Published in final edited form as:

J Neurosci Res. 2012 April ; 90(4): 887–894.

The effect of vagus nerve stimulation during transient focal cerebral ischemia on chronic outcome in rats

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Abstract

The aim of this study was to investigate the effect of vagus nerve stimulation (VNS) on infarct volume and neurological recovery up to three weeks following transient focal cerebral ischemia. Transient ischemia was produced by filament occlusion of the proximal middle cerebral artery (MCA) in rats. The right vagus nerve was stimulated starting 30 minutes after MCA occlusion and consisted of 30 second pulse trains (20 Hz) delivered to the animal's right vagus nerve every 5 minutes for a total period of 60 minutes (n=10). All the procedures were duplicated but no stimulus was delivered in a control group (n=10). Neurological evaluations were performed in all animals at 24 hours, 48 hours, 1 week, 2 weeks and 3 weeks after MCA occlusion, and animals were euthanized and neuronal damage evaluated in H&E stained sections. The ischemic lesion volume was smaller in the VNS-treated animals in comparison to the non-stimulated group ($p<0.02$). Although the functional score in both treated and untreated groups improved over the three week observation period ($p<0.001$), there was still a statistically significant improvement due to VNS treatment compared with control animals ($p<0.05$). Cerebral blood flow changes in the MCA territory during ischemia did not differ between the VNS-treated animals ($31.9\pm10.4\%$ of baseline) and control ($29.9\pm9.1\%$) animals ($p=0.6$). Stimulation of the vagus nerve for only a brief period early in ischemia provides neuroprotection in transient ischemia with neuroprotection persisting for at least three weeks.

Keywords

vagus nerve; cerebral ischemia; neuroprotection; cerebral blood flow

Introduction

According to the National Stroke Association, stroke is the third leading cause of death in the United States and a leading cause of adult disability. Neuroprotective treatment strategies that may have the potential to reduce damage and/or enhance recovery of function have appeared to work in animals, but few methods have been established in clinical practice (O'Collins et al., 2006). There has been recent interest in the effect of vagus nerve stimulation (VNS) in a wide variety of disorders. VNS is known as a safe and effective treatment for epilepsy (Beekwilder and Beems, 2010) and treatment resistant depression (Nahas et al., 2007), and is likely to serve as an effective treatment for protection against circulatory shock (Czura et al., 2010; Guarini et al., 2003), myocardial ischemia (De Ferrari and Schwartz, 2011; Kawada et al., 2008), ischemic stroke (Mravec, 2010) and ischemic

brain injury (Sun et al., 2011). Mechanisms of these effects of VNS are not fully understood. We have previously shown that VNS is neuroprotective in models of both transient (filament occlusion of the middle cerebral artery) and permanent (photothrombosis of the middle cerebral artery) focal ischemia (Sun et al., 2011). These studies, however, examined infarct volume, neurobehavioral indices and cerebral blood flow only one day after occlusion. Many neuroprotective treatments which reduce ischemic injury in animals have been found to be beneficial following both short-term and long-term outcome (Li and Zuo, 2009; Sakai et al., 2007; Sheng et al., 2009), while some neuroprotective methods applied after brain ischemia may improve short-term but not improve the long-term neurological outcome (Colbourne et al., 1999; Dietrich et al., 1993; Valtysen et al., 1994; Wang et al., 2002). Because investigation made in the first few days after brain ischemia may not correctly characterize the neuroprotective potential of various agents, it is important to assess the effectiveness of the long-term neurological outcome in animal studies. The current study was designed to investigate the effect of VNS on infarct volume and neurological recovery up to three weeks following transient focal cerebral ischemia. As far as we know, our results are the first report of VNS induced neuroprotection more than a few days following cerebral ischemia.

Materials and Methods

Animal preparation

All procedures performed were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Adult male Sprague-Dawley rats (259–350g) from Charles River Laboratory (Wilmington, Mass) were anesthetized with isoflurane (4% for induction, 1.0–1.5% for maintenance) in a mixture of nitrous oxide and oxygen (7:3). The body temperature was monitored by a rectal probe and maintained at $37.3 \pm 0.2^{\circ}\text{C}$ with a heating pad (ATC1000, World Precision Instruments, Sarasota, FL). Body temperature was supported until the animal was awake and ambulatory at which time he was able to maintain his own temperature. The tail artery was cannulated with a polyethylene catheter (PE-50) for the measurement of arterial pressure and for arterial blood gas sampling. Arterial blood pressure was monitored using a pressure transducer and recorded on a computer based recording system at 100 Hz (PowerLab, ADInstruments, Colorado Springs, CO). Arterial blood gas was measured at two points: immediately before, and one hour after MCA occlusion. The head was then placed in a stereotaxic holder, a midline scalp incision was made and the frontoparietal region of the skull was exposed. A 2-mm diameter circular lesion of the skull overlying the core of the right middle cerebral artery (MCA) territory (centered 1 mm posterior and 5 mm lateral to the Bregma) was thinned using a saline-cooled dental drill (Star Dental) for replacement of a laser-Doppler Flow (LDF) probe. Following two hours period of ischemia, cortical blood flow was monitored for 30 min after reperfusion. After reperfusion 0.25% bupivacaine was topically applied to the head, neck and tail wounds to decrease postoperative pain and Gentamicin (4mg/kg) was applied intramuscularly for 3days. To prevent dehydration and weight loss 10 ml of lactated Ringers solution was administered subcutaneously daily for three days. Additionally 2019 Teklad Global 19% Protein Extruded Rodent Diet (Harlan Laboratories, Indianapolis, IN) was softened with water to make it easier to eat and was provided for three days post occlusion.

A third group of animals, the sham ischemia group, was prepared identically to that described above. However, the filament was not inserted and so these animals were not made ischemic.

Measurement of Laser-Doppler Flow response

A LDF probe attached to a flowmeter (PeriFlux 4001; PeriMed) was affixed over the area of thinned skull using dental cement so as to obtain a continuous measurement of relative CBF during the experiment.

Transient MCA occlusion

Focal cerebral ischemia was induced using the filament model as described previously (Luckl et al., 2008; Shimazu et al., 2005). Briefly, through a midline neck incision, the right common carotid artery (CCA) was isolated from the surrounding connective tissue. The right external carotid artery was ligated and a 0.39-mm silicone-coated nylon filament (Docol Corporation, Redlands, CA, USA) was inserted through the CCA into the internal carotid artery. The filament was gently advanced until LDF indicated adequate MCA occlusion by a sharp decrease in ipsilateral blood flow to 20–30% of baseline. After 120 min of occlusion, the suture was withdrawn to allow for cerebral reperfusion. Prompt and proper reperfusion was monitored by LDF for 30 min.

Experimental protocol and vagus nerve stimulation

During exposure of the right CCA, the right vagus nerve was isolated from the surrounding connective tissue, and a stimulating electrode was placed around the nerve and held in place by suturing it to the sternocleidomastoid muscle (Smith et al., 2005; Sun et al., 2011). Three groups were studied: control (MCA occlusion, no stimulation), VNS treated (MCA occlusion with stimulation of right vagus nerve), and sham ischemia VNS treated (preparation for MCA occlusion, but no occlusion, with VNS stimulation) (Fig. 1). In all groups, CBF was monitored 10 minutes before MCA occlusion (or sham occlusion) to establish a baseline level and 150 minutes after MCA occlusion to observe effects from VNS treatment. In the treatment group (n=10), VNS started 30 minutes after MCA occlusion, and consisted of 30 second pulse trains (0.5 mA square pulses with width 0.3 msec and repetition rate of 20 Hz) delivered to the animal's right vagus nerve every 5 minutes for a total period of 60 minutes. The control group (n=10) was prepared exactly the same way, including the placement of the stimulating electrode on the vagus nerve, except that no stimulation was delivered. The sham ischemia group (n=8) was prepared identically and stimulated for 60 minutes, except that the MCA was not occluded.

Neurological evaluation

Neurological evaluations were performed in all animals at 24 hours, 48 hours, 1 week, 2 weeks and 3 weeks after MCA occlusion according to the protocol of Belayev et al. (1996). Postural reflex, visual placing in forward and sideways directions, tactile placing of the dorsal and lateral paw surfaces and proprioceptive placing were tested. These 6 tests were each scored from 0 to 2, and neurological function was graded on a scale of 0 (no deficit) to 12 (maximum deficit) as the sum of the scores of the individual tests. The sham ischemic animals were scored only at 24 hours and then sacrificed for histological analysis.

Infarct volume measurement

Animals were sacrificed 3 weeks after MCA occlusion. They were perfused transcardially with 0.9% saline and then with 4% paraformaldehyde. The brains were removed and after overnight fixation, were cut in 10 μ m sections at 200 μ m intervals and stained with hematoxylin-eosin (H&E). The sections were imaged under a light microscope (Eclipse E600 Nikon) and digitized with a CCD camera (SPOT version 4.5.9.9, Diagnostic Instrument Inc). Areas of damaged brain tissue along with left and right hemispheric areas were determined at each cross-sectional level selected by reference to the corresponding level in an atlas of the rat brain and were delineated using a computer-based image analyzer

(AIS 6.0; Imaging Research) by one of the investigators blinded to the treatment conditions. Ischemic damage volumes were obtained by subtracting the volume of the right infarcted hemisphere from that of the left (contralesional) hemisphere thus correcting for atrophy (Bland et al., 2000; Li et al., 2004). The damaged areas were summed and multiplied by the distance between slices to give infarct volumes.

Statistical analysis

Results are expressed as mean $\pm$ SD. Significant differences between groups were determined with Student *t* test for blood gases (pH, PCO₂, PO₂) and infarct volumes. Differences between groups were analyzed by repeated measures ANOVA followed by a Tukey test for physiological measurements (blood pressure (BP), heart rate (HR)), and LDF. The effect of vagus nerve stimulation was determined with a paired *t*-test. The neurological data was analyzed with non-parametric statistics. To examine changes in neurological score across time the Wilcoxon Signed Rank Test was used, and to examine the effect of stimulation, the Mann-Whitney Rank Sum Test was employed. A *p*-value of <0.05 was considered statistically significant.

Results

Blood gas data

Blood gas parameters were within normal physiological range both before and during ischemia. Furthermore, there were no statistical differences either between groups or within groups, as a result of stimulation (Table I).

Blood pressure and heart rate data

Although arterial blood pressure was monitored continuously, it was examined at the following 5 points: before MCA occlusion (Baseline), following MCA occlusion but before VNS (0–30 minutes following MCA occlusion), during VNS (30–90 minutes following MCA occlusion), following VNS but before reperfusion (90–120 minutes following MCA occlusion), and following reperfusion. Blood pressure in the baseline period prior to temporary MCA occlusion was 95.1 ± 14.1 mm Hg in the control group, 92.9 ± 9.0 mm Hg in the VNS stimulation group, and 91.3 ± 9.7 mm Hg in the sham group. Blood pressure showed no significant change throughout the MCA occlusion (Fig. 2). When blood pressure during the 30 seconds epochs of stimulation was compared to the blood pressure four minutes prior to stimulation it decreased by 8.6 ± 12.4 mm Hg ($p=0.056$). In the sham group stimulation produced a 14.2 ± 12.1 mm Hg decrease in blood pressure ($p<0.05$). Following reperfusion, blood pressure decreased to approximately 87–89 mm Hg in both treated and untreated stroke groups, a statistically significant decrease ($p<0.02$; two-way repeated measures ANOVA). Heart rate decreased to 84% of control during the period of stimulation in the stroke group and to 81% of control in the sham ischemic group, and then increased to the pre-stimulus level following the termination of VNS ($p<0.02$) (Fig. 3).

Cerebral blood flow (CBF)

CBF measured with LDF fell to 25–28% of control immediately after MCA occlusion in both groups (Fig. 4). By 80 minutes after MCA occlusion, LDF increased to 31–35% of control and remained close to this level until the release of the occlusion. There was no significant difference in the time course of the LDF between the control group and the group receiving VNS, and there was no difference in the degree of ischemia between the two groups. Stimulation of the vagus nerve produced a transient decrease in LDF to $92 \pm 13.3\%$ of the pre-stimulus flow in the stroke group and to $82.6 \pm 18.2\%$ in the sham ischemic group with no significant difference between the two stimulation groups.

Infarct volume

Animals receiving vagus nerve stimulation during MCA occlusion showed significantly smaller histological damage than animals not receiving VNS (Fig. 5). The infarct volume in animals receiving VNS was $51.1 \pm 42.8 \text{ mm}^3$ compared with $119.4 \pm 63.6 \text{ mm}^3$ in the control group ($p < 0.02$). There was no histological damage seen in any brains from the sham ischemic group.

Neurological Score

The functional score in both control and treated groups improved over the three week observation period ($p < 0.01$; Wilcoxon Signed Rank Test) (Fig. 6), and in addition there was a statistically significant improvement due to VNS treatment compared with control animals ($p < 0.02$; Mann-Whitney Rank Sum Test). The treated animals showed a lower score than the controls at all time points (1, 2, 7, 14 days: $p < 0.05$; 21 days: $p < 0.01$). No deficits were seen in any of the animals in the sham ischemia group.

Discussion

To investigate whether VNS has a chronic neuroprotective effect, we examined infarct volume and neurological recovery three weeks after transient MCA occlusion. The finding of our study is that VNS during MCA occlusion produced a chronic phase of neuroprotection in transient ischemia. The ischemic lesion volume was smaller in the VNS-treated animals in comparison to the non-stimulated group (42.8% decrease, $p < 0.02$), and the neurological scores improved three weeks after MCA occlusion.

The effects of VNS on cerebral ischemic injury observed in the current study are consistent with previous reports of VNS-mediated acute neuroprotection (Ay et al., 2009; Ay et al., 2011; Masada et al., 1996; Miyamoto et al., 2003; Sun et al., 2011), whereas no data are available concerning the effect of VNS on the long-term neurological and histological outcome. In our recent study, we reported that in both transient and permanent experimental models, the animals in the VNS-treated groups exhibited smaller infarct sizes, with to a 56.3% decrease in total infarct volume in transient MCA occlusion and a 38.4% decrease in permanent MCA occlusion (Sun et al., 2011). Similarly in the transient ischemia model neurological scores of VNS-treated animals were better than in the control group. Although the previous study provided useful information about VNS as a strategy for improving neurological outcome in multiple experimental ischemia models, it did not provide information on the persistence of the neuroprotection as it examined neurological outcome only one day after occlusion. There are studies suggesting that some neuroprotective methods may just delay cell death after brain ischemia (Kawaguchi et al., 2000; Trescher et al., 1997). Although previous studies suggest ongoing changes in stroke damage can persist for at least 1 year (Karki et al., 2010), ongoing cell death peaks at 24–48 hours and plateaus at three weeks after brain ischemia in rats (Li et al., 1995). In our study we evaluated the brain infarct volume and neurological recovery at three weeks after brain ischemia in rats. Our findings suggest that the VNS during MCA occlusion can improve long-term neurological outcome.

Stroke is a medical emergency and prompt treatment is crucial as early action can minimize brain damage and potential complications. The FDA-approved clot-busting drug tissue plasminogen activator (t-PA) is well recognized as the most promising treatment for ischemic stroke but must be administered within three hours of the beginning of stroke symptoms to be effective (Adams, Jr. et al., 2007; Albers et al., 2000). Although it is necessary that VNS be examined at much later times following ischemia, in order to confirm the prospective potency we started the treatment 30 minutes after ischemia was induced.

According to the American Stroke Association, generally, only 3 to 5 percent of those who suffer a stroke reach the hospital in time to be considered for this treatment. Our results suggest that VNS has the potential to be an effective translational treatment for acute ischemic brain stroke. However, in order for VNS to be useful in stroke therapy, further studies are needed to examine different stimulation parameters including duration of stimulation, frequency and pulse amplitude of stimulation, and different timings of VNS applications such as post acute stage and chronic stage of an ischemic stroke. In addition, it is essential to consider easier and less invasive procedures for application of VNS such as the use of a transcutaneous vagus nerve stimulator (Kraus et al., 2007).

Our study was not designed to determine the mechanisms by which VNS for only a brief period early in ischemia provides neuroprotection in transient ischemia. However there are many potential mechanisms. As shown in this study the CBF changes in the core of the MCA territory during ischemia do not differ between the VNS-treated animals and control animals and in addition there were no differences in CBF between the groups during the early reperfusion period. These results suggest that neuroprotective effect of VNS is not mediated through any changes in cerebral blood flow in the cortex. There are similar reports from our laboratory and others that the neuroprotection seen with VNS is not due to any alterations in tissue perfusion elicited by the stimulation (Ay et al., 2009; Ay et al., 2011; Sun et al., 2011). There are studies dealing with CBF change during acute VNS treatment using positron emission tomography (PET), functional MRI (fMRI), functional transcranial Doppler (fTCD) or single-photon emission computed tomography (SPECT) in patients with epilepsy or depression. In PET, fMRI and SPECT studies VNS increases CBF in several parts of the brain while decreasing CBF in other parts of the brain (Conway et al., 2006; Conway et al., 2011; Henry et al., 1998; Henry et al., 1999; Lomarev et al., 2002; Vonck et al., 2000), whereas in a fTCD study VNS did not produce significant increases in CBF (Neu et al., 2005). There are some limitations to these studies, however almost all of them had in small sample sizes, and in addition, patients enrolled in these studies were taking various medications concurrently. Interactions between existing medications and the alterations in regional CBF induced by VNS is not clear making it difficult to compare these studies with the results of the present study.

Additionally, these reports do not provide information on the effect of VNS on CBF under ischemic conditions. It should be noted that during the stimulus trains (13 stimulation epochs lasting 30 seconds each), LDF decreased transiently by approximately 8% during the stimulation and then recovered to the pre-stimulus level. If anything, this decrease in blood flow in the treated group would make the stimulated animals worse, not better.

An additional potential mechanism that has been suggested for VNS-induced neuroprotection involves the afferent vagal pathway. The vagus nerve consists of both afferent and efferent fibers with 80% of the afferent impulses originating in the thoracic and abdominal visceral organs (Foley and DuBois, 1937). This afferent nerve activity is relayed to the nucleus tractus solitarius which has projections to the locus coeruleus (LC) (Fahy, 2010; Ricardo and Koh, 1978) and is likely to play an important role in VNS therapy through the release of norepinephrine (NE) and 5-Hydroxytryptamine (5-HT) (Cheyuo et al., 2011). NE, activated by VNS, has anti-inflammatory effects (Madrigal et al., 2005) and can stimulate the release of 5-HT (Manta et al., 2009). Data showing that agonists of 5-HT can reduce the release of glutamate in cerebral ischemia (Mauler et al., 2001) indicating that 5-HT attenuates excitotoxicity by inhibiting glutamate release (Marcoli et al., 2004). These afferent nerve pathway effects could contribute to the effectiveness of VNS in brain ischemia.

Another proposed mechanism that has been suggested for VNS-induced neuroprotection operates through the efferent vagal pathway. The cholinergic anti-inflammatory pathway (CAP) is activated by the central cholinergic system in the brain via the efferent fiber of the vagus nerve (Pavlov et al., 2006; Tracey, 2007). The anti-inflammatory effect of the vagus nerve is mediated by the $\alpha 7$ nicotinic acetylcholine receptor, which is activated by acetylcholine. There are studies suggesting a neuroprotective effect of CAP in cerebral ischemia (Kutsuna et al., 2010; Ottani et al., 2009; Wang et al., 2008). Also, electrical stimulation of the vagus nerve leading to activation of the CAP (Tracey, 2009) may suppress brain inflammation, leading to neuroprotection in ischemic stroke. The similarity of the time course of recovery in the VNS stimulated animals (Fig. 6) and that found in rats also subject to filament MCA occlusion and treated with Reparixin (Villa et al., 2007), which reduces inflammation through the CXCL8 receptor, suggests that the anti-inflammatory hypothesis deserves further investigation.

In summary, we have shown that VNS for only a brief period early in ischemia provides neuroprotection in transient ischemia with this neuroprotection persisting for at least three weeks following the ischemia. Although the precise mechanism of this effect remains to be determined, it undoubtedly contributes to the beneficial effect of VNS on infarct volume and neurological recovery following cerebral ischemia.

Acknowledgments

This work was supported by NIH RO1 NS057400 and NS060653.

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Fig. 1.

Experimental procedure for the three groups of animals. Laser Doppler flowmetry (LDF) was monitored prior to middle cerebral artery (MCA) occlusion (BL), during MCA occlusion (before stimulation (BS), during stimulation (DS) and after stimulation (AS)), as well as following MCA occlusion and early reperfusion (RP). In the control ischemia group, vagus nerve stimulation (VNS) was not performed; in the VNS stimulated group the vagus was stimulated every five minutes starting 30 minutes into MCA occlusion for a period of 60 minutes, and in the sham ischemia group, the MCA was not occluded, but the vagus nerve was still stimulated for 60 minutes. The animals in the control ischemia group and the VNS stimulated groups were scored neurologically at the indicated times and were sacrificed at 21 days for histological damage. The sham ischemia group was scored neurologically only at 24 hours and was then sacrificed for histological analysis.

Fig. 2. Arterial blood pressure (ABP) at various times during the acute (monitoring) phase of the study. See legend for Fig. 1 for abbreviations. * $p < 0.05$, † $p = 0.056$: paired t-test. Values are expressed as the mean $\pm$ SD.

Fig. 3. Heart rate (HR) during stimulation (30–90 min). PS: 4 min prior to stimulation, ST: 30 sec epochs of stimulation. * $p < 0.02$ in comparison to PS. Values are expressed as the mean $\pm$ SD.

Fig. 4.

Sequential changes of laser-Doppler Flow (LDF) during and after occlusion of the MCA in animals receiving stimulation of the vagus nerve (closed circles) and without stimulation (open circles), as well as in sham ischemic animals (open diamonds). Changes in flow are shown as a percent of the baseline (pre-ischemia) flow. The LDF data during the period of stimulation (30–90 minutes) was obtained following the stimulus trains, not during a stimulus train. There were no significant differences between the two ischemic groups. Values are expressed as the mean $\pm$ SD.

Fig. 5.

Boxplot for cerebral infarct volumes three weeks after temporary MCA occlusion with (closed circles) and without (open circles) vagus nerve stimulation. Box plots are shown along with the raw data. Circles show infarction volume of each animal and the ends of the boxes define the 25th and 75th percentiles, with a line at the median and error bars defining the 10th and 90th percentiles. Infarct volumes were smaller in the animals receiving vagus nerve stimulation. * $p < 0.02$ in comparison to control group. Values are expressed as the mean $\pm$ SD.

Fig. 6. Neurological scores at 24 hours, 48 hours, 1 week, 2 weeks and 3 weeks after MCA occlusion in animals receiving stimulation of the vagus nerve and in animals without stimulation. There is a statistically significant affect of treatment ($p < 0.02$) and time ($p < 0.01$). * $p < 0.05$, † $p < 0.01$ in comparison to the control group; Mann-Whitney Rank Sum test. Values are expressed as the mean $\pm$ SD.

Table I**Blood gas data**

Control			
	pH	PCO ₂ (mmHg)	PO ₂ (mmHg)
Before MCA occlusion	7.44 ± 0.021	34.3 ± 5.7	106 ± 15.9
During MCA occlusion	7.43 ± 0.035	38.8 ± 8.2	97 ± 14.2
Vagus nerve stimulation			
	pH	PCO ₂ (mmHg)	PO ₂ (mmHg)
Before MCA occlusion	7.43 ± 0.029	37.1 ± 6.5	102 ± 20.7
During MCA occlusion	7.43 ± 0.028	37.3 ± 5.1	102 ± 19.1