ONLINE SUPPLEMENT

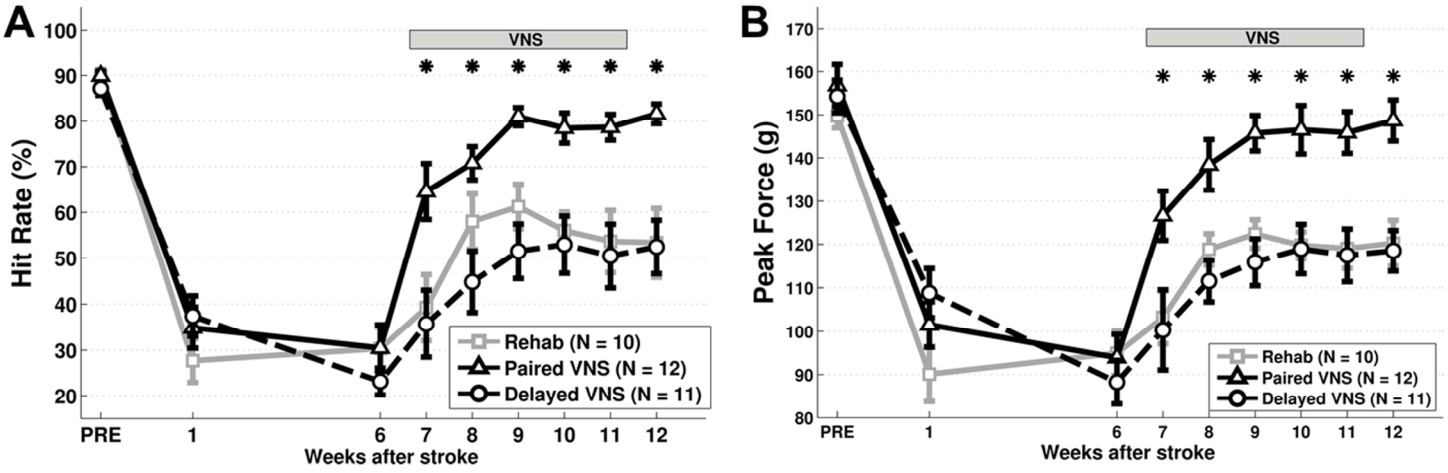
Vagus Nerve Stimulation During Rehabilitative Training Improves Forelimb Recovery after Chronic Ischemic Stroke in Rats

Navid Khodaparast, Ph.D.[1,2], Michael P. Kilgard, Ph.D.[1,2], Reema Casavant, Ph.D.[3], Andrea Ruiz, M.S.[1], Patrick D. Ganzer, Ph.D.[1,4], Iqra Qureshi[2], Robert L. Rennaker II, Ph.D.[1,2,4], Seth A. Hays[1,2,4]*, Ph.D.

Contents

I.1. Exclusion of subjects

We employed four primary exclusion criteria for the study. 1) Rats died as a result of surgical complications or stroke. 16 rats were excluded based on this criterion. 2) Rats failed to display a statistically significant reduction in hit rate compared to pre-lesion at week 1 or 6 after stroke. 14 rats were excluded based on this criterion. 3) Rats were too impaired to perform trials for ten days beginning on week 6. 2 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 could not bias 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). 4 rats were removed for technical issues related to cuff or headcap function (Rehab, n = 1; Paired VNS, n = 2; Delayed VNS, n = 1). These exclusions 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 no effect on the significance of any comparison (Fig. I).



Supplementary Figure I. Performance data including all subjects for (A) hit rate and (B) maximal pull force. * denotes $p < 0.05$ between Rehab and Paired VNS at each time point. Error bars indicate mean ± SEM.

I.2. Group Assignment

Following week 6 assessment of performance, rats were sorted into balanced groups based on hit rate. The first rats were randomly assigned to a treatment group. For subsequent rats, the 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.3. Raw behavioral statistical comparisons

The table below contains the statistical comparison for all t-test comparisons for all time points and across groups for subjects included in the main text.

Supplementary Table I

	Comparison	Group	Wk 6	Wk 7	Wk 8	Wk 9	Wk 10	Wk 11	Wk 12
Hit rate	PRE v. Week	Rehab	2.98×10^{-7}	2.06×10^{-4}	0.0023	0.0010	1.27×10^{-5}	7.52×10^{-4}	0.0011
		Paired VNS	3.42×10^{-6}	0.0024	0.0026	0.0108	0.0287	0.0193	0.0113
		Delayed VNS	4.60×10^{-9}	9.56×10^{-5}	1.01×10^{-4}	2.18×10^{-4}	4.37×10^{-4}	5.46×10^{-4}	1.52×10^{-4}
	Week 6 v. Week	Rehab	n/a	0.1150	0.0045	0.0017	0.0029	0.0175	0.0231
		Paired VNS	n/a	0.0069	8.49×10^{-6}	1.04×10^{-5}	3.68×10^{-5}	3.78×10^{-5}	6.41×10^{-6}
		Delayed VNS	n/a	0.1818	0.0237	0.0029	0.0025	0.0070	0.0015
	Paired v. Rehab	n/a	0.8153	0.0207	0.0419	0.0018	1.36×10^{-5}	0.0027	0.0027
	Paired v. Delayed	n/a	0.3991	0.0217	0.0017	5.12×10^{-4}	0.0020	0.0023	5.85×10^{-4}
	Rehab v. Delayed	n/a	0.4910	0.7685	0.1079	0.2516	0.9463	0.7472	0.9243
Maximal Force	PRE v. Week	Rehab	7.51×10^{-6}	2.61×10^{-4}	7.06×10^{-5}	3.28×10^{-5}	3.48×10^{-6}	5.46×10^{-4}	0.0015
		Paired VNS	2.69×10^{-5}	9.30×10^{-4}	0.0050	0.0244	0.0994	0.1047	0.0309
		Delayed VNS	2.68×10^{-6}	5.75×10^{-4}	4.89×10^{-5}	2.19×10^{-4}	8.55×10^{-4}	0.0013	4.55×10^{-5}
	Week 6 v. Week	Rehab	n/a	0.0346	5.52×10^{-4}	6.13×10^{-4}	0.0028	0.0064	0.0062
		Paired VNS	n/a	0.0076	2.56×10^{-4}	7.81×10^{-5}	1.28×10^{-4}	8.19×10^{-5}	3.85×10^{-5}
		Delayed VNS	n/a	0.2068	0.0046	0.0025	5.12×10^{-4}	0.0025	9.87×10^{-4}
	Paired v. Rehab	n/a	0.8017	0.0239	0.0075	1.88×10^{-4}	3.05×10^{-4}	4.90×10^{-4}	0.0012
	Paired v. Delayed	n/a	0.4809	0.0504	0.0011	1.89×10^{-4}	0.0016	0.0012	3.34×10^{-4}
	Rehab v. Delayed	n/a	0.6089	0.8621	0.1901	0.3449	0.8446	0.8414	0.8088

I.4. Isometric force task

The isometric force task was used to measure volitional forelimb strength as previously described[1,2]. 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, TX). Force measurements were sampled at 100 Hz and measured with ± 1 gram accuracy. 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, Sachse, TX) to deliver a sucrose pellet (45 mg dustless precision pellet, BioServ, Frenchtown, NJ) to a receptacle in 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 minutes each and were conducted twice daily, five days a week, with sessions on the same day separated by at least 2 hours. Once proficient, rats were held until they had 10 successive sessions averaging over 85% success rate. The pre-lesion data (PRE) reported in this study is compiled from these 10 sessions. Rats were then given a unilateral ischemic lesion followed by seven days of recovery, after which they returned for testing until they had 4 sessions with greater than 10 trials each (compiled as Week 1). Following forelimb assessment, rats were returned to home cage for 5 weeks, and were implanted with a vagus nerve cuff on week 4. Rats returned for assessment on week 6, and were assigned to

balanced groups based on performance (Online Supplement). Rats then proceeded to the therapy stage where VNS was delivered as appropriate for each group for 5 weeks. All rats underwent 1 week of additional training without stimulation (Week 12) to assess persistent effects of VNS.

I.5. Histological procedures

Rats were anesthetized with sodium pentobarbital (50 mg/kg, IP) and transcardially perfused with 250 mL of 0.02% heparin/0.1 M phosphate-buffered (PB) solution, followed by 450 mL of 4% paraformaldehyde/0.1 M PB solution. Brains were removed and postfixed in 4% paraformaldehyde/0.1 M PB solution, and then cryoprotected in a 30% sucrose/0.1 M PB solution. The tissue was sectioned at 40 μm intervals and processed with Cresyl Violet and myelin stains. Image J was used to determine lesion size. Nissl-myelin histology could not be performed on 5 of the 29 included subjects.

MAP-2 immunohistochemistry was performed similar to previous studies[3]. 40 μm sections of brain tissue were randomly selected for staining. Floating sections were incubated in 0.3% H_2O_2 & 50% ethanol in PBS for 30 mins, and then rinsed in PBS. Sections were blocked 2% normal horse serum (NHS) and 0.1% Triton in PBS for 1 hour. Sections were then incubated in monoclonal mouse anti-MAP2 primary antibody (Sigma; clone AP20; MAP2a +2b) diluted 1:500 in PBS containing 2% NHS and 0.1% Triton incubated for 24 hours at 4°C. Sections were rinsed in PBS and incubated in biotinylated horse anti-mouse secondary antibody (Vector Labs) diluted 1:200 in PBS containing 2% NHS for 2 hours at room temperature. After PBS rinse, sections were treated with Elite ABC reagent (Vector Labs) for 2 hours, PBS rinsed, and exposed to 3-3' diaminobenzidine tetrahydrochloride (DAB; Sigma) for 5 minutes. Sections were mounted on slides, dehydrated with a series of alcohol washes, covered in DPX mounting medium (Sigma), and coverslipped.

Photomicrographs of brain sections that were stained with nissl/myelin or MAP-2 were taken with a NanoZoomer (Hamamatsu, Tokyo, Japan). MAP-2 analysis was performed in the following regions of interest: perilesional cortex layer II/III and layer V (4 images), homotopic contralesional motor cortex layer II/III and layer V (2 images), and contralesional insular cortex (1 image). Digitized images were converted to 8-bit grayscale. Images were processed in Image J using custom software, similar to previous studies[4]. Background values were calculated and subtracted within images. The cutoff threshold value was kept constant for all images. The number of pixels above the cutoff threshold divided by the total number of pixels in the image (Area Fraction) was calculated for each ROI.

II. Supplementary references

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(2) Pruitt D, Hays S, Schmid A, Choua C, Kim L, Trieu J, et al. Controlled-cortical impact reduces volitional forelimb strength in rats. Brain Res. 2014.

(3) Bury SD, Jones TA. Unilateral sensorimotor cortex lesions in adult rats facilitate motor skill learning with the "unaffected" forelimb and training-induced dendritic structural plasticity in the motor cortex. J Neurosci. 2002;22:8597-8606.

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