Acute Vagus Nerve Stimulation Spares Motor Map Topography And Reduces Infarct Size After Cortical Ischemia

by

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ABSTRACT

Stroke remains a leading cause of adult disability in the United States. In recent studies, chronic vagus nerve stimulation (VNS) has been proven to enhance functional recovery when paired with motor rehabilitation training after stroke. Other studies have also demonstrated that delivering VNS during the onset of a stroke may elicit some neuroprotective effects as observed in remaining neural tissue and motor function. While these studies have demonstrated the benefits of VNS as a treatment or therapy in combatting stroke damage, the mechanisms responsible for these effects are still not well understood or known. The aim of this research was to further investigate the mechanisms underlying the efficacy of acute VNS treatment of stroke by observing the effect of VNS when applied after the onset of stroke. Animals were randomly assigned to three groups: Stroke animals received cortical ischemia (ET-1 injection), VNS+Stroke animals received acute VNS starting within 48 hours after cortical ischemia and continuing once per day for three days, or Control animals which received neither the injury nor stimulation. Results showed that stroke animals receiving acute VNS had smaller lesion volumes and larger motor cortical maps than those in the Stroke group. The results suggest VNS may confer neuroprotective effects when delivered within the first 96 hours of stroke.

ACKNOWLEDGMENTS

I am so grateful to all of my committee members, and especially to Dr. Jeffrey Kleim and all of the members of Kleim lab—both past and present—who have helped me grow as both a researcher and a person. I could not have done it without the guidance and assistance from everyone.

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CHAPTER 1

INTRODUCTION

Stroke

Stroke continues to be a leading cause of death and a major cause of disability in the United States (American Stroke Association, 2019). While the mortality rate of stroke has been on the decrease in recent years, a majority of stroke survivors will experience long term disability (Feigin et al., 2014). Thus, it is becoming increasingly important to continue examining new therapies that may aid in recovery from or protect from damage incurred by stroke. It is particularly valuable to address ischemic stroke, as the majority of patients suffer from ischemic strokes (Center for Disease Control and Prevention, 2017). During an ischemic stroke the blood supply to part, or all of the brain is obstructed. This prevents the tissues in that area from receiving the oxygen they need which will lead to cell death.

Current emergency treatment methods of ischemic stroke typically involve either clot-busting medication, such as tissue plasminogen activator (tPA), or intra-arterial therapy. However, these options must be administered within a very limited time window. tPA must be delivered within 4.5 hours (Mayo Clinic, 2019), while intra-arterial therapy, such as mechanical thrombectomy, typically must be done within a 6 hour time window (Abou-Chebl, 2010). Additional limitations to tPA treatment eligibility include age, severity and location of stroke, as well as general health (American Stroke Association). Once the effective time frame for these treatments has passed, the remaining option is physical therapy (motor rehabilitation) to try to aid in motor function recovery after the stroke has taken its course. Fortunately, motor

rehabilitation has been long established as an effective way of restoring motor function following neural injury, and many studies have demonstrated the benefits of motor rehabilitation following stroke (Dimyan et al., 2011; Nudo, Barbay and Kleim, 2000).

Rat model of stroke

There has been some debate over the clinical relevancy of using rodents to model human neurological disorders due to the obvious neuroanatomical differences. However, there are a multitude of reasons justifying the rat as a suitable choice to model neurological disorders in humans. Rats have been studied the most out of all animal models of neurological diseases. Despite the extensive amount of prior research using rat models, this does not compensate for the fact that there are indeed some challenges when modeling human neurological diseases in animals. This is because the same disorder may cause different physical symptoms across species. In spite of this, these challenges can be overcome by focusing on changes in function rather than physical changes associated with the neurological disease under study. It has been found that functionally, rats and primates have a lot of similarities. (Cenci et al., 2002; Kleim, Boychuk and Adkins, 2007)

While there are several commonly used methods for modeling stroke (focal ischemia) in rats, injection of Endothelin-1 (ET-1) is recognized as a suitable model due to its similarities to the stroke experienced by human patients (Cenci et al., 2002). An intracortical injection of ET-1, a powerful vasoconstrictor (Yanagisawa et. al., 1988), will reduce local blood flow for over an hour (Fuxe et al., 1997). Cerebral blood flow (CBF) in the ipsilesional cortex stays depressed and does not recover to contralesional cortex levels until 16-22 hours. This delayed, gradual reperfusion more closely models the

ischemic stroke that human patients experience in comparison to other stroke models. In addition, the manner in which the infarct gradually grows is also similar to that which takes place in human stroke patients (Biernaskie et al., 2001).

Vagus Nerve Stimulation

In recent years, attention has shifted towards vagus nerve stimulation (VNS) due to its potential for a non-invasive treatment alternative to a variety of neural injuries and disorders. The ability to non-invasively stimulate the vagus nerve in human patients has been proven by Clancy et al. (2014) who demonstrated a method to effectively transcranially stimulate the auricular branch of the vagus nerve. As such, there is worth in examining the efficacy of direct VNS as a treatment for a variety of conditions in hopes of eventually developing a non-invasive approach with the same effect.

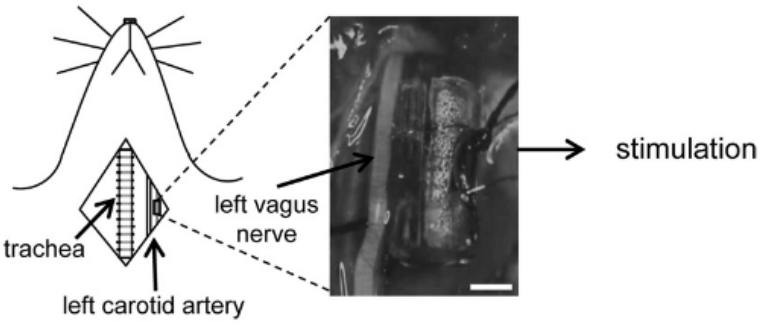

Figure 1: Vagus nerve stimulation cuff implant. Depiction of the relative location of the left vagus nerve in a rat (left) and photograph of the implant of vagus nerve stimulator (right) (Nichols et al., 2011)

Vagus nerve stimulation is an approved treatment for epilepsy and there have been several clinical investigations of the viability of VNS in treating anxiety and Alzheimer's Disease, with some studies reporting some success for depression treatment (Daban et al., 2008; Groves et al., 2005; Sackeim et al., 2001). There have also been a multitude of experiments in rats demonstrating the viability of VNS paired with

rehabilitation as a method of augmenting functional recovery following neural injury, such as stroke (Khodaparast et al., 2013; Porter et al., 2011) and traumatic brain injury (Hays et al., 2013; Pruitt et al., 2016).

Research objective

The goal of this study was to investigate the effects of applying acute VNS following an ischemic stroke. Previous studies have focused on the effects observed when VNS paired with rehabilitative training as a therapy following stroke. Some studies have determined that without pairing VNS with rehabilitation training, VNS alone is not significantly effective in motor function recovery, however it must be noted that these experiments were all done at a time point of one week or more following the stroke (Khodaparast et al., 2014; Porter, et al., 2011). The authors of these studies believe there are separate mechanisms behind their findings and the neuroprotective effects reported when applying VNS stimulation during, or relatively soon after (within 2 hours of) the brain injury, postulating that the results seen when starting VNS therapy a week or more after the stroke are due to VNS promoting neuroplasticity (Khodaparast et al., 2016). Several studies investigating the efficacy of VNS during the onset of a stroke (Hiraki, et al., 2012; Sun et al., 2012), or shortly after—30 min after the stroke induction (Ay et al., 2009)—have found that animals receiving VNS had smaller lesions and overall better functional scores, implying the presence of potential neuroprotective effects of VNS when applied at the time of stroke. Yet the time period after the stroke has completed its course, and before rehabilitative measures begins, has still not been closely examined. Thus, this study aimed to address the questions of whether similar effects would still be observed when delivering acute VNS beginning within 48 hours after the injury.

CHAPTER 2

METHODS

Subjects

Fifteen adult (90 days), male Long-Evans rats were assigned to one of three groups: Control (n=6), Stroke (n=4) or VNS+Stroke (n=5). Animals in the VNS+stroke group received both a cortical ischemic stroke and VNS cuff implant on day 1, while animals in the Stroke group received only stroke on day 1 (see figure 2 below of experimental timeline). Animals in the control group received no VNS nor stroke. Animals in the VNS+Stroke group were given stimulation for three consecutive days beginning within 48 hours after the injury. On day 6, all animals were mapped via ICMS and perfused following the mapping procedure. Throughout the entirety of the experimental timeline, each animal's weights were maintained to be above a minimum of 85% of its original weight. All procedures involving animal subjects were approved by the Arizona State University Institutional Animal Care and Use Committee.

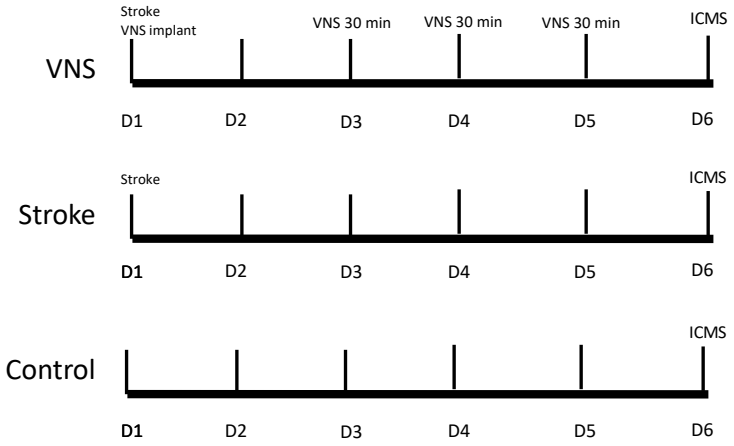

Figure 2: Experimental timeline for each group

VNS cuff electrode and connector head stage construction

Bipolar electrode stimulation cuffs were constructed by threading platinum-iridium (PI) wire through medical grade microtubing (Micro-Renathane 0.040" x 0.025"). Tubing was cut to approximately 4mm measurements and an incision made down the length of the tubing. Platinum-iridium wire (Medwire .006" braided platinum iridium wire) was cut to lengths of approximately 6.5 centimeters. One end of each wire was soldered to a gold press pin and the other threaded perpendicularly through the tubing (see Figure 3 below). Exposed ends were then trimmed and coated with UV cured glue (Loctite light cure adhesive) to eliminate any exposed, uninsulated wire along the exterior of the cuff. Cuff construction methods mostly followed methods as previously reported in (Nichols et al., 2011; Porter et al. 2011) and protocols shared by Dr. Michael Kilgard and lab members at University of Texas at Dallas' Texas Biomedical Device Center. However due to the lab's past concerns regarding proper contact between the electrode and nerve (Hillebrand, 2018), the design used in this experiment was modified for a closer, more secure fit around the vagus nerve, and as such deviates from previously described methods in literature by using a smaller tubing (.040" x .025" rather than .08" x .04").

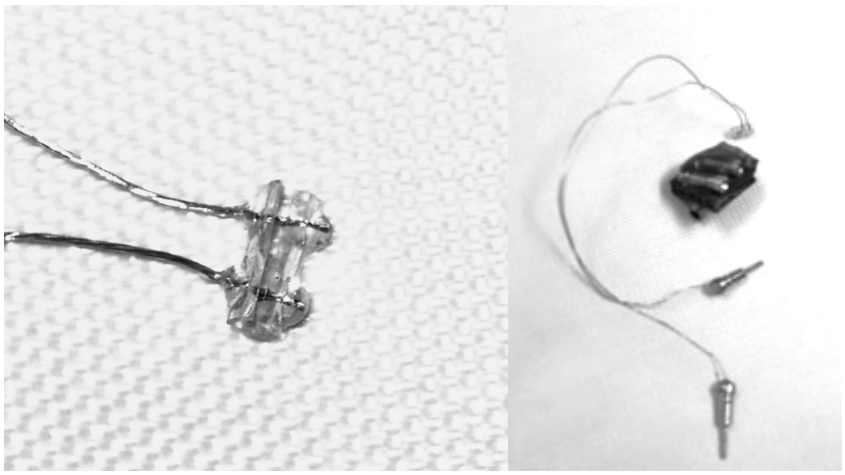

Figure 3: Image of VNS cuff and head stage. Left: magnified image of tubing cuff. Right: image of full VNS cuff with leads and connector head stage.

Two channel connector head stages were constructed by adding wire leads between connector pins and gold press pins. All of the wires and exposed metal were then encased in acrylic and left to air-cure for at least 24 hours before testing. All head stages were tested by connecting to a stimulator and verifying the signal on an oscilloscope.

Each stimulation cuff was tested at two separate points in time. Cuffs were first verified electrically by observing an expected output waveform when connected to both stimulator and oscilloscope. The tubing portion of the cuff was submerged in saline solution to complete circuit before turning on the stimulator. Stimulator machine was set to same parameters as used in experimental stimulation (see VNS stimulation section). Each cuff was tested once more during surgery, at time of implant. (see VNS cuff implant section). Any stimulator cuff electrodes or head stages that generated errors were discarded and not used for any experiments.

Surgical procedures

VNS cuff implant

Animals were anesthetized under 5% isoflurane gas and maintained at levels between 1.5% and 3%. Subjects were placed in a stereotaxic instrument to secure head at a level position and placed on a heating pad to help maintain body temperature of approximately 37.4 °C. Animals' blood oxygen level and heartrate were also monitored via pulse oximeter paw cuff (MouseSTAT Jr) throughout the surgical procedure. Implant procedure was similar to methods as described in previous literature (Dorr and Debonnel, 2006; Nichols, 2011). After anesthesia induction, the rat was laid on its back with its ventral side exposed. A rostral to caudal incision was made over the left neck area, and muscle tissue blunt dissected to expose the carotid sheath. The carotid sheath was teased apart along with any facia along the nerve to completely expose the vagus nerve. The vagus nerve was then fit into the VNS cuff and tested. VNS cuffs were confirmed to be working properly if sudden decrease in heart rate was observed upon turning on stimulation. After confirming the cuff's proper operation, the opening of the VNS cuff was sealed using dental filling composite (Silmet Profil flow) and cured via UV light gun. The wire leads of the cuff were then tunneled subcutaneously towards an opening created on the dorsal side of the head. The ventral opening was then sutured closed and antibiotic ointment applied over the suture line. Upon testing, average VNS induced heart rate decrease was 119.2 ± 70.898 BPM.

Cortical Ischemia

Immediately after the cuff implant, animals were maintained under 5% isoflurane gas and maintained at levels between 1.5% and 3%. For animals who received VNS implant, they were left on maintenance levels of isoflurane while immediately moving on

to the ischemia induction phase. An anterior to posterior incision was made to expose the skull before creating a burr hole. A Hamilton syringe was lowered into the burr hole to deliver 4μl of Endothelin-1 (prepared as 1mg/mL) as an intracranial injection. Endothelin-1 was injected at 1μl/min and allowed to sit for 5 minutes to allow for proper perfusion. After the injection was completed and the Hamilton syringe removed completely, the hole was sealed with bone wax.

Head stage placement and recovery

Following injection of Endothelin-1, five indentations were drilled into the skull, all well posterior to the Endothelin-1 injection site. Bone screws were inserted into these areas and acrylic used to affix the head stage over the bone screws. The pin ends of wire leads were plugged into the head stage, then additional acrylic was layered over and around the head stage to ensure sturdy fixation. After allowing the acrylic to set and confirming stable bonding, the opening around the head stage was sutured closed and antibiotic ointment applied over the suture line.

At the completion of surgery, all animals were given 4.0cc ringers and 0.03cc buprenorphine (Buprenex 0.3mg/mL) and allowed to recover for several minutes on the heating pad before being returned to the home cage.

VNS Stimulation

48 hours following the initial surgery, unanesthesized animals were given 30 minutes of vagus nerve stimulation for three consecutive days. VNS was delivered at 1Hz, 500ms train of 15 pulses (0.8mA). During stimulation, animals were monitored and allowed to do as they pleased and move freely within the cage space.

Motor cortex mapping (Intracortical Microstimulation)

Animals were anesthetized with an IP injection of ketamine hydrochloride (approximately 90mg/kg). Animals were also given 0.15cc xylazine (AnaSed, 20mg/mL, IP) approximately 5 minutes following the injection of ketamine. A craniotomy was performed on the left hemisphere and dura removed to expose rostral and caudal forelimb areas of the motor cortex.

Following methods similar to (Kleim, Barbay, and Nudo, 1998; Warraich, 2013), a 500μm grid was superimposed over an image taken of the exposed cortex. Using a hydraulic microdrive, a glass microelectrode was lowered (approximately 1550μm) at points along the grid lines. At each site the current was gradually increased (≤60μA) until a movement was elicited and both the movement type and threshold recorded. If no movement was observed at 60μA, the site was deemed no response. Movement category, thresholds and their corresponding locations were recorded for each animal's caudal forelimb area (CFA) and rostral forelimb area (RFA). The resulting motor cortex map was then analyzed using pixel to area measuring software, and thresholds were sorted by category (distal and proximal) before analyzing averages for each animal.

Histology

Immediately following the cortical mapping procedure, animals were given an overdose of pentobarbital (Euthasol, 290mg pentobarbital sodium/mL) and perfused with 0.1M phosphate buffer solution, followed by 4% paraformaldehyde solution. Neural tissue was extracted and left to rest in 4% paraformaldehyde for at least 24 hours before being transferred to 30% sucrose solution. Tissue was left in 30% sucrose for a minimum of 48 hours before being sectioned coronally at a thickness of 40μm on a microtome.

After collecting tissue sections into 0.1M phosphate buffer solution, every seventh section was mounted onto poly-l-lysine subbed slides for a sample approximately every 240μm interval and left to air dry before staining. Slides were stained with cresyl violet (for Nissl substance staining) and Myelin stains. For every other section, ipsilesional and contralesional cortex areas were traced in imageJ and lesion volumes calculated.

Statistics

All data in this study were reported as the mean and standard error of the mean. Statistical significance was determined using a one-way analysis of variance (ANOVA) with Group as a between subject factor and subsequent Fisher's Least Squares Difference (LSD) post hoc tests.

CHAPTER 3

RESULTS

Motor Maps

A One-Way ANOVA revealed a significant main effect of Group on total motor map area [$F(2,12)=34.569$; $p<0.01$]. Subsequent multiple comparisons revealed the Control animals to have significantly larger total motor maps than both the VNS+Stroke and Stroke animals. Further, VNS+Stroke animals had larger motor maps than the Stroke animals (Figure 5). The same effect of Group was found for both the RFA [$F(2,12)=5.893$; $p<0.05$] and CFA [$F(2,12)=23.881$; $p<0.01$]. Further, a main effect of Group was also found for total movement threshold [$F(2,12)=26.716$; $p<0.01$], CFA-Distal movement threshold [$F(2,12)=8.605$; $p<0.05$] and CFA-Proximal movement threshold [$F(2,12)=24.544$; $p<0.01$]. Stroke animals had significantly higher total, CFA-Distal and CFA-Proximal movement thresholds than both the Control and VNS+Stroke animals (Figure 6). There was no statistically significant difference between Control and VNS+Stroke movement thresholds on any of the three measures. Most VNS+Stroke group animals had no movement responses throughout the entire RFA. Thus, RFA movement thresholds were not compared between groups due to insufficient threshold data.

Lesion Volume

A one-way ANOVA revealed a significant main effect of Group on lesion volume [$F(2,12)=34.569$; $p<0.01$]. Subsequent multiple comparisons revealed the Stroke animals to have significantly larger lesion volumes than both the VNS+Stroke and Control animals (Figure 7).

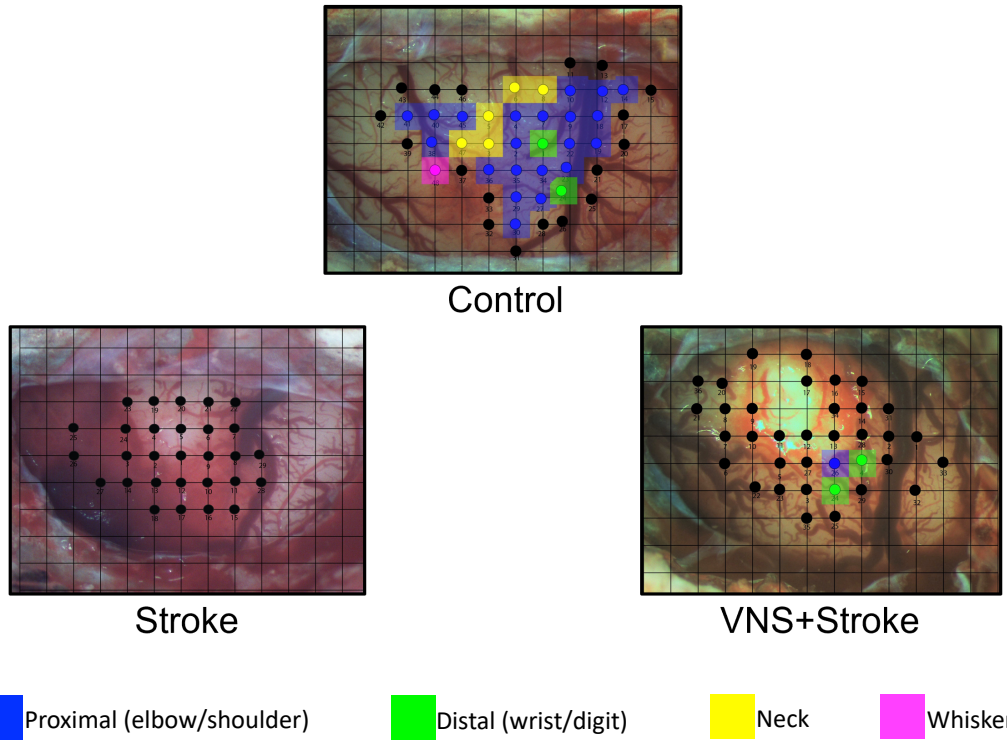

Figure 4: Representative motor maps. Representation of (ipsilesional) caudal forelimb area (CFA) and rostral forelimb area (RFA) of Control, Stroke, and VNS+Stroke groups. Proximal and distal forelimb movement sites were denoted with blue and green markers respectively. No response sites were indicated by black markers.

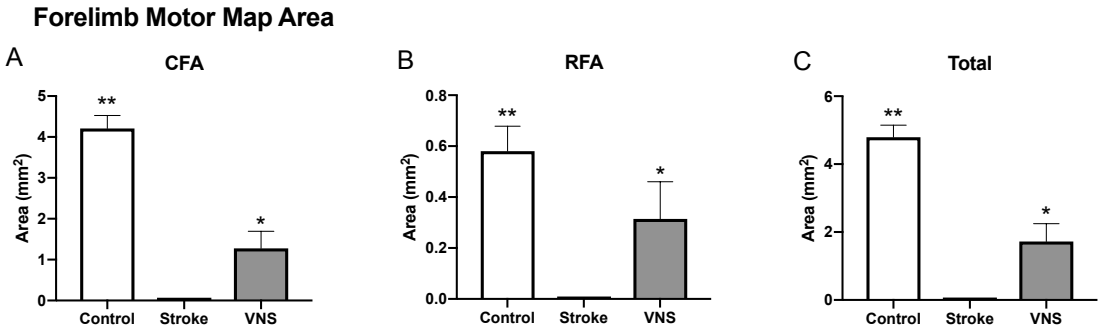

Figure 5: Average forelimb motor map areas for each group (A: Caudal forelimb area B: Total forelimb area).

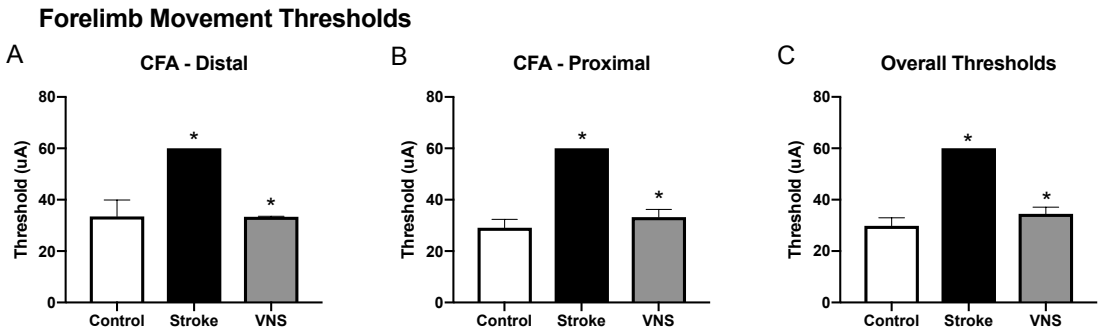

Figure 6: Forelimb movement thresholds. Forelimb movement thresholds as measured by ICMS (A: Caudal forelimb area (distal movements) B: Caudal forelimb area (proximal movements) C: Overall thresholds)

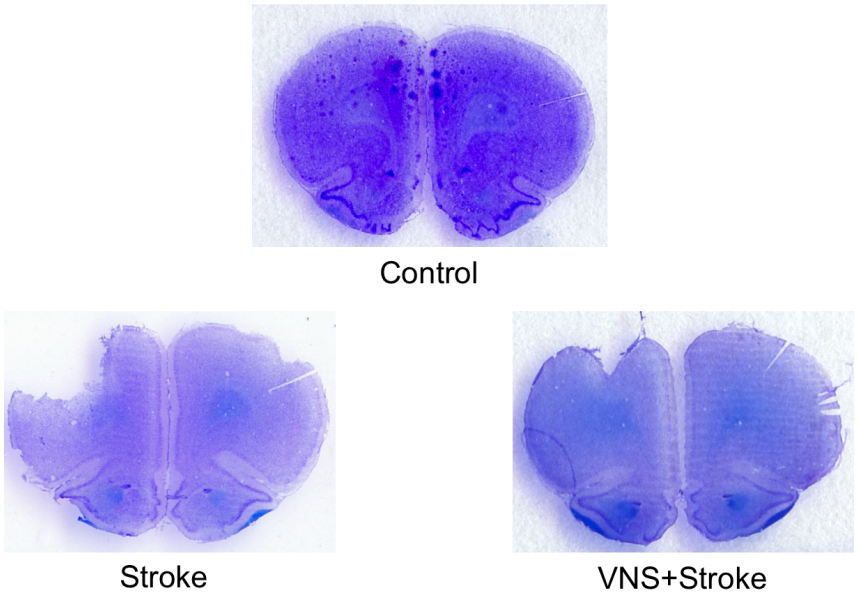

Figure 7: Representative coronal sections for each group (Control, Stroke, and VNS+Stroke) to demonstrate lesion volume. Sections were stained with cresyl violet and myelin stains.

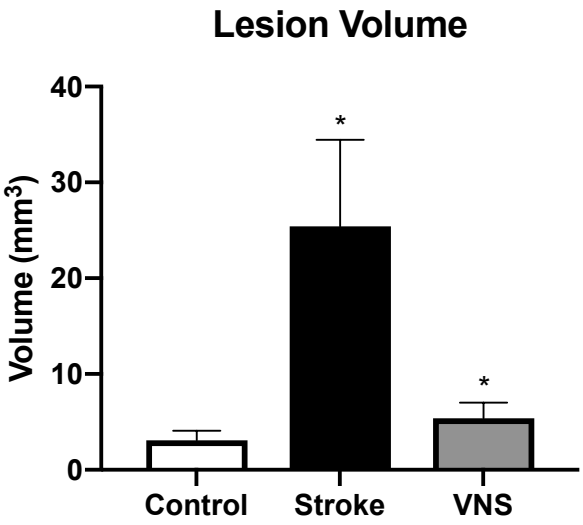

Figure 8: Volumetric analysis of infarct size. Lesion volume estimation calculated by finding the difference in ipsilesional cortical volume and contralesional cortical volume.

CHAPTER 4

DISCUSSION

The aim of this project was to determine the effects of VNS had when applied daily during the 96 hours after an ischemic stroke. This was accomplished by measuring the effects of 3 days of VNS on motor cortex map integrity and lesion volume. By comparing these data sets for groups of Control, Stroke, and VNS+Stroke animals, we were able to observe the effects of VNS stimulation in preventing damage to tissue and helping to preserve motor map integrity.

Animals in the VNS+Stroke group showed significantly smaller lesion volumes in comparison to animals in the stroke-only group. Animals who received VNS also each exhibited significantly lower motor movement threshold than those of the stroke group. Additionally, though the motor map size of the VNS+Stroke group was significantly larger than the map size of the stroke-only animals. While all animals in the stroke group measured no response across entire forelimb motor cortex (Figure 4), all animals in the VNS+Stroke group had at least one or more map sites that elicited a forelimb response.

These results imply that the delivery of VNS within 48 hours post-stroke is able to help to preserve animals' motor function and help mitigate lesion development. To examine potential mechanisms responsible for these observations, we can first review what effects of the stroke occur during the timepoint at which we begin VNS.

Biernaskie et al. (2001) demonstrated that stroke induced lesions continue to develop in size until 48 hours after the injury, thus lesions are still developing when VNS is applied within 48 hours following stroke. Within the first few hours after a stroke, the neural tissue affected by the stroke can be categorized into two regions—regions of

irreversible damage (the ischemic core) and regions of reversible damage (the tissue neighboring the ischemic core). In the reversible damage zone tissue, the neurons are not directly impacted by the injury but do border the ischemic core and may also die (Murphy et al., 2009). The smaller lesion size in the animals receiving VNS during this period of time suggests that VNS is able to halt or slow the lesion development and neuronal death that would otherwise normally occur in the progression of an ischemic stroke.

To examine what might be driving these neuroprotective effects, we can examine what other changes are observed in the brain at this time point in the progression of stroke. A compilation of human and animal data concluded that the changes observed in the brain after a stroke can be broken up into three time windows. The first time window, ranging from the hours immediately after the stroke up to days after the stroke, have shown changes including cerebral blood flow (CBF), cerebral metabolic rate of oxygen ($CMRO_2$) and inflammation (Cramer, 2004).

It has been reported that in an ET-1 model of stroke, CBF levels in the ipsilesional cortex will return to levels matching the contralesional cortex at 16-22 hours after the injection of ET-1 (Biernaskie et al., 2001). Recent work in the lab (Hillebrand, 2018) has demonstrated a profound decrease in both heart rate and regional cerebral blood flow immediately following VNS onset. The decrease is followed by an enduring increase in blood flow lasting beyond the cessation of stimulation. However, by the time VNS was delivered (48 hours following ET-1 injection), CBF levels should have recovered from ET-1 induced vasoconstriction, but animals may still be in some way benefiting from the increased CBF levels.

It is also known that the vagus nerve's cholinergic anti-inflammatory pathway can be activated via VNS. While it is still uncertain what overall effect VNS induced activation of anti-inflammatory elements has on the overall inflammation (Mravec, 2010), it has been reported that VNS is able to reduce inflammation levels in animals (Borovikova et al., 2000), and also has had some effects, though somewhat limited, on neurogenic inflammation levels in human patients (Kirchner et al., 2006).

It is reasonable to postulate that either of these two effects of VNS could play a part in the protective effects observed in this experiment. Further work using a different model of ischemia may help to determine the mechanism by which VNS confers neuroprotective effects.

It is also important to note that although animals receiving VNS showed both spared tissue and preserved motor map integrity, the amount of animals' motor map that was spared was smaller in comparison to the amount of tissue spared. This suggests that while there is indeed more tissue spared, not all of the tissue is functional. More of this tissue could potentially become functional again after motor rehabilitation. Thus, there may be potential in VNS as a stroke treatment to address the period of time after current clinical treatment options are no longer effective, and before the damage has completely taken its course as a means of limiting damage. VNS applied during this time may be worthwhile as a precursory measure before motor rehabilitation begins. Further work repeating this experimental procedure with an added element of motor rehabilitation following the VNS treatment would serve to more thoroughly investigate the full potential of this treatment.

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