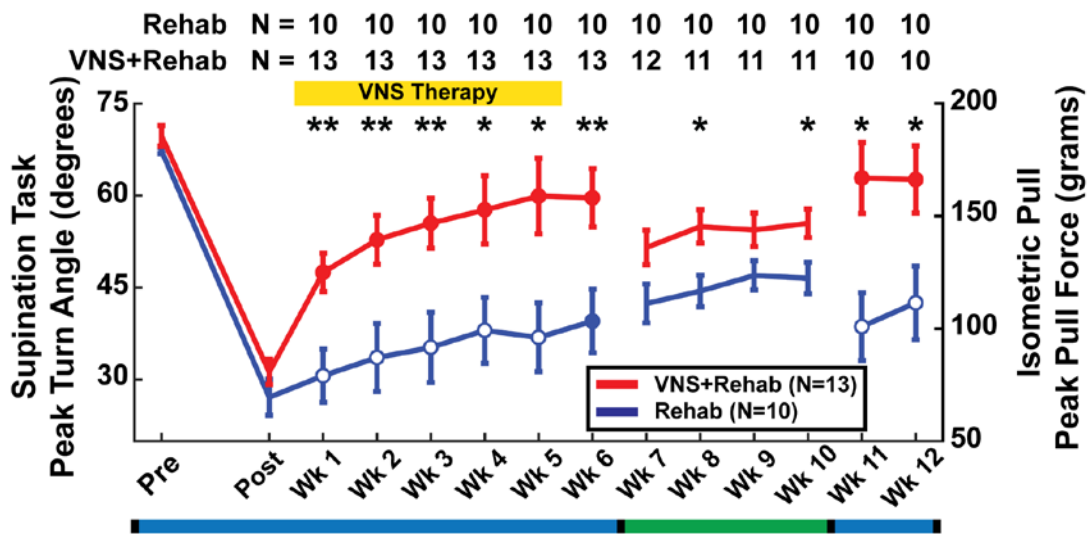
Figure I. Forelimb performance data including all subjects for peak turn angle and peak pull force. Group sizes are indicated at the top of the figure for each timepoint.
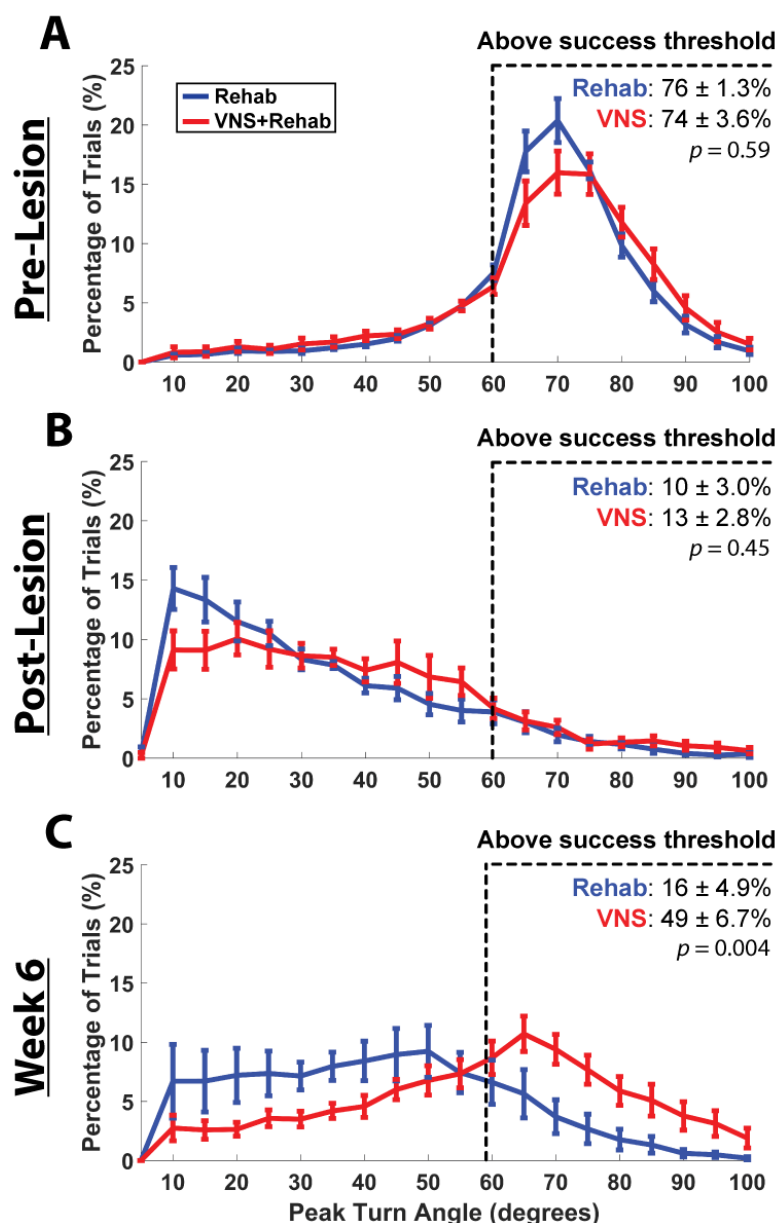
Figure II. Distribution of turn angles. Probability distribution histograms of peak turn angles during **(A)** Pre-Lesion training, **(B)** Post-Lesion testing, and **(C)** on the sixth week of therapy. The numbers in the dashed box indicate the percentage of trials that exceed the 60 degree threshold for each group. Note the rightward shift in the VNS group following six weeks of VNS therapy.

IV. Supplemental Videos

Video I. Supination performance prior to stroke

Representative example of a rat (**Rehab Rat01**) performing the supination assessment task prior to stroke. Note that the rat is highly proficient at the task and displays excellent forelimb motor control.

The plot on the bottom left displays the turn angle of the manipulandum plotted in real time for an individual trial. The horizontal green line indicates turn angle threshold to trigger delivery of a reward pellet. The triangle indicates peak turn angle on a trial. The plot on the bottom right shows performance over the course of the session. Each circle indicates peak turn angles from an individual trial. Green circles indicate trials in which the peak turn angle exceeded the reward threshold. White circles indicate trials in which peak force did not exceed the reward threshold.

Video II. Supination performance after stroke

Representative example of a rat (**Rehab Rat01**) performing the supination task one week after stroke. The rat displays a notable reduction in peak turn angle, consistent with a reduction in forelimb supination, and clear deficits in motor control.

The plot on the bottom left displays turn angle of the manipulandum on an individual trial. The horizontal green line indicates turn angle threshold to trigger delivery of a reward pellet. Note that the threshold adaptively scales based on the median turn angle of the ten antecedent trials. The triangle indicates peak turn angle on a trial. The plot on the bottom right shows performance over the course of the session. Each circle indicates peak turn angle from an individual trial. Green circles indicate trials in which the peak turn angel exceeded the reward threshold. White circles indicate trials in which peak force did not exceed the reward threshold.

Video III. Supination performance after rehabilitation alone

Representative example of a rat (**Rehab Rat01**) performing the supination task at the conclusion of six weeks of rehabilitative training alone. Note the sustained impairments in forelimb supination ability and motor control despite intensive rehabilitative training.

The plot on the bottom left displays turn angle of the manipulandum on an individual trial. The horizontal green line indicates turn angle threshold to trigger delivery of a reward pellet. Note that the threshold adaptively scales based on the median turn angle of the ten antecedent trials. The triangle indicates peak turn angle on a trial. The plot on the bottom right shows performance over the course of the session. Each circle indicates peak turn angle from an individual trial. Green circles indicate trials in which the peak turn angel exceeded the reward threshold. White circles indicate trials in which peak force did not exceed the reward threshold.

Video IV. Supination performance after rehabilitation paired with VNS

Representative example of a rat (**VNS Rat02**) performing the supination task at the conclusion of six weeks of rehabilitative training paired with VNS. Note the improvements in motor control and increase in forelimb supination ability compared to after stroke.

The plot on the bottom left displays turn angle of the manipulandum on an individual trial. The horizontal dashed green and red line indicates turn angle threshold to trigger delivery of a reward pellet and VNS delivery. Note that the threshold adaptively scales based on the median turn angle of the ten antecedent trials. Vertical red lines indicate VNS delivery when pull force exceeds the stimulation threshold. The triangle indicates peak turn angle on a trial. The plot on the bottom right shows performance over the course of the session. Each circle indicates peak turn angle from an individual trial. Green circles with a red border indicate trials in which the peak turn angel exceeded the reward and stimulation threshold and a pellet and VNS was delivered. White circles indicate trials in which peak force did not exceed the reward threshold.

Video V. Performance on the isometric pull task after rehabilitative training on the supination task

Representative example of a rat (**Rehab Rat01**) performing the isometric pull task at the conclusion of four weeks of isometric pull training (Week 10 of the study). The rat displays a notable reduction in peak pull force, consistent with forelimb paresis, and clear deficits in motor control.

The plot on the bottom left displays pull force applied to the handle on an individual trial. The horizontal green line indicates the adaptively scaled force threshold to trigger delivery of a reward pellet. Note that the threshold adaptively scales based on the median peak force of the ten antecedent trials. The triangle indicates peak pull force on a trial. The plot on the bottom right shows performance over the course of the session. Each circle indicates peak force from an individual trial. Green circles indicate trials in which pull force exceeded the reward threshold. White circles indicate trials in which peak force did not exceed the reward threshold.

Video VI. Performance on the isometric pull task after rehabilitative training on the supination task paired with VNS

Representative example of a rat (**VNS Rat02**) performing the isometric pull task at the conclusion of four weeks of isometric pull training (Week 10 of the study). Note that VNS was only delivered on the supination task, and no VNS was delivered during isometric pull testing. The rat displays improvements in motor control and increase in forelimb pull force.

The plot on the bottom left displays pull force applied to the handle on an individual trial. The horizontal green line indicates the adaptively scaled force threshold to trigger delivery of a reward pellet. Note that the threshold adaptively scales based on the median peak force of the ten antecedent trials. The triangle indicates peak pull force on a trial. The plot on the bottom right shows performance over the course of the session. Each circle indicates peak force from an individual trial. Green circles indicate trials in which pull force exceeded the reward threshold. White circles indicate trials in which peak force did not exceed the reward threshold.

V. Supplemental References

1.   Fisher M, Feuerstein G, Howells DW, Hurn PD, Kent T a., Savitz SI, et al. Update of the stroke therapy academic industry roundtable preclinical recommendations. *Stroke.* 2009;40:2244–2250.

2.   Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, et al. Heart disease and stroke statistics-2016 update a report from the American Heart Association. 2016.

3.   Meyers EC, Granja R, Solorzano BR, Romero-Ortega M, Kilgard MP, Rennaker RL, et al. Median and ulnar nerve injuries reduce volitional forelimb strength in rats. *Muscle Nerve.* 2017;12:133–150.

Stroke Online Supplement

Table I. Checklist of Methodological and Reporting Aspects for Articles Submitted to *Stroke* Involving Preclinical Experimentation

Methodological and Reporting Aspects	Description of Procedures
Experimental groups and study timeline	☐ The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study. ☐ An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated. ☐ An overall study timeline is provided.
Inclusion and exclusion criteria	☐ A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article.
Randomization	☐ Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided. ☐ Type and methods of randomization have been described. ☐ Methods used for allocation concealment have been reported.
Blinding	☐ Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible. ☐ Blinding procedures have been described with regard to masking of group assignment during outcome assessment.
Sample size and power calculations	☐ Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided.
Data reporting and statistical methods	☐ Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups. ☐ Baseline data on assessed outcome(s) for all experimental groups have been reported. ☐ Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms. ☐ Statistical methods used have been reported. ☐ Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures.
Experimental details, ethics, and funding statements	☐ Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described. ☐ Different sex animals have been used. If not, the reason/justification is provided. ☐ Statements on approval by ethics boards and ethical conduct of studies have been provided. ☐ Statements on funding and conflicts of interests have been provided.