

DEVELOPMENT OF A VAGUS NERVE ELECTRICAL STIMULATION AND
RECORDING SETUP FOR ULTRASOUND CHARACTERIZATION

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POOJA MEHTA

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DR. HUBERT H. LIM

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Dedication

I dedicate this thesis to my mother, Mrs. Rashmi R. Mehta, who would have been thrilled that one of her children is now pursuing a career related to the life sciences, a circumstance she deemed impossible in a family of engineers who regrettably valued physics, chemistry and mathematics more than biology.

Abstract

The objective for my MS Thesis was to develop a stable electrical stimulation and recording preparation for the vagus nerve in guinea pigs so that ultrasound stimulation on this pathway can be studied robustly. In particular, the plan was to electrically stimulate the vagus nerve and characterize the response patterns (i.e., different components and peaks of the evoked compound action potential corresponding to different subpopulation of fibers) using electrodes wrapped around the nerve. Ultrasound stimulation of the vagus nerve can then be performed and the corresponding response patterns can be compared with those caused by electrical stimulation to assess how ultrasound is activating the vagus nerve. Ultrasound stimulation will also be performed while electrically stimulating the vagus nerve to assess suppressive or modulatory effects of ultrasound. To further demonstrate proof-of-concept of US neuromodulation in a simplified preparation, I assessed how ultrasound stimulation with electrical stimulation of the vagus nerve modulates the heart rate in guinea pigs. The long term goal is to develop a non-invasive method for modulating vagus nerve activity that can have clinical applications for treating inflammatory disorders, seizures, depression, tinnitus, and other conditions that may be modulated by the vagus nerve.

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List of Abbreviations

VNS	Vagus Nerve Stimulation
FUS	Focused Ultrasound
EEG	Electroencephalography
US	Ultrasound
EMG	Electromyography
CAP	Compound Action Potential
fMRI	Functional Magnetic Resonance Imaging
TNF	Tumor Necrosis Factor
IL-1	Interleukin-1
HMGB-1	High-Mobility Group Box 1 protein
DARPA	Defense Advanced Research Projects Agency
UltRx	Ultrasound Prescriptions
LCAMP	Larynx Compound Muscle Action Potentials
ILM	Intrinsic Laryngeal Muscle
TA	Thyroarytenoid
RLN	Recurrent Laryngeal Nerve
I.V.	Intravenous
ElectRx	Electrical Prescriptions
GP	Guinea Pig
LV	Left Vagus
RV	Right Vagus
LR	Left Recurrent laryngeal
RR	Right Recurrent laryngeal
LS	Left Sympathetic
RS	Right Sympathetic
LVN	Left Vagus Nerve
RVN	Right Vagus Nerve
LSN	Left Sympathetic Nerve
RSN	Right Sympathetic Nerve

Chapter 1: Introduction and Background

1.1 Motivation for the Proposed Master's Thesis

Vagus nerve stimulation (VNS) is a medical treatment that involves delivering electrical impulses to the vagus nerve. It is currently approved for and used as an adjunctive treatment for certain types of intractable epilepsy and treatment-resistant depression. Invasive VNS received level C evidence (i.e. at least fair scientific evidence suggests that there are benefits provided by this clinical service, however the balance between benefits and risks are too close for making general recommendations) for achieving >50% seizure reduction [1]. It exerts anti-epileptic or anti-epileptogenic effect possibly through neuromodulation of certain monoamine pathways [2]. VNS is currently at various levels of investigational use as a potential treatment for depression [3], anxiety disorders [4], migraine [5] and Alzheimer's disease [6]. Beyond these, VNS is also under investigation for the treatment of inflammation, asthma and pain [2]. VNS influences the production of inflammatory cytokines to dampen the inflammatory response [7]. It triggers the systemic release of catecholamines that can alleviate an asthma attack [8]. VNS induces anti-nociception by modulating multiple pain-associated structures in the brain and spinal cord affecting peripheral/central nociception, opioid response, inflammation process, autonomic activity, and pain-related behavior [9]. Progression in VNS clinical efficacy over time suggests an underlying disease-modifying neuromodulation, which is an emerging field in

neurology. With multiple potential clinical applications, further development of VNS is encouraging.

Traditionally, scientists have modulated the nervous system to treat various disorders with chemicals and by electrical stimulation. Recently, more non-invasive techniques are being investigated such as using magnetic fields to drive electric currents in the brain, and using focused ultrasound (FUS). FUS has been shown to be effective to ablate tissue for decades [10-12], however its use to excite or inhibit neural tissue is a relatively new direction for the field. If ultrasound could be used to excite the vagus nerve non-invasively, this paints a bright future for treating many disorders without patients having to undergo surgeries.

Therapeutic ablative ultrasound has been used in treating neurological disorders including tremors, epilepsy, chronic pain, and brain tumors [13]. The ultrasound used for ablation requires high intensities, however it has also been shown that ultrasound at low intensities (e.g. 130 mW/cm²) focused to the thalamic areas of the brain can suppress chemically-induced acute epileptic electroencephalography (EEG) activity. Ultrasound (US) can also serve as a non-invasive tool to modulate brain activity rather than to surgically ablate brain tissue. An *in vivo* study performed on rats showed that US stimulation of the motor cortex can elicit muscle twitching and limb movements as indicated by electromyography (EMG) or visual assessment [14]. Another study showed that

US can reversibly suppress sensory-evoked potentials in the cat primary visual cortex after US stimulation of the lateral geniculate nucleus [15].

US has also been shown to have an effect on nerves as well as various receptors and muscle fibers. There have been several pioneering studies on US modulation of nerve conduction [16-18]. Furthermore, US has been demonstrated to differentially stimulate the mechanoreceptors or thermoreceptors on the fingertip of humans as indicated by EEG and functional magnetic resonance imaging (fMRI) [19]. Another study showed that the sensation induced in humans with US stimulation correlates with the density of their mechanoreceptors [20].

In regard to targeting nerves, there have been several studies that have reported excitation of nerves by US, but these results have been inconsistent. One study found that US could excite the abducens nerve non-invasively in rats by eliciting eye movements [24]. However, control experiments were not performed to confirm the nerve was directly activated and/or muscles or other structures were stimulated to induce the eye movements. Another *in vitro* study suggested that US can not only modulate the compound action potential (note that the compound action potential (CAP) is a summation of a group of almost simultaneous action potentials from individual axons of a nerve, and thus a measurement indicative of induced nerve activity) but also elicit the CAP on crab leg nerves [25]. While these results suggest that US can excite nerves, other

studies suggest that only transient elevation of the CAP is possible. For example, one *in vitro* study on the bullfrog sciatic nerve found that the conduction velocity of the nerve increases by 5-20% and the amplitude of the CAP increases by 8% with a 3.5 MHz US transducer at 1 W/cm² [26]. Another *in vitro* study shows that frog sciatic nerves can have an early enhancement response to US stimulation since their CAPs increased by 80% within 10 ms and returned to the baseline after 20 ms following a 500 second single-pulsed stimulus at 400 W/cm² [27]. Therefore, it still remains unclear if US can directly excite nerves and/or enhance nerve activity especially in an *in vivo* animal preparation.

In comparison to US excitation, there is more convincing data available supporting US suppression of nerve activity [18, 21, 22, 27, 36]. One of the earliest studies on nerves from cat, monkey, earthworm and man (with *in vitro* and *in vivo* preparations) showed that US was able to reversibly or irreversibly block nerve conductivity and decrease the CAP responses depending on the applied parameters [18]. Two recent *in vitro* studies on the frog sciatic nerve suggested that nerves could be blocked by tens of seconds of pulsed US stimulation [21] or have decreased electrical excitability with a single, short-pulse, low intensity US stimulus [27]. Another study on the rat vagus nerve also revealed that nerve activity could be inhibited following tens of seconds of pulsed US stimulation [22], although this study did not control for muscle activity interference (details in Chapter 2). Despite these studies which have

demonstrated the ability to suppress nerve activity with US stimulation, there has not yet been a systematic study identifying an effective range of parameters that can suppress nerve activity, and several of these previous studies have presented contradicting or mixed results. However, these findings do suggest that US can be used as a potential approach for inhibiting nerve activity.

Many disabling medical conditions are characterized by undesirable or hyperactive nerve activity, resulting in unwanted sensation or muscle activity. If the action potentials travelling along these abnormally firing nerves could be modulated, the disabling condition could be minimized or eliminated. Therefore, an effective and quickly reversible means of blocking nerve conduction would have many important clinical applications, such as blocking chronic peripheral pain and stopping unwanted motor activity, such as muscle spasms, spasticity, tics and choreas. Although there are many existing methods for surgically or pharmacologically blocking nerve impulses, all of these methods have significant disadvantages such as: non-specificity, serious side-effects, low success rates and nerve destruction. High frequency electrical stimulation has been tried for nerve blocking [23, 48] but has not proven to be very specific and it is not consistently effective. Therefore there is a widespread clinical need for a safe, reliable and reversible nerve blocking technique. US to block nerves could be useful in these scenarios.

Particularly for the vagus nerve, selective suppression of certain peaks found in the CAP can be useful since the most common side effects of VNS are hoarseness and throat burning sensations. Hoarseness is caused by A-alpha fibers of the vagus nerve and throat burning sensations are caused by C fibers of the vagus nerve [28]. Selective inhibition of specific nerve fibers could potentially allow VNS to be functional while minimizing side effects. VNS causes tracheal muscle activity in *in vivo* preparations when the muscles aren't blocked or the recurrent laryngeal nerve isn't cut [47]. If certain CAP peaks that relay neural firing to the muscles can be suppressed/blocked, then muscle activation may be prevented, which may reduce or eliminate common VNS side effects. Furthermore, the vagus nerve is involved with a multitude of bodily and neural functions, as described above. Being able to modulate a subset of specific fibers within the vagus nerve could enable more specific treatment of a given health condition while avoiding other unwanted physiological and neural side effects.

One new and exciting direction of neuromodulation is the potential for using VNS to treat inflammatory disorders. Work performed by Kevin Tracey and colleagues [29] has shown that vagus nerve stimulation can reduce inflammation in a manner that depends on nicotinic receptors containing the alpha-7 subunit and on acetylcholine-synthesizing T lymphocytes via the spleen, which has been termed the cholinergic anti-inflammatory pathway. Their research has shown that vagus nerve stimulation inhibits the release of TNF, IL-1, HMGB1 and other inflammatory cytokines in rats. In my advisor's lab (the Lim Lab run by Dr. Hubert

Lim), we are trying to modulate this pathway using ultrasound, to potentially treat inflammation in a mouse model of arthritis provided by our collaborator Dr. Bryce Binstadt at the University of Minnesota. This arthritis treatment project is a recently funded grant from DARPA called Ultrasound Prescriptions (UlTRx). The purpose of this thesis project is to develop an electrical stimulation and recording setup of the vagus nerve to characterize which parameters of US work for exciting and suppressing the nerve. To this end, we are first working *in vivo* in guinea pigs since the animal is larger than the mouse and will allow easier surgical exposure and stimulation of the vagus nerve, and also the Lim Lab has extensive experience with neuromodulation experiments in guinea pigs. Afterwards, the lab will translate the findings from guinea pigs to the mouse model of arthritis. When ultrasound has been sufficiently characterized for invasive nerve modulation *in vivo*, the eventual goal is to translate the findings to be able to achieve vagus nerve modulation non-invasively to treat arthritis. We are also trying to investigate the underlying mechanisms of how ultrasound modulates nervous tissue.

1.2 Organization of the Thesis

This thesis is organized into six chapters. Chapter One is an introduction to VNS, US neuromodulation, and the problem that motivates this project. Chapter 2 is a literature review describing the techniques that have been used for electrical stimulation and recording of the vagus nerve and the research that has

been done in regards to US neuromodulation. Chapter Three is a description of the anatomy of the vagus nerve in the guinea pig and the challenges I faced in identifying the vagus nerve based on previous anatomical studies.

The main results of this thesis will be described in Chapters Four and Five. Chapter Four describes the setup for recording of the CAP from the guinea pig vagus nerve, and the challenges involved with getting true nerve recordings. Chapter Five describes the results obtained using an alternative readout to show US modulation of vagus nerve activity (due to the challenges described in Chapter Four), in which US was used to modulate heart rate changes caused by electrical stimulation of the right vagus nerve. Finally, in Chapter Six, I present future directions for this project and a description of how my Master's thesis paves the way for future research in our lab for the recently funded DARPA project.

Overall, the main goal of this Master's thesis was to develop a new *in vivo* setup in guinea pigs to be able to electrically activate and record from the vagus nerve while being able to apply US stimulation to the nerve. I achieved my goal in which my setup can now be used by future researchers in the Lim Lab to systematically characterize a wide range of ultrasound parameters on the ability to modulate the vagus nerve invasively and eventually non-invasively for treating various health disorders, initially focusing on arthritis treatment for the DARPA UltrRx project.

Chapter 2: Literature Review

2.1 Electrical Stimulation and Recording of Nerves

There has been interest in recording nerve activity for several decades. Recording activity from nerves serves as an indicator of assessing viability and regeneration if there has been damage. It is also a technique employed to implement closed loop control of nerve stimulation, in particular vagus nerve stimulation (VNS). In an experimental setting, nerve recording is of value since it is a method to obtain insight as to the mechanisms of nerve stimulation. Recordings from nerves can also help assess the fiber composition of different nerves, the conduction velocities of different fibers, and thresholds of activation of different fiber subpopulations. Such recordings are greatly beneficial for confirming if nerve stimulation is actually working to activate or suppress the nerve, for neuromodulation applications. It is also helpful to assess mechanisms of action of different stimulation and nerve blocking paradigms.

2.1.1 Vagus Nerve Stimulation and Recording

There have been quite a few animal studies describing peripheral nerve structures, fiber composition, diameters of fibers and conduction velocities [33, 34]. For the vagus nerve in particular, most of these studies have been *in vitro* studies. There have been *in vitro* studies to assess the effect of temperature on vagus nerve conduction [35] and the effect of local anesthetic agents on the

conductive properties of the vagus nerve [33]. For such studies, having an *in vitro* setup is justified since they deal with properties of the nerve structure itself. However, for assessment of nerve function, it is critical to have an *in vivo* setup since nerve activity can affect and can be affected by other physiological activity or conditions. In this regard, there have been only a few *in vivo* studies stimulating and recording from the vagus nerve. These studies have focused on the ability to use VNS on experimentally induced seizures in rats [37, 38], to implement closed loop VNS control of heart rate in pigs [39], to selectively activate and block vagus nerve fibers in pigs [40, 41] and rats [42, 43], to study the different direct and indirect components in an evoked compound action potential (CAP) in pigs and thus study organs innervated by the vagus nerve [44, 45], to check for nerve activation using larynx compound muscle action potentials (LCAMP) in rats [46], to differentiate between nerve and muscle activity in rats [47], and to characterize the recruitment of myelinated and unmyelinated fibers in the vagus nerves of dogs [48]. There have also been a couple of human studies to assess the functionality of VNS by intraoperative recordings [49, 50]. Overall, there is a dearth of studies describing nerve function *in vivo*.

2.1.2 Properties of Electrical Propagation in a Nerve

In a healthy nerve, stimulation of a nerve fiber membrane produces a conducted impulse or CAP which is the cumulative response of action potentials of individual axons, when the stimulus intensity exceeds the fiber's threshold.

Various axons which respond to lower intensity stimuli do so because of their membrane properties. In general, medium-sized fibers have the lowest threshold, the large fibers have the second lowest threshold, and finally the fine or small fibers have the highest threshold [51]. CAP amplitude can vary depending on the intensity of the stimulus and, therefore, the number of fibers stimulated. If the stimulus is supramaximal in intensity then CAP amplitude and its integral (area covered by the CAP) will be maximal. Threshold for stimulation and therefore ability to evoke a CAP depends on both the duration and magnitude of the applied stimulating current. Both of these parameters interact. If stimulus durations are shorter than the rheobase value (the rheobase value is the minimal current amplitude of infinite duration (in a practical sense, about 300 ms) that results in the depolarization threshold of the cell membranes being reached), a compensatory increase in stimulus current is required to change membrane behavior and produce the CAP. This interaction of stimulus duration and current is useful in differentially activating larger, myelinated fibers as opposed to small, unmyelinated fibers. In addition to being less likely to activate small unmyelinated fibers, the short-duration stimulus pulse also produces less stimulus artifact. Further, short-duration pulses are desirable since the clinical concerns need to be directed toward the status of medium or large, myelinated fibers at a stage of regeneration when these should be present in significant number at both the stimulating and recording sites. The finest unmyelinated fibers, including regenerating fibers, have a much higher threshold than large axons and may

require substantially greater stimulation to evoke a CAP [51]. Even high current stimulation may not evoke a response in some abnormal fibers without also increasing stimulus duration. Thus, many factors may influence the responsiveness of nerve fibers to electrical stimulation.

In a myelinated axon, impulse conduction occurs when a region of active membrane, often involving more than one node of Ranvier, excites adjacent nodes. The mechanism of the action potential "jumping" to the next node in a myelinated nerve is affected by the diameter of the axon, the thickness of myelin, and the distance between nodes. There is a remarkably constant proportional relationship between these three factors with various sized myelinated fibers [51]. Thus, the distance between nodes, the thickness of myelin and the number of nodes responding as a unit, as well as axonal diameter, determine the axon's conduction velocity. Another factor in determining conduction velocity is the time required to reproduce the action potential at each node. Distribution studies of conduction velocities have shown a relationship between CAP size and shape and the axonal composition of the whole nerve. Certainly, the presence of a CAP indicates the presence of variable axons. Without extreme amplification or averaging, direct stimulation and recording seems to require at least 4,000 moderate to large fibers with some degree of myelination [51].

2.1.3 Differences between Animal Models

It is also important to note the variations between different animal models since the vagus nerve has been shown to have some dissimilarities among human, large animal models and small animal models. For example, in humans and large animals such as pigs, cats, rabbits, and dogs, there can be large distances between the stimulating and recording electrodes (both on the cervical vagus nerve), ranging from 20 mm [49] to 47 mm [52], whereas in small animal models, the general distance between the stimulating and recording electrodes is around 5 mm [38]. These large differences in lengths of the vagus nerve and distances between the stimulating and recording electrodes can partially explain the variability in results across species. Also, the vagus nerves of different species may vary in fiber composition, and may receive afferent innervation from distinct muscles. This can explain the variability of shapes of CAPs recorded in different animal models.

2.1.4 Stimulation Artifact

It is difficult to stimulate and record from an intact nerve trunk because the stimulating current follows two paths: the usual short path between the two adjacent stimulating electrodes and another longer path through the nerve and the body fluids; that is, the two regions where the nerve re-enters the body are “shorted” together by the body fluids. In the longer path, a substantial portion of the stimulating current flows past the recording electrodes and generates a huge

shock artifact that totally obscures the CAP [38]. When the distance between the stimulation and recording electrodes is greater, there is a smaller stimulation artifact, and the latency of the compound action potential is greater compared to when the distance between the electrodes is shorter. Therefore, in large animal models the greater distance between stimulation and recording electrodes decreases the likelihood of activity being mixed with artifact. Successful recordings without an overlapping artifact are less likely in small animal models where the distance between electrodes is minimal, and this artifact is probably why there are much fewer *in vivo* studies performed in rodents as compared to larger animals such as pigs and dogs. Some studies have attempted to use three stimulating electrodes with a potentiometer to obtain the least possible stimulation artifact [38]. This method however, can reduce the amplitude of the artifact but not the duration, which does not solve the problem of nerve activity being covered or masked by the artifact (a problem reported in [40]), so this issue still remains a challenge.

2.1.5 Muscle Activity versus True Nerve Activity

Stimulation of the vagus nerves elicits various collateral effects [53]. The most common of these is the co-activation of the intrinsic laryngeal muscles (ILMs) as well as the thyroarytenoid (TA) muscle, with effects ranging from trembling of the voice and hoarseness to complete long-term paresis of the vocal chords [54, 55]. The ILMs and the TAs, co-activated during VNS, receive their

innervation from a branch of the vagus, the recurrent laryngeal nerve (RLN). Anatomical studies have confirmed that these fibers account for the vast majority of the large and thick myelinated fibers in the main trunk of the cervical vagus, differentiating them from the parasympathetic fibers, which are thin myelinated and unmyelinated fibers. The RLN in most species contains afferents from these muscles, in which the activity can spread through the tissue or fluids via conduction back to the nerve, and can be misinterpreted as nerve activity. The threshold for the fibers that activate these muscles is the lowest amongst all the fibers, therefore these muscles are almost always activated when other nerve fibers are activated. This muscle activity feeding back through the nerve can interfere with or mask true nerve activity, and there is a general lack of addressing of this issue in the scientific literature.

Some studies [40, 48] take this muscle artifact issue into consideration and use techniques to differentiate nerve versus muscle activity. Grill et al.'s study in dogs [48] used a neuromuscular block called succinylcholine (injected I.V.) to eliminate the muscle activity, and they also recorded electromyography (EMG) signals from the laryngeal muscle to show the difference between nerve and muscle activity. On the EMG, the activity is of ~5 mV in amplitude, and it propagates through the tissue or fluids back to the nerve, and back through the nerve to the recording electrode, as an artifact signal of ~20 μ V in amplitude. Tahry et al.'s study in rats [40] used a muscle-paralyzing agent (vecuronium) on

the laryngeal muscles to differentiate between nerve and muscle activity. Another study [45] recorded activity from the contralateral vagus nerve to the nerve being stimulated and found a peak on that nerve (as well as on the ipsilateral nerve, but with a slightly different shape and latency), which is an indirect artifact component since the two nerves are not directly connected. The authors conducted several controls and concluded that this activity originates from the periphery and not the brainstem. They hypothesized that it is transmitted via the neural network of the heart. Since the gastrointestinal tract has an intrinsic nervous system, an indirect component from the periphery could also originate there. However, vagal abdominal fibers have a diameter of less than 6 μm , which does not match the threshold, velocity and latency of the component they saw. The lungs, larynx and the esophagus are other organs that are innervated by the vagal nerve and whose fibers are present at the cervical level. They ruled out involvement of the lungs in this component since efferent bronchial fibers have a diameter of less than 4 μm . A response coming from these fibers would have a much higher threshold. The larynx also cannot be involved, since it has little to no afferent vagal innervation (in the cat) [56]. Afferent fibers of the esophagus have a lower velocity than found for this component. This leaves the heart as the most likely source of this indirect component. Controls like these are necessary to determine what is truly being activated. These controls are particularly important for our study since we are trying to alter nerve function with ultrasound, and thus we must truly be able to tell apart nerve activity from all other types of interfering

activity. In general, activity (recorded differentially across a nerve) having greater amplitudes than a few tens of μV , or durations longer than a few ms, is most likely muscle activity, and we must be cautious of this, and must implement adequate controls (like neuromuscular blocks and EMG recordings) to claim true nerve activity.

2.2 Ultrasound Stimulation

2.2.1 Ultrasound Modulation of Nerve Activity

There have been studies where the effect of focused ultrasound (FUS) on nerve tissue has been tested [21, 22, 58], but most of these studies are *in vitro* studies and most of these studies are on the sciatic nerve, or other thick and long nerves. Unpublished work from the Lim Lab found suppression (and also enhancement in some cases) of CAP activity with US in the guinea pig sciatic nerve. There is a study on vagus nerve modulation using FUS [22], but to my knowledge, this is the only study of its kind. This study was conducted on the left vagus nerve of rats, and they showed suppression of CAP with US of various intensities, and therefore concluded that there is a proportional relationship between acoustic intensity and the level of inhibition. They used a needle hydrophone to determine the acoustic intensity generated by the transducer for a given input voltage, and a thermocouple to measure the change in temperature. The time scale of the observed CAP in this paper however, was ~ 20 ms, which is too large to be true nerve activity as shown in earlier studies. Recording details

aren't discussed in their publication, and the amplitude of the CAP obtained is ~400 μ V which is again too high if this was a differential recording across the nerve. There aren't any controls conducted for elimination of muscle activity; therefore, there is a need for a better-controlled study investigating whether it is truly nerve activity that is modulated with US.

2.2.2 Mechanisms of Action of Ultrasound

Potential underlying mechanisms of US modulation of nerves are still unclear, and we are trying to investigate these mechanisms in the Lim Lab in conjunction with our into the CAP modulation effects. There are at least two factors proposed for stimulation of peripheral nerve structures - one is known as radiation force of US while the other is called an ultrasonic bio-effect, which can be either a thermal or cavitation mechanism [59]. There have been many biomedical applications of radiation force of US, including shear wave elasticity imaging, assistance with targeted drug and gene delivery (via opening up the blood brain barrier) and manipulation of cells and particles [60]. A few studies believe that it is the mechanical force modulating the excitability of stretch-activated ion channels because the US parameters used in these studies did not significantly increase the nerve temperature [60, 27]. One radiation force model was developed to explain the suppression of nerve activity [61]. Furthermore, several nerve compression experiments provide indirect evidence that the mechanical force of US could potentially inhibit nerve activity. These studies

show nerve elongations [62] or transverse compression [63], which may cause transient changes in membrane ion channels or myelin disruption, to suppress nerve activity. To investigate the role of a thermal mechanism to suppress nerve activity with US stimulation, several studies use thermocouples for monitoring the temperature changes in the nerve in real time. It was shown that the CAP decreased as the temperature increased from 20°C to 45°C due to US heating effects [21]. The blocking of CAP was reproducible by application of graded amounts of heat to small segments of nerves [18]. This modulation of CAP activity that correlates with temperature changes is possibly due to temperature effects on ion channels. However, the modulation of nerve activity did not appear to be due to cavitation induced by US because the intensity used in a study was far lower than the cavitation threshold in live tissue [64]. Overall, it is very likely that both radiation force and thermal mechanisms play a combined role in modulating nerve activity.

2.3 Relevance of Literature to this Project

All these findings indicate that it is possible to electrically stimulate the vagus nerve and record CAP activity of the nerve, however it is important to be cautious of potential issues such as the length of the nerve used to build this setup, and the interference of artifacts such as the stimulation artifact and muscle activity artifacts. Appropriate controls should be taken to be convinced of true nerve activity. *In vivo* preps are crucial to fully understand the effects of nerve

stimulation. There are several studies showing the ability to use US to modulate nerve activity, but very few of these are conducted *in vivo*, and there is only one showing modulation of vagus nerve activity. More importantly, results have been inconsistent and it is unclear whether true modulation occurs. Therefore, it is critical in this field to develop a preparation to do electrical VNS and modulate the thus induced activity with US, while avoiding artifact issues during recording, to enable further studies to characterize US effects in detail. My thesis will address this.

Chapter 3: Anatomy of the Vagus Nerve

The guinea pig model isn't one that is typically used for nerve stimulation and recording preparations, in contrast to the commonly used rat model. The Lim Lab is primarily an auditory research lab. The structure and frequency hearing range of the guinea pig ear is similar to that of humans. Guinea pigs also display the Preyer reflex used in checking for deafness, in which the outer ear moves in response to a whistle. Guinea pigs are therefore a preferred animal model for studying the auditory system [65]. This is why our lab has been using guinea pigs for experimentation. While the Lim Lab has developed a novel method to induce neuroplasticity via Multimodal Synchronization Therapy (mSync) in which auditory and sensory pathways are stimulated in a coordinated fashion, we have not directly recorded from the peripheral nervous system in these experiments. Moving to nerve stimulation and recording was a new direction for our lab, so the experimental setup and protocols had to be newly developed for our recently funded project (UltRx in collaboration with Medtronic, funded by the DARPA ElectRx (Electrical Prescriptions) program), which started at the end of 2015. Eventually, the goal of the project is to stimulate, via US alone or using US to modulate electrical stimulation, the nerves and organs of mice with arthritis (arthritis model is available from collaborator Dr. Bryce Binstadt's lab at University of Minnesota). Our lab made the decision to first investigate and optimize the US parameter space invasively in guinea pigs before moving the setup to mice because mice are extremely small, and the surgical setup to

access the nerves in a mouse (the vagus nerve, in particular) is uniquely challenging.

The vagus nerve is the tenth cranial nerve, and interfaces with parasympathetic control of the heart, lungs, digestive tract and other visceral organs. The vagus nerve are paired (i.e. left and right vagus nerves); however, they are normally referred to in the singular. It is the longest nerve of the autonomic nervous system in the human body. Guinea pig cervical nerve anatomy is not as clearly articulated as other rodents such as rats and mice. The following figure (Figure 3.1) depicts the nerves in the neck of a guinea pig.

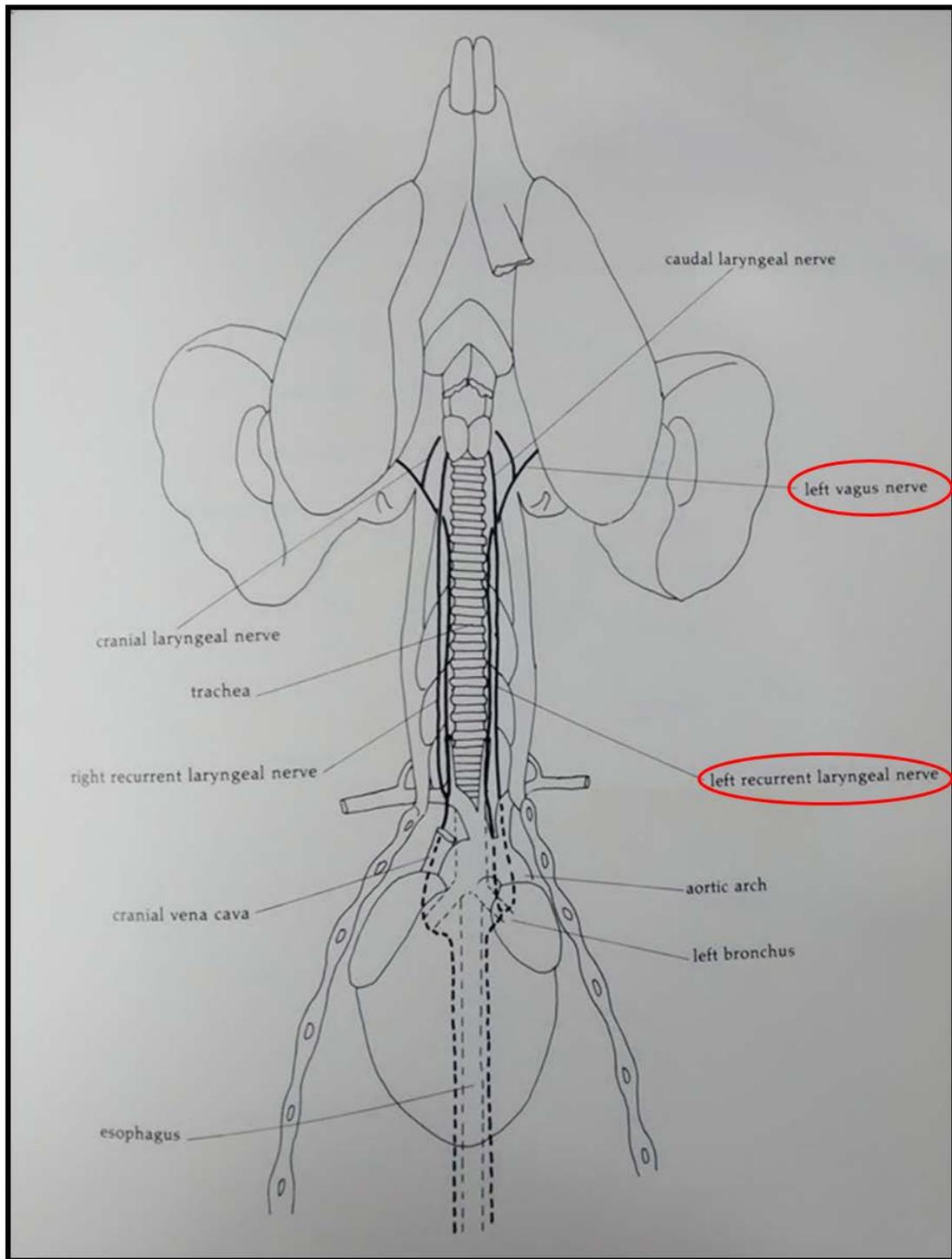


Figure 3.1: Published guinea pig neck anatomy Source: Anatomy of the Guinea Pig – Cooper & Schiller [66]. The two nerves of interest have been encircled in red.

Figure 3.1 does not correctly represent the anatomy of the nerves in the guinea pig. It shows two nerves: the left vagus nerve and the left RLN (encircled in red in Figure 3.1). When I performed the surgery, there were 3 nerves instead of 2 nerves, i.e. one more in addition to the RLN and the vagus nerve as shown in Figure 3.2 below.

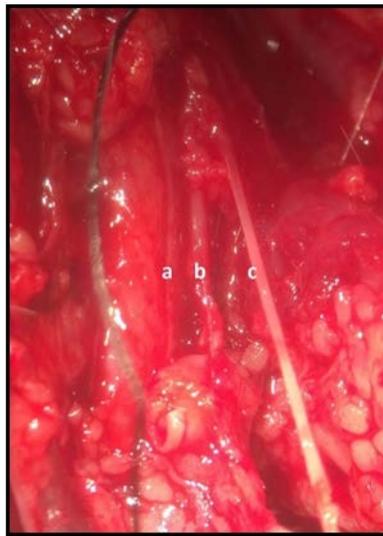


Figure 3.2: Photo from my surgery of nerves in the guinea pig neck: a is the recurrent laryngeal nerve and either b or c is the vagus nerve (photo from guinea pig (GP) 1)

There are detailed studies describing the anatomy both in the rat and the mouse. Unfortunately, the rat and mouse anatomy is different from that of the guinea pig, so it was not possible to transfer the knowledge from those species to the guinea pig. Figure 3.3 below shows the mouse anatomy. LV and RV represent the left and right vagus nerve, LR and RR represent the left and right recurrent laryngeal nerves and LS and RS represent the left and right sympathetic nerves; they are circled in red on the figure. When I performed the

surgical preparation in a mouse, the anatomy resembled that shown in Figure 3.3, with the sympathetic nerve far removed from both the vagus and RLN, and this made it possible to avoid confusion between the sympathetic and vagus nerves.

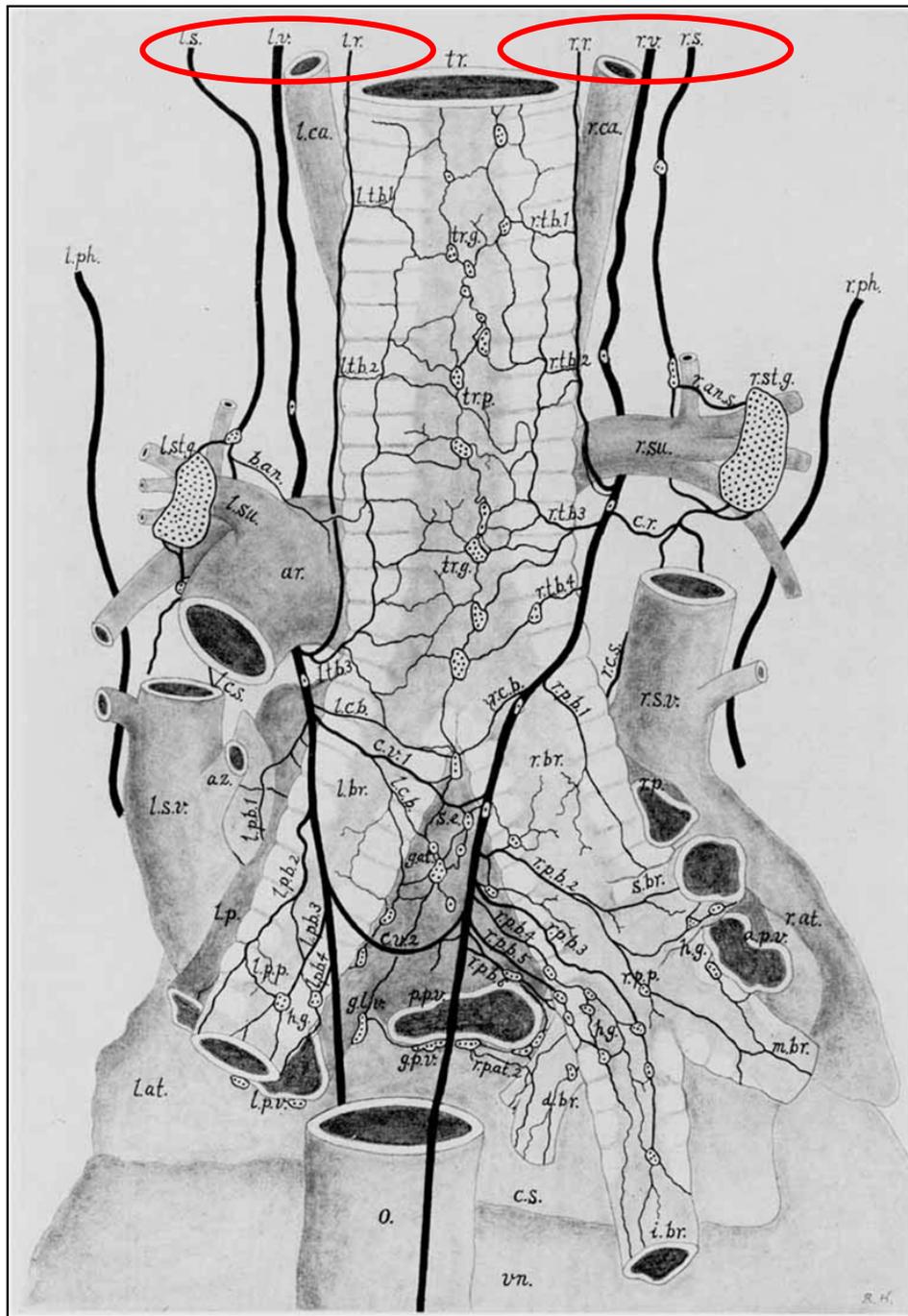


Figure 3.3: Dorsal view of the respiratory organs and the heart and vessels of the mouse, showing the distribution of the tracheal and pulmonary branches of the vagi (LV and RV), sympathetic nerves (LS and RS), recurrent laryngeal nerves (LR and RR) and the tracheal and pulmonary ganglia. [67]

In order to attempt to identify the vagus nerve in guinea pigs, I sought additional publications and resources that were not limited to anatomical studies. There have been several studies describing the inhibitory innervation of the guinea pig trachealis muscle [68-70]. These studies illustrate the anatomy of the guinea pig, and they define three pairs of nerves, with the sympathetic nerve lying between the vagus nerve and the RLN.

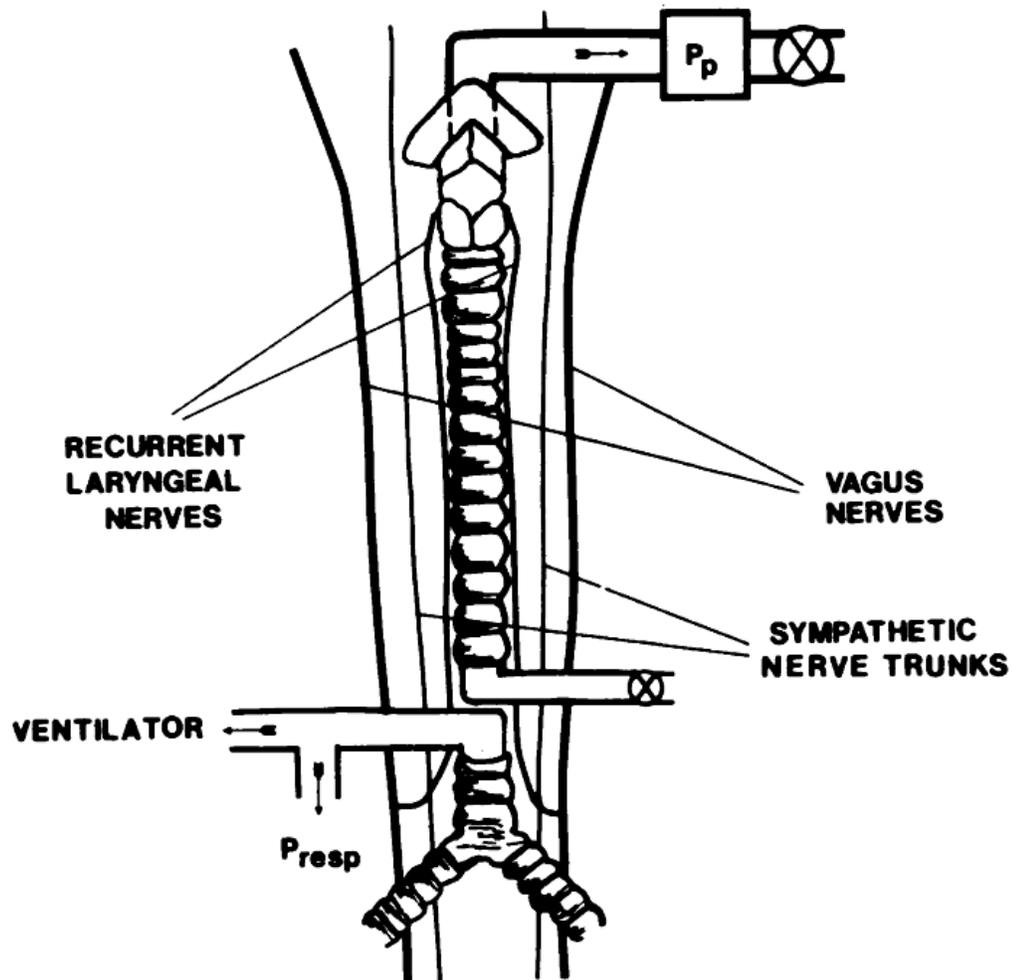


Figure 3.4: Anatomy of the guinea pig cervical nerves showing three instead of just two pairs of nerves [68]

Since the anatomy shown in Figure 3.4 is in contradiction to the anatomy in the rat/mouse (mouse anatomy shown in Figure 3.3) in that the positions of the vagi and the sympathetic nerves are reversed, and because the papers describing the anatomy in Figure 3.4 are not recent (1970s-1980s), we decided to verify the nerves in the guinea pig via an experimental setup. In this experiment, described below, I separately stimulated the two nerves on the right side of the animal and observed any modulation in heart rate.

Traditionally, stimulation of the vagus nerve has been reported to decrease heart rate (bradycardia) [84], since it has parasympathetic control of the heart, and cardiac sympathetic nerve stimulation is supposed to increase heart rate (tachycardia). I used biphasic square pulse stimuli (frequency: 10 Hz, duration: 500 μ s – 5 ms, amplitude: 1 mA – 5 mA) to stimulate either nerve in 5 animals, expecting that stimulation of one nerve would cause tachycardia and the other would cause bradycardia. However, stimulation of both nerves caused tachycardia of about 15-30%.

It is generally recognized that the two divisions of the autonomic nervous system exert antagonistic effects on various aspects of the performance of the heart. However, these opposing influences are not algebraically additive; complicated interactions exist [77]. There have been studies reporting that VNS has caused tachycardia instead of bradycardia [72-81]. Symons et al. [74] claimed that the animal that best exhibits this opposite effect is the cat. They

conducted many controls to prove that this effect isn't due to current that spreads to the sympathetic nerve. They hypothesize the presence of sympathetic fibers in addition to parasympathetic fibers within the vagus nerve itself. It has been shown in other studies of the guinea pig [69], that the sympathetic and vagus nerves are connected through the superior cervical ganglion and the proximal ganglion. However, when these ganglia were removed by Symons et al. [74], tachycardia still occurred. These results suggest that certain species have vagus nerves of mixed function and fiber type, and that the response to VNS depends on which fibers can dominate in certain conditions. Some studies report tachycardia occurring after the initial bradycardia. One study in dogs [72] suggests that the tachycardia occurring after bradycardia caused by VNS (called postvagal tachycardia) results from the excitation of cholinergic parasympathetic fibers that leads to liberation of catecholamines possibly from chromaffin cells. Another study in cats [73] claimed that postvagal tachycardia is not mediated by sympathetic adrenergic mechanisms. They suggest instead that the observed tachycardia is dependent upon the preceding vagal bradycardia and may be related to an increase in net sodium influx into pace-maker cells initiated by the hyperpolarization of the pace-maker cell membrane during and immediately after vagal stimulation. There can also be central mechanisms at play which cause VNS to indirectly affect the sympathetic system to compensate for the over-activity of the direct parasympathetic activity. However, many studies rule this out by stimulation of the nerve post cutting or crushing the nerve rostral of the

stimulation (i.e. eliminating the pathway going up to the brain that comes back down through the sympathetic pathway). As such, there may be many factors that lead to the vagus nerve causing an increase instead of a decrease in heart rate as I observed in the guinea pigs.

Therefore, this method of monitoring heart rate was not conclusive in helping to confirm that it was the vagus nerve that was stimulated. I traced the nerves downwards after the animal was euthanized in three animals, to see which nerve curves back up under the aorta to form the recurrent laryngeal nerve. In two animals, the branch of the vagus that looped upward to form the recurrent laryngeal nerve was the medial nerve, and in one animal, it was the lateral nerve. The orientations (medial versus lateral) of the nerves could have changed while I was manipulating them, thus making the results of tracing the nerves inconsistent. It is difficult to trace the nerve through the ribcage and lungs, without damaging blood vessels. When the blood vessels break, it becomes more challenging to trace the nerves; therefore, this tracking method did not work in more than three animals.

The left vagus nerve crosses in front of the left subclavian artery to enter the thorax between the left common carotid and subclavian arteries. It descends on the left side of the aortic arch, which separates it from the left pleura, and travels behind the phrenic nerve. It courses behind the root of the left lung and then deviates medially and downwards to reach the esophagus and form the

esophageal plexus by joining the opposite (right) vagus nerve [71]. Therefore, another thought was to stimulate both nerves in the neck individually and record activity from the left vagus nerve on the esophagus (below the diaphragm) to determine which stimulation causes activity in the vagus nerve further downwards in its pathway. The recordings from that experiment are shown below in Figure 3.5.

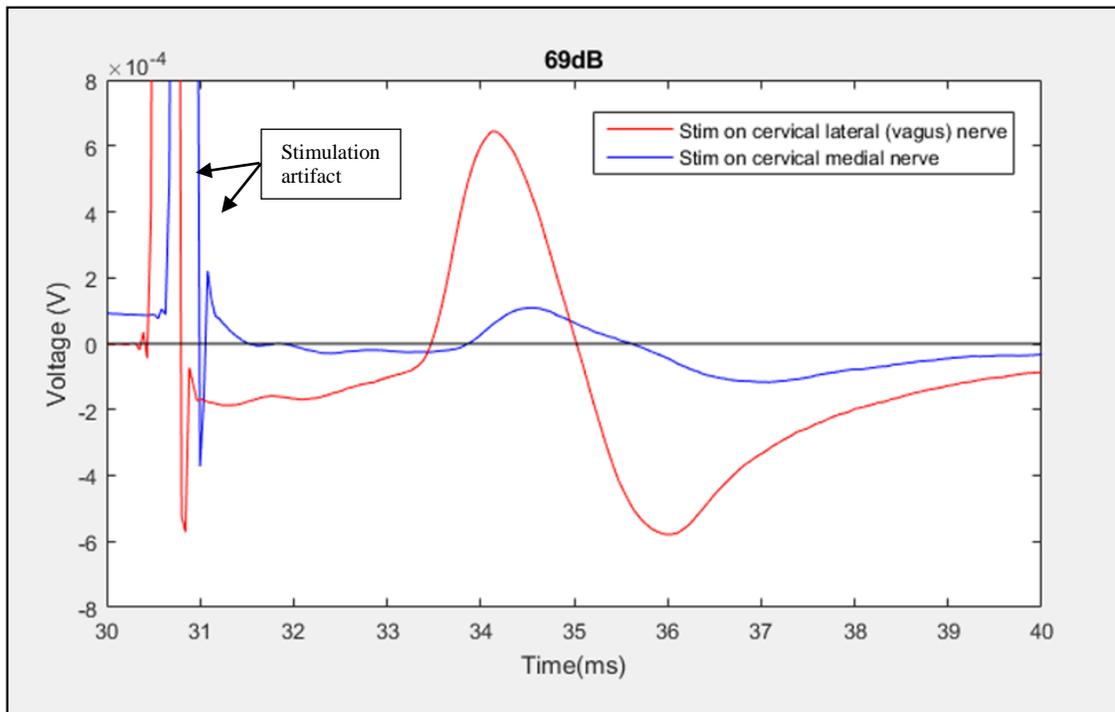


Figure 3.5: Recordings from the esophageal vagus nerve while stimulating the cervical medial and lateral nerves (GP 1). The first response as pointed out is the stimulation artifact and the response of interest begins after 33 ms.

Both nerves when stimulated showed activity on the esophageal vagus nerve. That both nerves would show activity is most likely due to muscle activation caused by the nerve stimulation, which I will describe in detail in Chapter 4. In Chapter 4, I explain how muscle activity can appear as an artifact on more than one nerve in the vicinity. Taking into account that both the traces in Figure 3.5 (red and blue) have a muscle activity artifact, the difference between the two traces indicate that the trace in red (lateral nerve stimulation) has larger net activity. Therefore, it can be inferred that the activity seen in red (lateral nerve stimulation) is a summation of true nerve activity and muscle activity, whereas the activity in blue (medial nerve stimulation) is muscle activity alone. This data suggests that the lateral nerve is the vagus nerve since true nerve activity would only occur when there is a direct connection between where the stimulation and recording occur. Further stimulation and recording details are discussed in Chapter 4.

Since the eventual animal model to be used in the DARPA UltRx project is the mouse (where there is no confusion regarding identification of the nerve), and since the primary goal in the guinea pig is to determine the feasibility of modulating nerve activity by US, I decided to use the lateral nerve for stimulation and recording based on my findings that the lateral nerve is likely the vagus nerve. Stimulation of the cervical nerves while recording from the nerves in the sub-diaphragmic esophageal area suggests the lateral nerve to be the vagus nerve, and the only papers describing the anatomy of the nerves in the neck in

the guinea pig seem to agree that the lateral nerve is the vagus nerve. Even if the previous publications indicating that the lateral nerve is the vagus nerve turn out to be incorrect, I can still achieve my main objective of this Master's thesis in demonstrating the ability to modulate nerve activity with US.

Chapter 4: Development of Nerve Setup to Characterize Ultrasound Neuromodulation

The goal of my Master's thesis project is to create a setup for enabling detailed characterization of ultrasound (US) modulation of nerve activity, preferably the left vagus nerve of the guinea pig. As described in Chapter 3, the laterally located nerve in the neck is most likely the vagus nerve and is the nerve targeted in my thesis research. The desired readout to demonstrate that US can modulate nerve activity is to record changes in the compound action potential (CAP), either showing direct excitation of activity or a decrease in activity that is initially induced from electrically stimulating the nerve. Chapter 4 will describe the various recording and stimulation setups to first identify ways to be able to record CAP activity from the nerve. Chapter 5 will leverage the findings from Chapter 4 to develop a final setup that can enable characterization of US neuromodulation of the vagus nerve.

4.1 Compound Action Potential

As action potentials propagate along an axon, they produce electric potentials that can be recorded from the surface of the nerve. When a nerve bundle is stimulated, many axons produce action potentials synchronously. The resulting electric responses recorded from the surface of the nerve are called CAPs to distinguish them from the action potentials generated by individual

axons. CAPs can be measured as shown in Figure 4.1 below [79]. Stimulation electrodes are applied to one end of the nerve; recording electrodes are located in a different location along the nerve. If the nerve is stimulated with a current pulse of sufficient amplitude, the action potentials produced in the fibers propagate toward the recording electrodes. The aggregate effect of the many action potentials is an extracellular wave of negative potential, moving along the surface of the nerve. If the recording electrodes are widely spaced (left panel of Figure 4.1), the wave of negative potential produces a negative peak in recorded voltage $v(t)$ as it passes the positive ('+' in Figure 4.1) recording electrode. At a later time, the negative wave of extracellular potential passes the negative ('-' in Figure 4.1) recording electrode, where it contributes a positive peak to the recorded voltage $v(t)$. If the electrodes are more closely spaced to each other (center panel of Figure 4.1), the negative and positive parts of the recorded voltage $v(t)$ merge, and the resulting waveform is called a diphasic CAP. The propagation can be blocked by a number of methods including mechanical methods (pressure applied to the nerve or crushing the nerve with forceps), electrical methods (passing a blocking level of current through the nerve), or chemical methods (applying potassium chloride, local anesthetics, cocaine, or tetrodotoxin to the nerve). If the CAP is blocked between the two recording electrodes (right panel of Figure 4.1) so that it does not reach the recording electrode, a monophasic CAP is recorded.

The monophasic CAP consists of one or more peaks of (negative) potential that last on the order of milliseconds. The maximum amplitude varies with the recording conditions but rarely exceeds a few millivolts. Note that there is a commonly used convention of plotting negative monophasic action potentials as upward deflections in physiological publications. Multiple peaks might represent repeated action potentials produced by the same fibers or alternatively, sub-populations of fibers that differ in their propagation velocities and thus produce components with different delays. The shape of the CAP depends on the population of excited fibers within the nerve. If all of the fibers in the nerve have similar diameters, then the propagation velocities for action potentials will be similar. The resulting monophasic CAP will be brief. If the nerve contains a heterogeneous mix of fibers with different diameters (as in the guinea pig vagus nerve), then the monophasic CAP will become broader as it propagates. If the distance between the stimulating and recording electrodes is sufficiently large, the monophasic CAP may consist of several peaks—each corresponding to a different subpopulation of fibers that conducts action potentials at a different velocity. Studying the electrical properties of the different peaks has led to insights about the thresholds, refractory periods, and velocities of propagation of action potentials in different subpopulations of fibers [80-82].

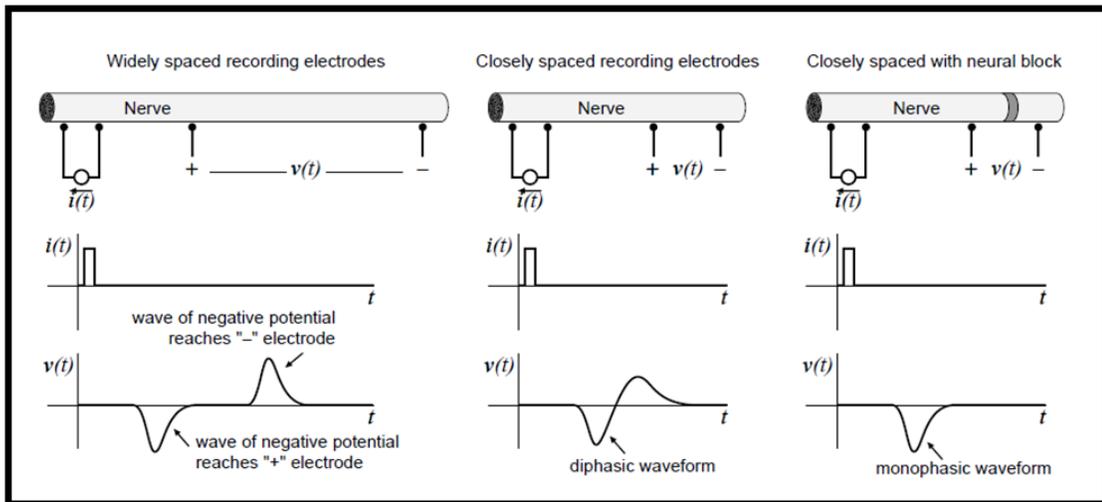


Figure 4.1: Three schemes for measuring CAPs. In each scheme, a current $i(t)$ is applied to two stimulus electrodes, and a voltage response $v(t)$ is measured across two recording electrodes (represented by '+' and '-' in upper panels). The nerve response to a pulse of current consists of two, temporally separated components, if the recording electrodes are widely spaced (left panels). A diphasic waveform results for more closely spaced recording electrodes (center panels). A monophasic waveform results if a portion of the nerve between the recording electrodes (shown as a dark band) is altered to block transmission of action potentials (right panels). [79]

4.2 Composition of the Vagus Nerve

There are three types of peripheral nerve fibers based on their diameter: A group, B group and C group fibers. Fibers of the A group have a large diameter, are myelinated, and have the highest conduction velocity of all the nerves in the body. The A group consists of four types of nerve fibers: A-alpha fibers (afferent or efferent fibers), A-beta fibers (afferent or efferent fibers), A-gamma fibers (efferent fibers), and A-delta fibers (afferent fibers). Nerve fibers in the B group are myelinated with a small diameter. Generally, they are the preganglionic fibers

of the autonomic nervous system and have a low conduction velocity. The C group fibers are unmyelinated and as the B group fibers have a small diameter and low conduction velocity. These fibers include postganglionic fibers in the autonomic nervous system and nerve fibers at the dorsal roots (IV fiber), which carry the sensory information of nociception (pain), temperature, touch, pressure and itch [83].

The cervical vagus is a mixed nerve. It contains motor, sensory, parasympathetic and afferent autonomic fibers. The intrinsic laryngeal muscles (ILMs), co-activated during VNS, receive their innervation from a branch of the vagus, the recurrent laryngeal nerve (RLN). Anatomical studies have confirmed that the fibers from the RLN account for the vast majority of the large and thick myelinated fibers in the main trunk of the cervical vagus, differentiating them from the parasympathetic fibers, which are thin myelinated and unmyelinated axons [40]. The fiber composition of the vagus nerve has been studied in several animal models as well as in humans. A representative figure from a cat model is shown in Figure 4.2 below. Figure 4.2 shows the average amplitude and durations of various fibers in the cat vagus nerve. A-alpha and A-beta fibers have the shortest duration but the largest amplitude. A-delta fibers have a short duration and a small amplitude. C-fibers are much longer in duration, and relatively large in amplitude as well.

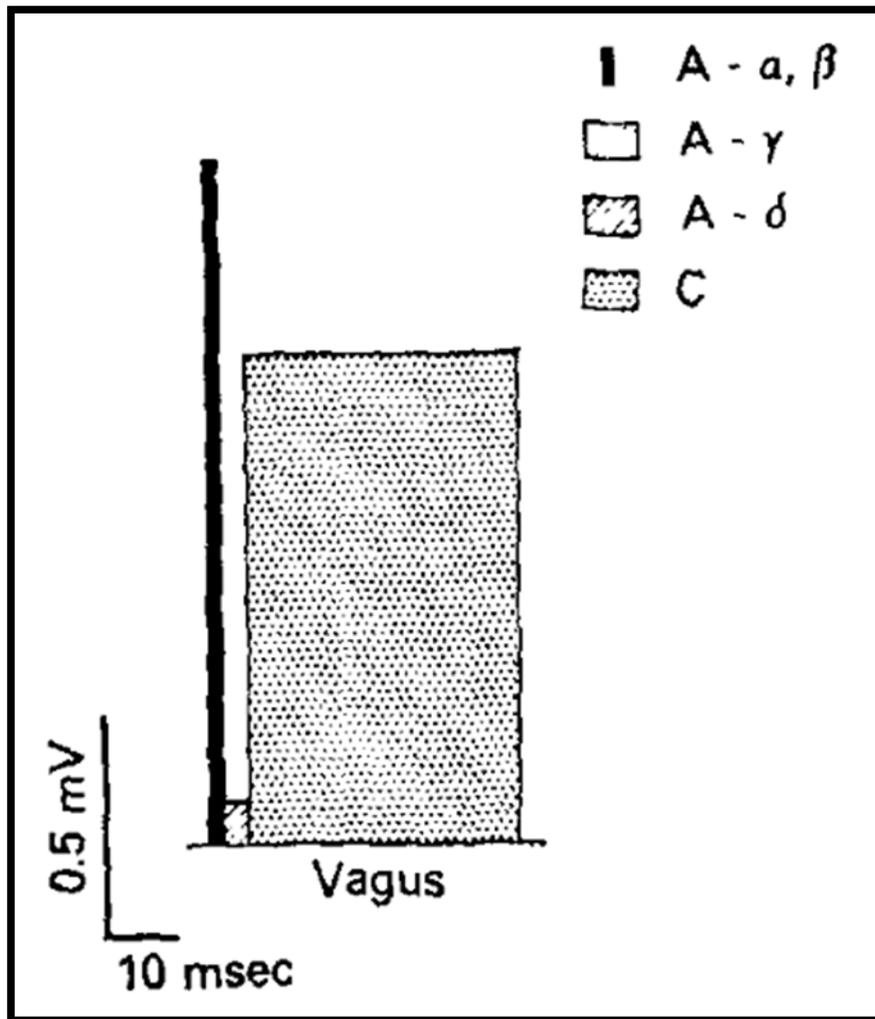


Figure 4.2: Average amplitude and duration of various components in the response recorded from 21 cat vagal nerves [33]. A-gamma fibers not found in the cat vagus nerve.

4.3 Stimulation and Recording Methods

I have performed many different setups for stimulation and recording of the vagus nerve. For stimulation, silver wires coated with Teflon, exposed at the tip, or tin coated copper wires were used. For recording, either flexible electrodes - gold or titanium nitride (TiN) (provided by Dr. Walter Voit from UT Dallas) or

silver wires coated with Teflon, exposed at the tip, or tin coated copper wires were used. In some cases, recordings were made from the muscle, which was achieved using needle electrodes. A picture of the typical setup is shown below in Figure 4.3.

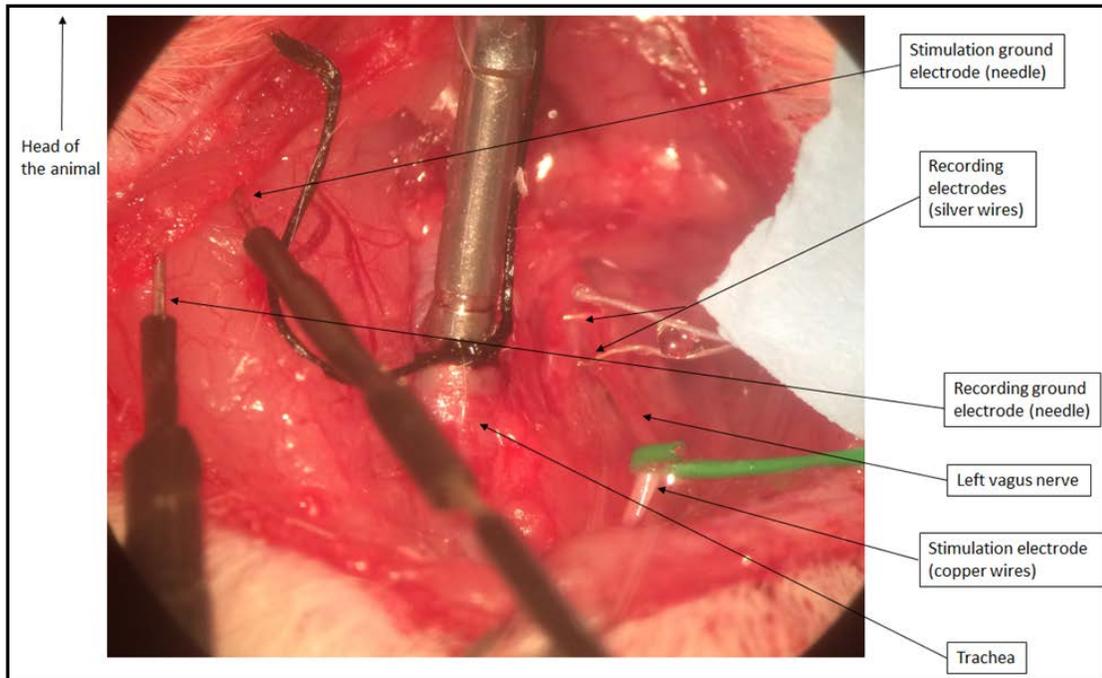


Figure 4.3: Nerve stimulation and recording setup

There was generally spacing of about 5 mm to 1 cm between the stimulation and recording electrodes. The nerve was bathed in mineral oil after the electrodes were placed to prevent the nerve from drying out. Three types of recording electrodes were used throughout the work presented in the thesis:

1. Flexible gold electrodes provided by Dr. Walter Voit's lab at UT Dallas

The flexible electrodes used are shown in Figure 4.4 below. The entire electrode is shown in the left panel of Figure 4.4, and a close-up schematic of the 4 channels are shown in the middle panel. They are temperature sensitive electrodes i.e. they soften up at about 40-45°C and can then be folded in to a convenient shape such as a groove so that the nerve can be wrapped in it (shown in right panel of Figure 4.4). When the nerve is positioned as desired, the flexible electrode is glued shut with a silicone epoxy, and can be folded when heated to and folds around nerve.

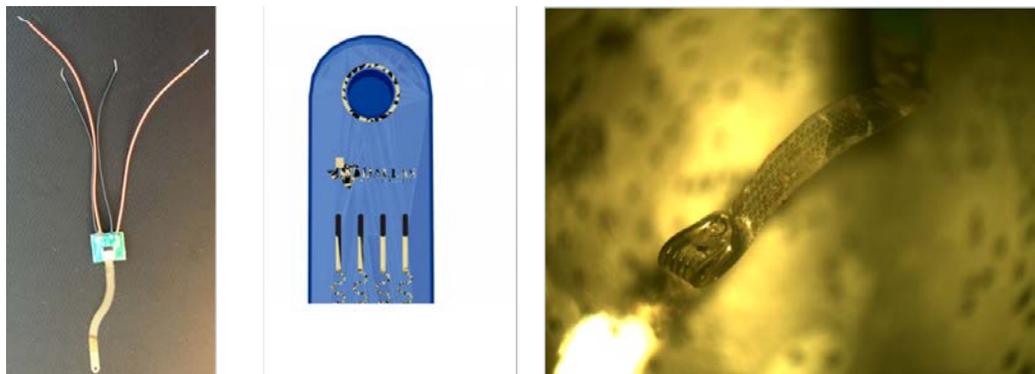


Figure 4.4: 4-channel gold flexible electrodes. The left panel shows the entire electrode with its connections. The middle panel is a schematic of a close-up of the 4 gold sites of the electrodes. The right panel shows the flexible electrode folded to a shape making it conducive for the nerve to fit in.

2. Tin coated copper wires

These are insulated wires partially etched to record from the tin coated copper. These are the green wires shown as the stimulating electrodes in Figure 4.3.

3. Silver wires insulated with Teflon

These are insulated wires where the Teflon coating is exposed at the tip to record from the silver. These are the silver wires shown as the recording electrodes in Figure 4.3.

4.4 Nerve Recordings to Different Nerve Stimulation Paradigms

Stimulation involved placing a stimulation wire on the nerve and the stimulation ground wire anywhere in the body (usually the skin/muscle in the neck to keep the electrical field generated confined to a minimum area). Ideally, stimulation ground should be on the nerve to ensure that the current flows through the nerve, however high artifacts were generated by this method most times. Stimulation was generally a biphasic square pulse, the two phases often being separated by 10 ms in order to evaluate how each phase of the pulse induces nerve activity. Stimulation was conducted 100 times (i.e. 100 trials), once every 500 ms (i.e. at 2 Hz). Stimulation strength is reported in decibels (dB). The dB to micro-amperes (μA) conversion is as below:

$$\mu A = 10^{dB/20}$$

For example, 20 dB is 10 μ A, 40 dB is 100 μ A, 60 dB is 1 mA and so on.

Recording from the nerve involved placing the recording electrode (on the nerve), reference electrode (on the nerve or shorted with the ground) and the ground electrode (usually in the skin/muscle in the neck). When the reference and ground wires were shorted to one another and placed in the neck muscle, this was referred to as a non-differential recording. When the reference was placed on the nerve, this enabled common mode rejection and was called a differential recording. The differential recording trace recorded was the difference of two electrodes placed on the nerve, with reference to the ground (i.e. *(signal - ground) - (reference - ground)*). This method helped to eliminate the common signals such as the heart beat signal that might otherwise interfere with the recorded nerve signals. For obvious reasons, differential recordings were smaller in magnitude than non-differential recordings. This is demonstrated in the figures below. Other figures below depict recordings of different stimulation and recording paradigms, effects of varying stimulus parameters on the recorded signals, and an evaluation of muscle versus true nerve activity.

4.4.1 Effects of Recording Configuration

All recordings shown are an average of 100 trials. GND refers to the ground electrode, REF refers to the reference electrode. The presented trace represents:

(signal - ground) - (reference - ground)

4.4.1.1 Non-differential Recording

Stimulation: Tin coated copper wires

Recording: 4-channel gold flexible electrode

Schematic:

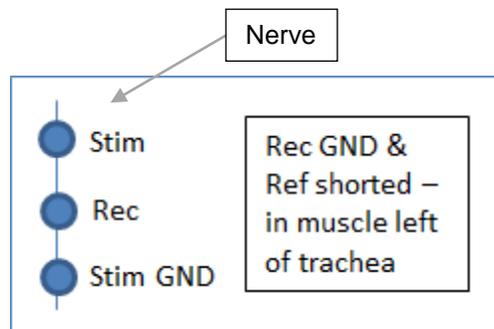


Figure 4.5: Schematic corresponding to Figure 4.6

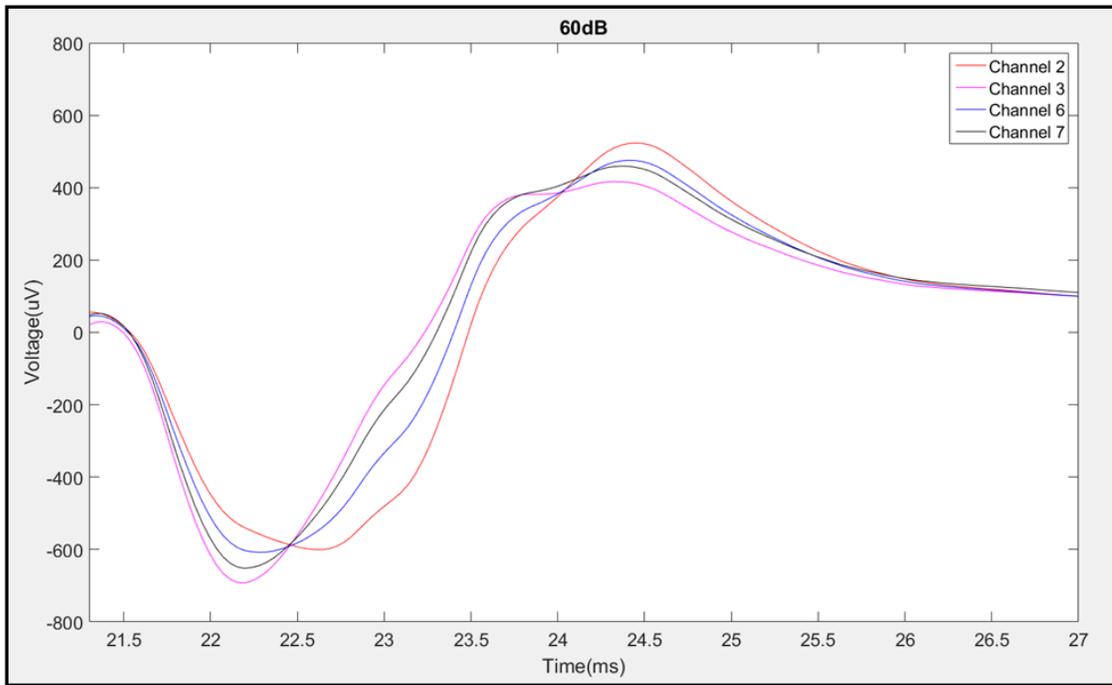


Figure 4.6: Non-differential recording from 4 sites of vagus nerve activity (GP 2). Activity is shown immediately at the offset of the stimulus artifact (hidden). The electrical stimulus onset is at 20 ms and stimulus pulse duration is 200 μ s. Note the peak to peak amplitude is \sim 1.1mV.

4.4.1.2 Differential Recording

Stimulation: Tin coated copper wires

Recording: 4-channel gold flexible electrode

Schematic:

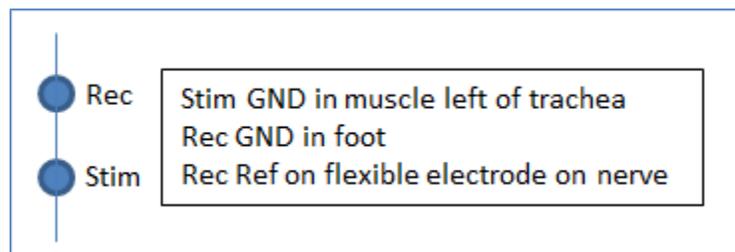


Figure 4.7: Schematic corresponding to Figure 4.8

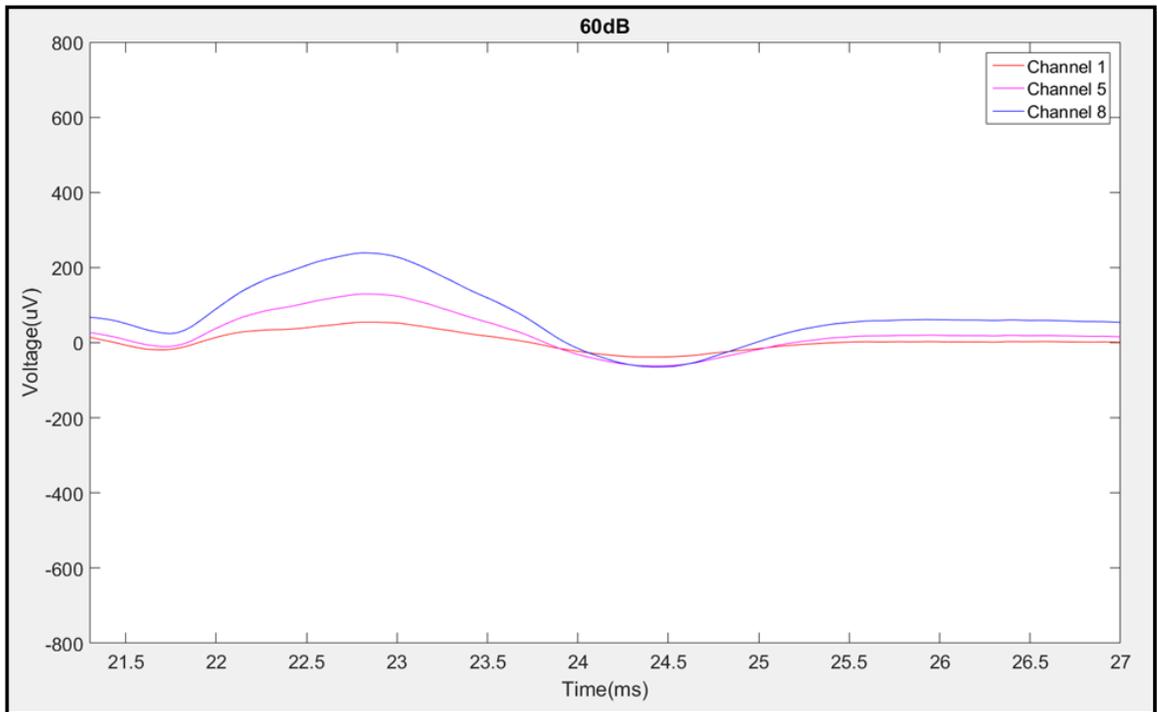


Figure 4.8: Differential recording from 3 sites (since the 4th is used as a reference electrode) of vagus nerve activity (GP 2). Activity is shown immediately at the offset of the stimulus artifact (hidden). The electrical stimulus onset is at 20 ms and stimulus pulse duration is 200 μ s.

Note the peak-to-peak amplitude for the differential recording in Figure 4.8 is $\sim 303.6 \mu$ V, approximately 3.7 times smaller than in the non-differential case shown in Figure 4.6. This is as expected since the reference is on the nerve for the differential case, thus lower amplitudes will be recorded.

4.4.2 Comparison of Different Recording Electrodes and Effects of Electrical Stimulus Polarity

The recordings in Section 4.4.2 were made using the 4-channel gold flexible electrode. In this section, I used tin coated copper wires. These wires are sometimes more convenient to use when the distance between two recording sites needs to be manually controlled. I compared wire recordings with flexible electrode recordings to justify the use of either, as was convenient for the protocol. Below is a figure of a typical recording from tin coated copper wires.

Stimulation: Tin coated copper wires

Recording: Tin coated copper wires

Schematic:

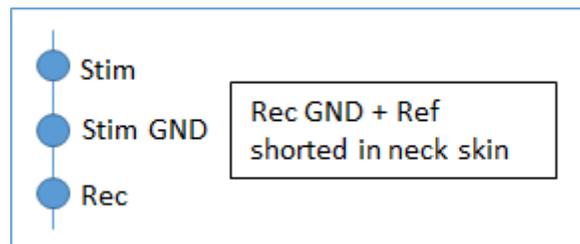


Figure 4.9: Schematic corresponding to Figure 4.10

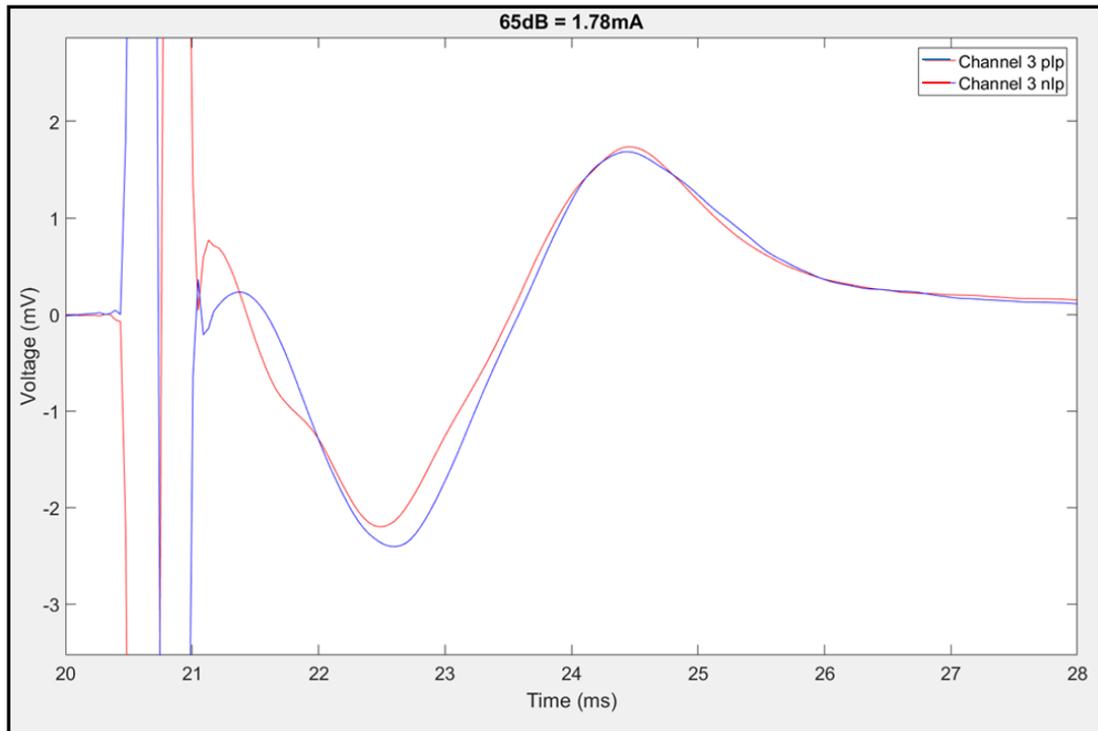


Figure 4.10: Non-differential recording from copper wires of vagus nerve activity (GP 3). plp in the legend refers to a biphasic electrical stimulus with a positive leading pulse (in blue), nlp refers to a biphasic electrical stimulus with a negative leading pulse (in red), as demonstrated by the artifact. Activity is shown immediately at the offset of the stimulus artifact (seen between 20.5 ms and 21 ms). The electrical stimulus onset is at 20 ms and stimulus pulse duration is 200 μ s. Level of stimulus pulse is shown at the top of the plot.

In Figure 4.10, the polarity of the stimulus has been reversed in the two conditions to check if the direction of the activity remains the same. This technique ensures that the activity observed is indeed activity and not a continuation of the stimulus artifact. Activity seen here is similar to that seen in the non-differential recording using the gold flexible electrode in Figure 4.6, except it is of higher magnitude due to the fact that the stimulus current is almost double (1.78 mA versus 1 mA). Therefore, the wires are as effective as the flexible electrodes for the purpose of recording such signals.

4.4.3 Comparison of Different Stimulation Electrodes

Three different stimulation paradigms were compared: stimulation with 3 sites of the flexible electrode, stimulation with 4 sites of the flexible electrode, and stimulation with tin coated copper wires. Note that each site has the same amplitude of current flowing through it. Thus, stimulation with 4 sites of the flexible electrodes delivers more effective current than that with 3 sites of the flexible electrode, which is why this was tested. Wires occupy less space on the nerve, therefore this stimulation electrode comparison was done to justify the use of any of the stimulation methods, depending on what was most convenient for a given experimental setup. Figure 4.12 below shows 4 subplots of increasing current levels from 45 dB to 52 dB, to show different threshold of activation by the different stimulation paradigms.

Stimulation: Comparison of 3 paradigms

Recording: 4-channel gold flexible electrode

Schematic:

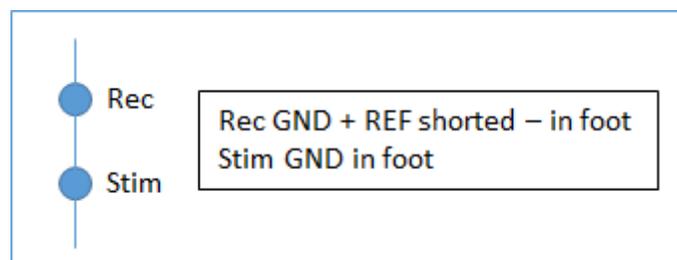


Figure 4.11: Schematic corresponding to Figure 4.12

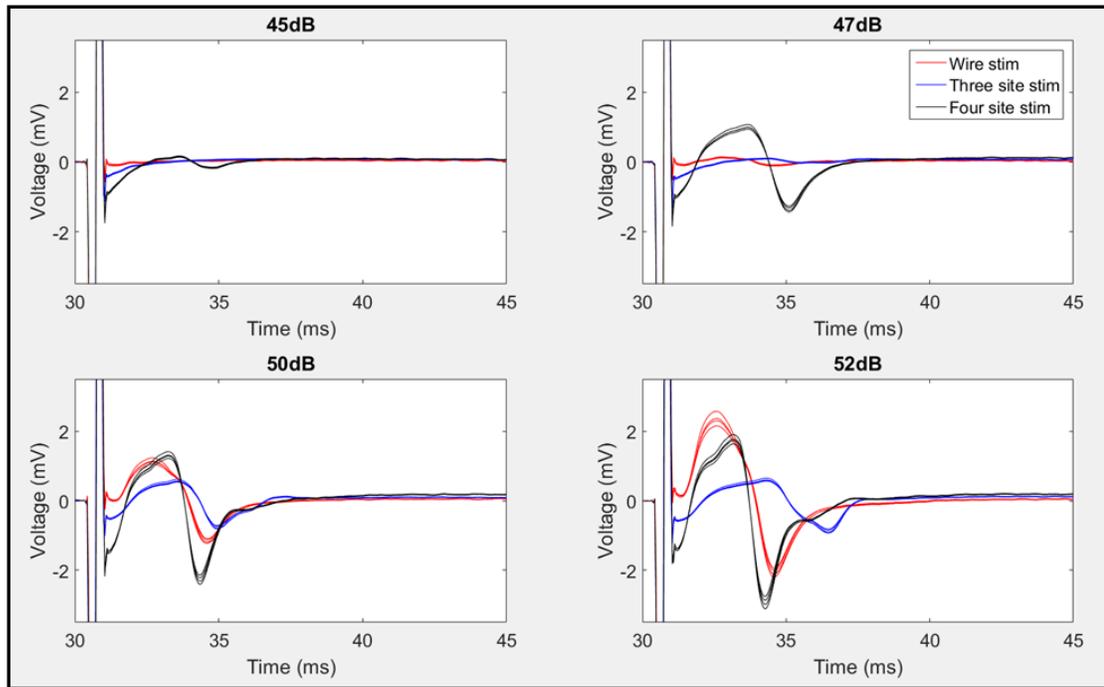


Figure 4.12 Comparison of flexible electrode stimulation and copper wire stimulation (GP 4). Activity is shown immediately at the offset of the stimulus artifact (seen shortly after 30 ms). The electrical stimulus onset is at 30 ms and stimulus pulse duration is 200 μ s. The four panels show increasing current from left to right in the upper panel and then left to right in the lower panel.

Figure 4.12 shows that four site stimulation (in black) was the most effective, as was expected, with the lowest threshold of activation at 45 dB. Three site stimulation (in blue) seems the least effective of the three. Wire stimulation (in red) has a higher threshold of activation (47 dB) than the four site stimulation, but proves as effective thereafter. Therefore using wires to stimulate seems as adequate as using the flexible electrode, and I made the decision to use wires for stimulation due to the ability to more easily position them on the nerve while requiring less space for them.

4.4.4 Effects of Stimulus Pulse Duration

Different pulse widths for stimulation were compared. There are studies showing that the threshold for some fibers of the vagus nerve is around 500 μs for currents up to 1 mA [57]. These fibers were shown not to be activated by 200 μs pulses, unless the amplitude was at least a few mA. Since our lab's stimulation setup for this experimental protocol doesn't exceed 3 mA, I decided to try stimulations of 500 μs pulse duration. Therefore, comparison between 200 μs and 500 μs pulse responses was performed.

Stimulation: Tin coated copper wires

Recording: 4-channel gold flexible electrode

Schematic:

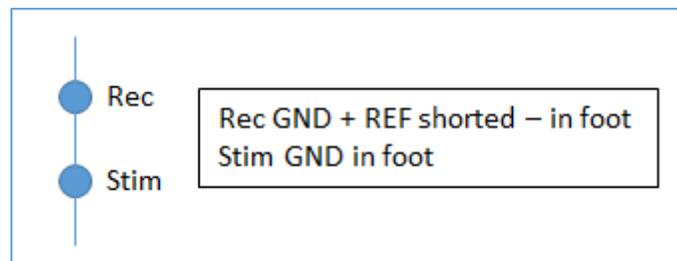


Figure 4.13: Schematic corresponding to Figure 4.14

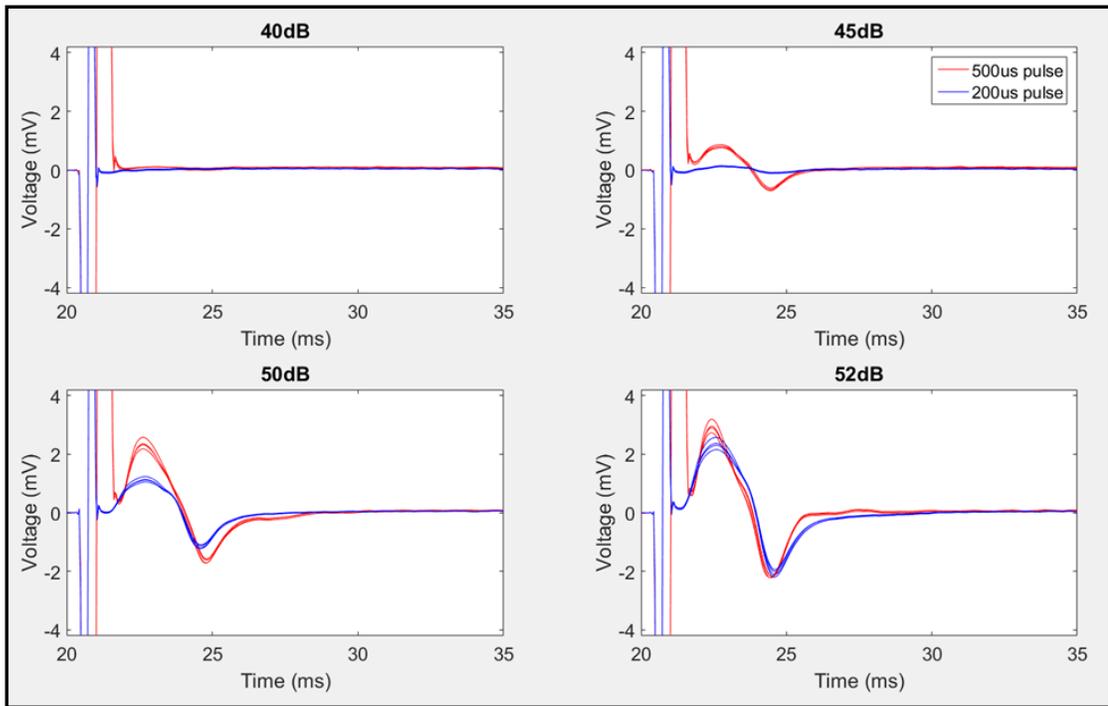


Figure 4.14: Comparison of responses from 200 μ s and 500 μ s stimulus pulse durations (GP 4). Activity is shown immediately at the offset of the stimulus artifact (seen shortly after 20 ms). The electrical stimulus onset is at 20 ms. The four panels show increasing current from left to right in the upper panel, and then left to right in the lower panel.

It can be seen that the threshold for activation is lower for the 500 μ s pulse (45 dB), as expected. However, the shape of the responses caused by the 500 μ s pulse is similar to that caused by the 200 μ s pulse. Therefore, it can be inferred that no additional fibers are getting activated. The shapes of the two responses may look similar also because it is not true nerve activity and just muscle activity, explained in detail in Section 4.4.5. However, since the 200 μ s induces a similar response to the 500 μ s pulse, 200 μ s pulses are used in all other recordings.

From Sections 4.4.1 to 4.4.4, the activity seen is similar in amplitude and in duration, and this is what I consistently observe in my recordings. When stimulation of the nerve is conducted, there is always some visible twitching of the trachealis muscles, intrinsic laryngeal muscles (ILMs) or thyroarytenoid (TA) muscles. This implies that muscle is being activated by stimulation of the nerve (directly by nerve (RLN) innervating these muscles or indirectly through spread of current). This muscle activity can flow back through the surrounding fluids and tissue and then propagate back up the nerve and appear as activity. Therefore, appropriate controls should be taken to eliminate this muscle contamination of nerve activity.

4.4.5 Evaluation of Muscle Activity versus True Nerve Activity

In all of these experiments above, there was always visible twitching of the muscle under the nerve (TA muscles) and at times, of the muscle near the trachea (trachealis muscle), which is why we suspected that we were seeing muscle activity propagate back up through the nerves that was obscuring the true nerve activity. At lower current levels when there is no twitching, this presumably nerve activity is also not seen. Most studies which have reliably demonstrated true nerve activity see amplitudes of $\sim 20 \mu\text{V}$. We see responses of much higher amplitudes, so it is unlikely that this is true nerve activity. We recorded from the twitching muscles to confirm this.

4.4.5.1 Recording from Muscles (EMG)

Stimulation: Tin coated copper wires

Recording: 4 channel gold flexible electrodes, and needle electrodes to record from muscles

Schematic:

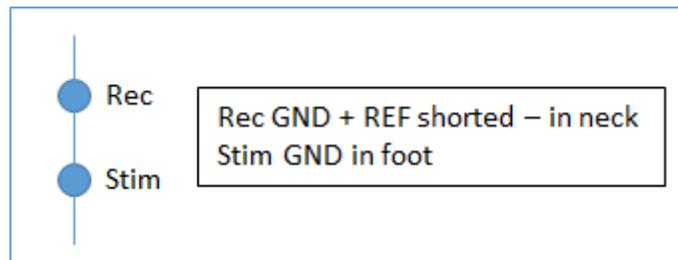


Figure 4.15: Schematic corresponding to Figure 4.16

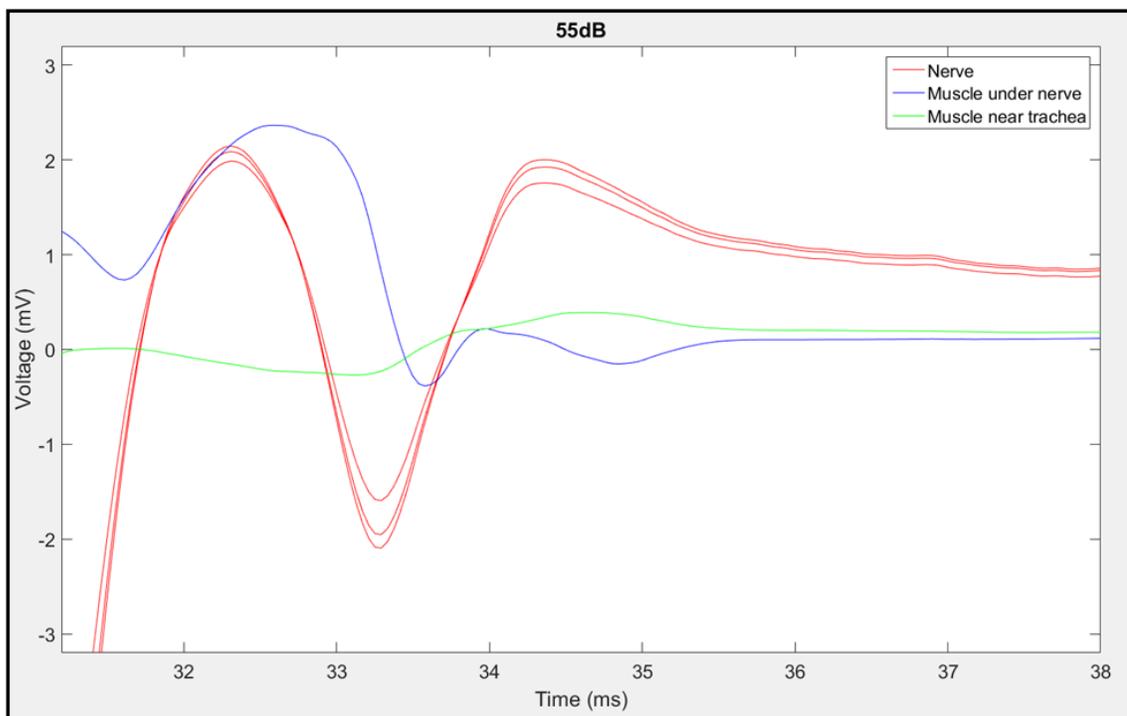


Figure 4.16: Recording from the nerve and the two twitching muscles while stimulating the nerve (GP 5). Activity is shown immediately at the offset of the stimulus artifact (hidden). The electrical stimulus onset is at 30 ms and the stimulus pulse duration is 200 μ s.

Figure 4.16 shows that the first blue peak aligns more or less with the first red peak, and the second red peak seems to be close to a sum of the second blue peak and the green peak. Thus, the nerve activity can be seen to align to a great extent with the muscle recordings. This confirms our suspicion that the recording is mainly muscle activity flowing back up through the nerves to our recording electrodes.

4.4.5.2 Direct Stimulation of Muscle

The next thing I tried was to stimulate the muscle itself and view the responses from the nerve, shown in Figure 4.18 below.

Stimulation: Needle electrodes

Recording: 4 channel gold flexible electrodes, and needle electrodes to record from muscles

Schematic:

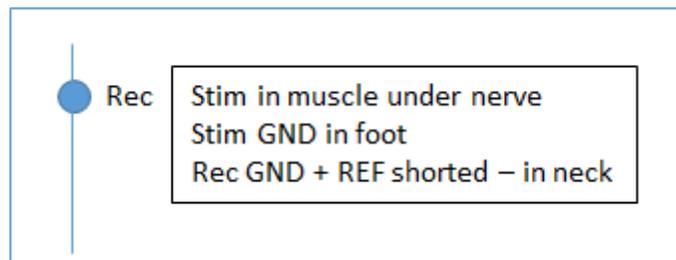


Figure 4.17: Schematic corresponding to Figure 4.18

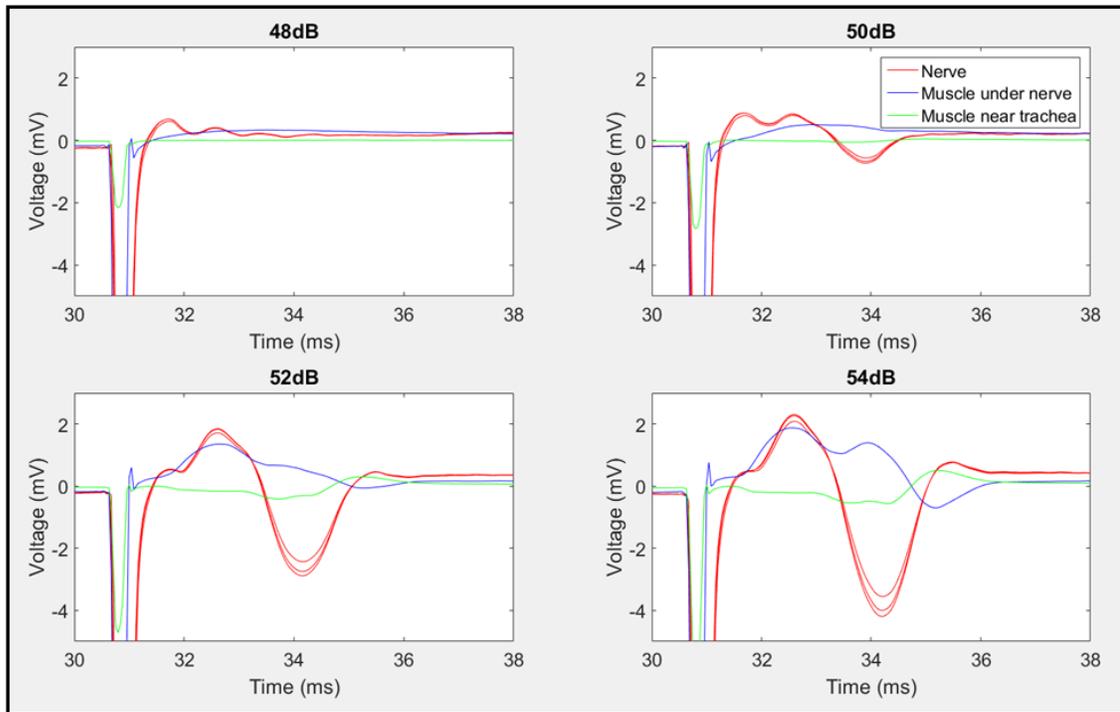


Figure 4.18: Muscle stimulation and muscle and nerve recording (GP 6). Activity is shown immediately at the offset of the stimulus artifact (seen at 31 ms). The electrical stimulus onset is at 30 ms and stimulus pulse duration is 200 μ s. The four panels show increasing current from left to right in the upper panel, and then left to right in the lower panel.

In Figure 4.18, as in Figure 4.16, the muscle and nerve activity align up, except for the negative peak seen at 34 ms. Ringing can be observed in the 48 dB case, which is a problem we have been facing with some recordings, and we are still trying to resolve that issue. It is clear that the recordings we see are not true nerve activity. There may be some nerve activity mixed with muscle activity and/or mixed within the stimulation artifact.

Calcium channel blockers (Diltiazem 5 mg/mL) were applied to the nerve and muscle area (since nerves use calcium channels to communicate at neuromuscular synapses) in the hope of eliminating muscle activity. This

increased the threshold for activation from 46 dB to 60 dB and decreased the amplitude of the observed activity. There was no activity at current levels that activity was seen earlier - this is further evidence that the only activity we were able to pick up was muscle activity. From a deeper literature study, neuromuscular blocks employed by other groups were discovered (succinylcholine and vecuronium) which were used for this purpose specifically. Figure 4.19 below shows a figure from the study in dogs by Grill et al. [32] where they used succinylcholine to block muscle activity. Figure 4.20 below shows a figure from the study in rats by Tahry et al. [47] where they use vecuronium to block muscle activity. They also lesion the nerve to control for the origin of the activity. The next step for the Lim lab to expand upon my work will be to use these neuromuscular blockers to check if there is any nerve activity masked by the muscle activity that I have seen in my nerve recordings.

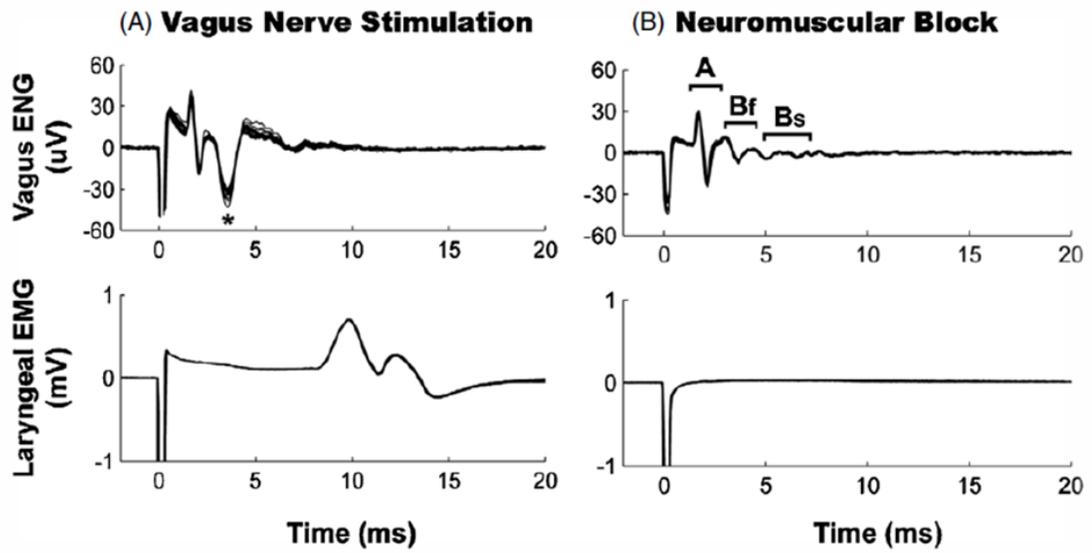


Figure 4.19: Recordings from Grill et al.'s study [32]. The left panel shows nerve recordings (top panel) and muscle recordings (bottom panel). The right panel shows the same recordings when the neuromuscular block was injected. It can be seen that the muscle activity (bottom panel) has disappeared and the nerve activity has changed, and now reveals more intricate details which were earlier masked by the muscle activity.

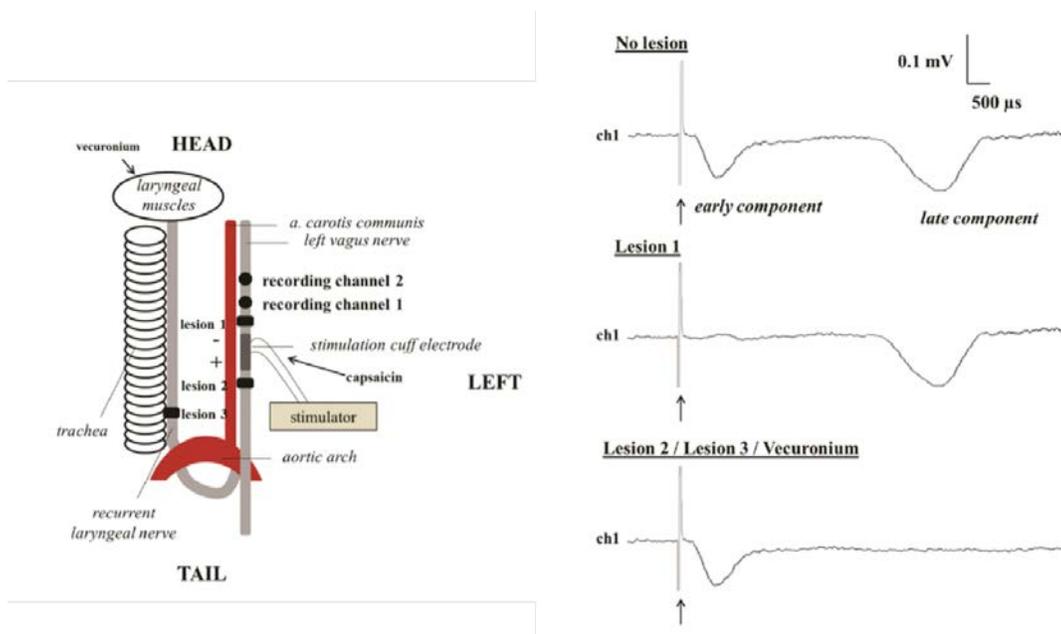


Figure 4.20: Recordings from Tahry et al.'s study [47]. The left panel shows the schematic of their setup, including where the lesions 1, 2, and 3 are made. The right panel shows the nerve recordings when there is no lesion (top panel), when lesion 1 is made between the stimulation and recording electrodes so as to eliminate direct nerve activity, and when lesion 2/3 is made or vecuronium is applied (bottom panel). This last check gets rid of the muscle activity.

I have tried cutting the nerve where lesion 2 is indicated in the left panel of Figure 4.20, and this does not eliminate the activity seen, but definitely changes it. I believe there are more muscles than just the laryngeal muscles getting activated, and that activity may originate central of the recording and not from where the lesion is made distally. Lesioning central to the recording also changes the activity, but does not eliminate activity, showing that the laryngeal muscles originate distally confirming what is shown in Figure 4.20. A better check would be to use the neuromuscular block, which would eliminate all muscle activity, regardless of its origin.

4.4.5.3 Recording from Multiple Nerves (Spread of Current versus Direct Muscle Activation)

It is possible that muscle was getting activated by spread of current from the stimulation electrode, and that the activity was not caused by direct muscle activation from nerve stimulation. This is unlikely since the preparation was well isolated and bathed in mineral oil, a non-conductor of electricity. However, to verify this, I recorded from all four nerves in the vicinity - the left and right vagus nerves and the left and right sympathetic nerves.

Stimulation: Silver wires, on LVN

Recording: Silver wires, from LVN, LSN, RVN, RSN

Setup: Stim GND & Rec (REF+GND shorted) in neck muscle

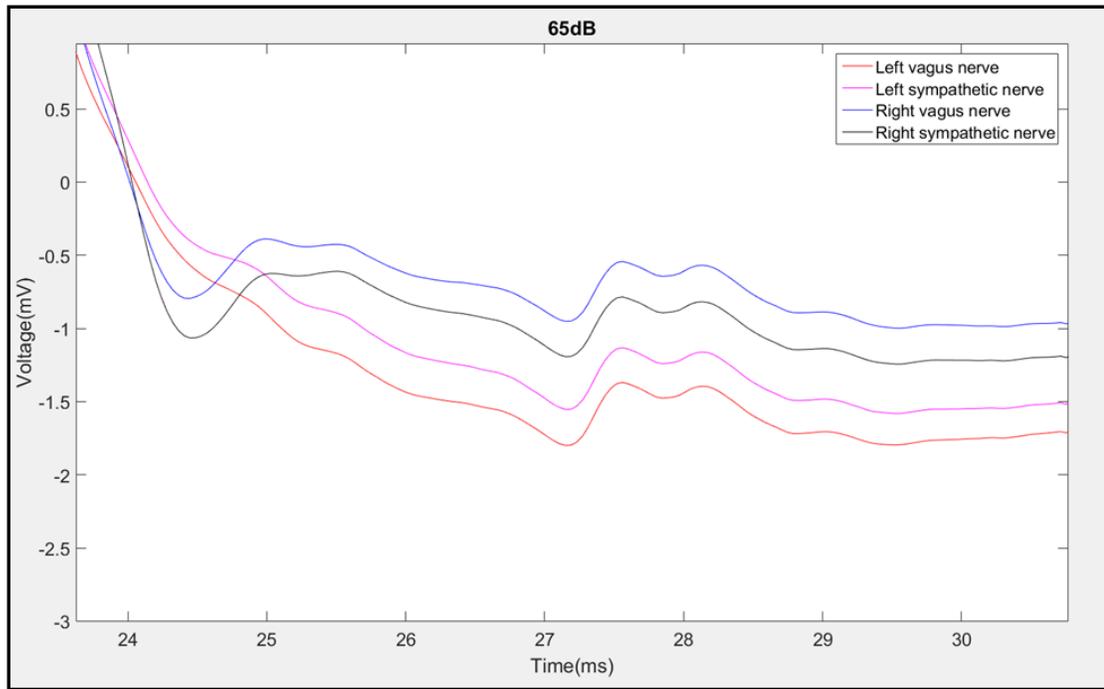


Figure 4.21: Recording from four nerves (LVN, LSN, RVN, RSN) while stimulation is on the LVN (GP 5). Activity is shown immediately at the offset of the stimulus artifact (hidden). The electrical stimulus onset is at 30 ms and the stimulus pulse duration is 200 μ s. There is a baseline shift in the recordings caused by the artifact, but that does not cause any discrepancy in the general shapes of the curves for all four cases.

Figure 4.21 shows activity on all four nerves, which further confirms that what we see on the LVN cannot be true nerve activity, otherwise it wouldn't have appeared at the same latency and with the same shape on the LSN and the other nerves. The peak just after 27 ms in Figure 4.21 is seen on all four nerves. If this was the only peak, then activity through current spread would not have been ruled out since activity through current spread should induce peaks on all 4 nerves at approximately the same latency. However, the nerves on the right (RVN and RSN), show an additional peak just after 24 ms in Figure 4.21. There has been an earlier study [45] where they show an additional peak on the

contralateral VN, which they call an “indirect component” which they propose arises from the neural network of the heart. Figure 4.22 is from this study.

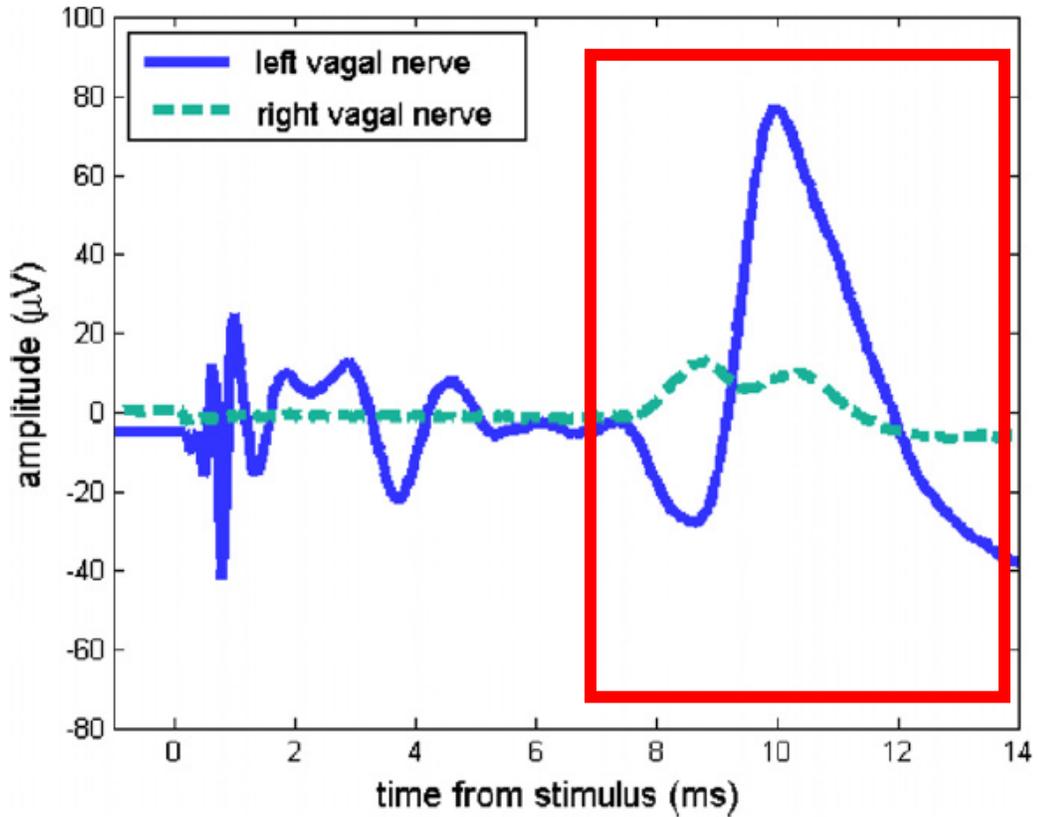


Figure 4.22: Recordings from Ordelman et al.'s study [45], region of interest enclosed in red. Two peaks seen on RVN, and one peak seen on LVN.

Figure 4.22 shows additional activity on the LVN (everything before 6 ms) that is most likely true nerve activity. Important to note is that this study was done in pigs, where the vagus nerve is much longer than in guinea pigs, which is probably why the latency of muscle activity seen is much greater (~8 ms in this

case). The nerve and muscle activity seen by them is well separated so as not to be confused with one another. It is likely that in a guinea pig model the two overlap, making it harder to distinguish one from the other.

4.4.6 Summary of Findings

I tried many different setups and ultimately found muscle activity dominated almost all activity that I observed. Also, another reason why we may not be able to see activity is that it gets lost in the stimulation artifact. The artifact in many cases is high enough to saturate the pre-amplifier we use for recording, which could have some impact on any activity recorded soon after the artifact. Issues of saturating our pre-amplifier and causing baseline shifts of artifact can be masking the nerve activity. A solution for this problem is to stimulate further away on the vagus nerve to reduce the electrical artifact. To this end, I stimulated the vagus nerve in the neck and recorded from the vagus nerve in the esophageal area. However, the activity in this case also appears to be muscle activity since it appears similar to all responses seen in this chapter, but just at a greater latency, which makes sense since the recording setup is further away (shown in Figure 3.5 in Chapter 3). In addition to these issues, our recording setup has high noise, up to a 100 μV at times, which can mask the activity since we anticipate (from literature) true nerve activity to be in the tens of μV s in case of a differential recording. This high noise would be an issue that averaging and

neuromuscular blocks may not be able to solve, in which case we need electrodes that pick up significantly lower noise than the signal.

After thinking through this setup, the only way we can be sure of true nerve activity is to use neuromuscular blocks in conjunction with electrodes having a lower noise floor, which will be pursued by another researcher in the Lim Lab. However, more immediately for my thesis project, I can measure heart rate changes, showing US neuromodulation on what is induced by electrical stimulation of the vagus nerve. This latter way doesn't permit me to evaluate how the CAP changes directly from US stimulation, but the advantage is that the readout is free of these recording artifacts, in which I realize now how challenging it is to create such a clean nerve stimulation-recording setup. US modulation of the vagus nerve observed by measuring the changes in heart rate is discussed in the next chapter, in addition to the preparation and hardware I developed to achieve that measurement.

Chapter 5: Using Ultrasound to Modulate Vagus Nerve Activity

From the previous chapter, it is clear that it has been challenging to obtain true nerve activity from the vagus nerve. Therefore, another thought was to use another readout to observe US modulation of vagus nerve activity. Since I observed that electrical stimulation of the right vagus nerve increases the heart rate, I thought of modulating right vagus nerve activity with US to see if US can excite the vagus nerve to increase heart rate or when paired with electrical stimulation, whether it can enhance/suppress the heart rate increase caused by electrical VNS.

5.1 Setup

The heart rate (HR) was monitored by a clip-on heart rate monitor I made in-house using a pulse sensor and an Arduino microcontroller board. An electrocardiogram (ECG) was recorded periodically to make sure the clip-on monitor was reporting the heart rate accurately. Photos of the setup are shown in Figures 5.1-5.3 below. ECG was recorded using three needle electrodes (a simpler version of the original 12-lead system) using the lead II placement. The positive electrode is the white wire in Figure 5.1 below; the negative electrode is the yellow wire, and the ground electrode is the green wire. This is a traditional placement of electrodes for recording ECG activity in animal experiments.

Ultrasound was transmitted using a 220 kHz transducer and a focusing cone (both provided by Medtronic). Energy was delivered to the transducer using a signal generator and a power amplifier (E&I). Electrical stimulation was done using a pair of silver wires, both hooked on to the nerve. In this case, the stimulation ground was placed on the nerve as well since that ensures maximum conduction through the nerve and I didn't have to worry about the interference of recording the stimulus artifacts in this setup.

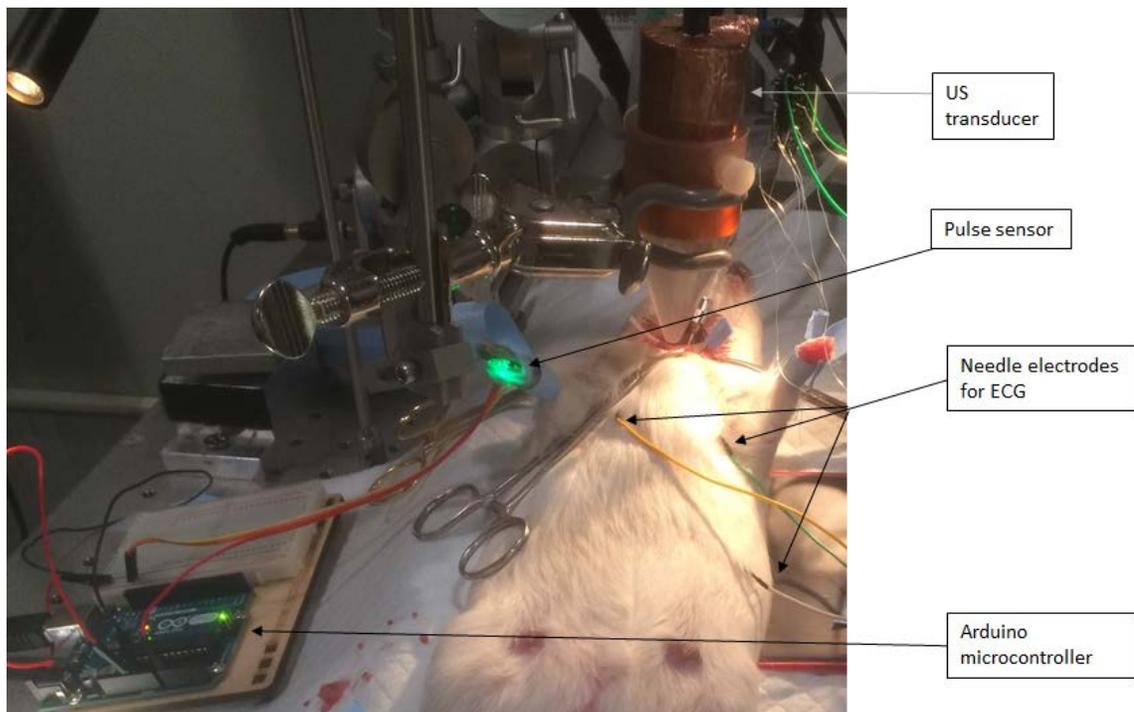


Figure 5.1: Setup for US modulation of the nerve and HR recording. Electrical stimulation electrodes cannot be seen in this figure, as they are covered by the US transducer.

The nerve is hammocked to the transducer as shown in Figures 5.2 and 5.3 below. A sheet of parafilm is introduced under the nerve, which is then used to lift the nerve up and secure it to the ultrasound cone.

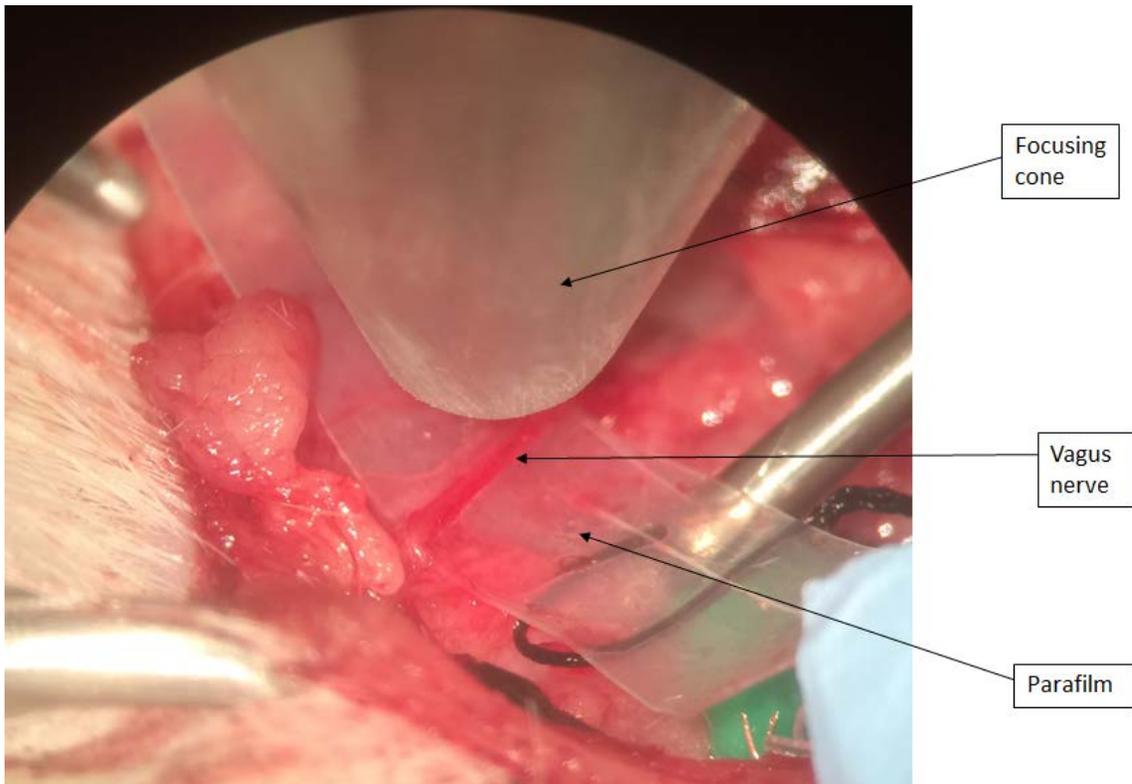


Figure 5.2: Setup showing parafilm to lift up the nerve to cone (yet to be lifted). This is a photo taken before the nerve is hammocked.

When the parafilm is taped to the cone by either end, the pocket is filled with agar (shown in Figure 5.3 below) to create a medium between the water-filled cone and the nerve since there should be no air gap between the US transducer and the target location, since air is a poor conductor of US.

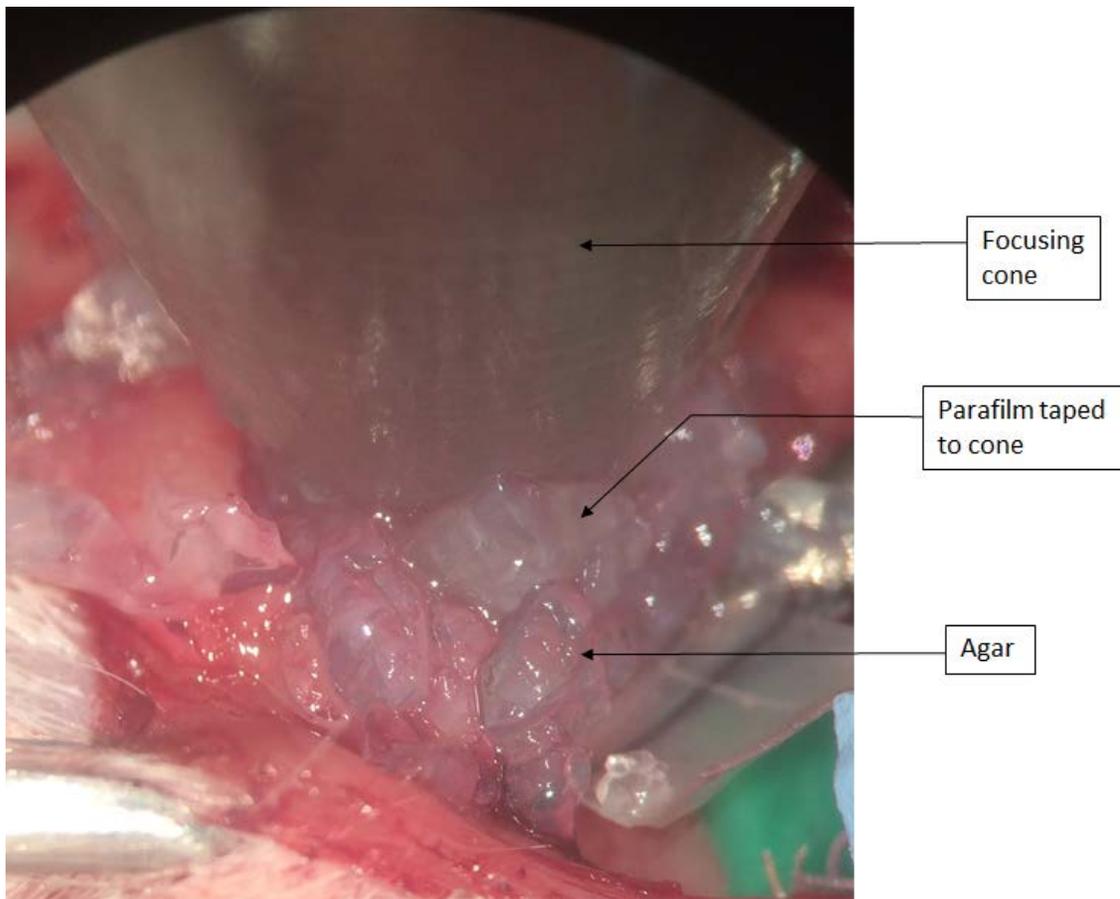


Figure 5.3: Setup showing hammocked nerve, with the interface covered with agar. Film is covered by agar so cannot be seen in this photo, but arrow points to its location under the agar. Nerve cannot be seen as its view is entirely obscured by agar in this photo.

US at various pressures (ranging from 300 kPa to 1800 kPa) and pulse durations (ranging from 10 ms to 500 ms) were presented to the vagus nerve to cause excitation, and thereby cause a change in heart rate. However, with the parameters tested, excitation did not occur. From unpublished data from the Lim lab, US did not cause excitation of the sciatic nerve either. However, US was able to cause suppression in sciatic nerve CAPs when the foot was stimulated, and in some cases even caused enhancement. Therefore, I hypothesized that

US should also be able to suppress and/or enhance the electrically-induced vagus nerve activity to the heart.

Figure 5.4 below shows an example of a typical raw ECG recording. This has been amplified 10 times by the pre-amplifier.

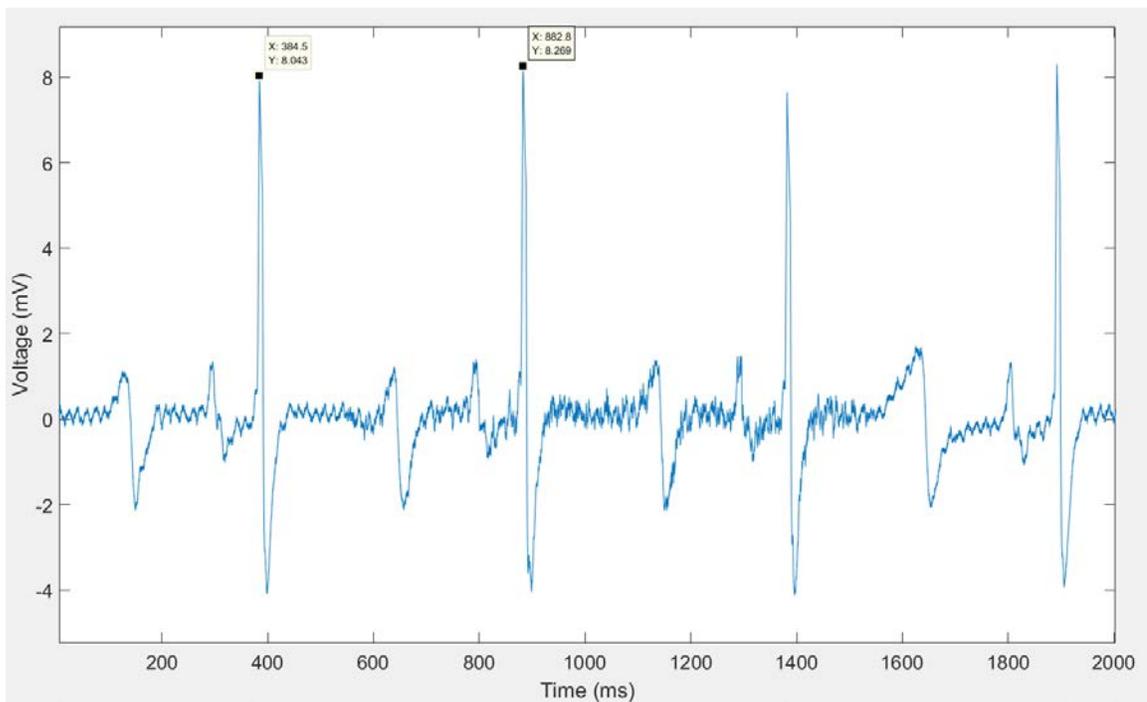


Figure 5.4: A typical raw ECG recording. Pointers indicate the two time points as 384.5 ms and 882.8 ms. Therefore the time between two consecutive beats is 498.3 ms, which makes the heart rate 120.4 beats per minute.

Recordings from the pulse sensor are not shown since direct calculations of the heart rate (calculated from inter-peak interval) were displayed on the monitor instead of the raw trace.

Electrical stimulation consisted of biphasic square pulses at 10 Hz frequency, 5 ms duration in GP 7, and 500 μ s duration in GP 8. Electrical stimulation was conducted on the vagus nerve with silver wires to increase the heart rate. The general protocol was to conduct electrical stimulation on the vagus nerve, observe how much heart rate increase was induced; then I would present US to the nerve for several minutes. Thereafter, I would conduct electrical stimulation on the vagus nerve again, to observe how much it increased the heart rate and then I would compare this with the original heart rate increase and noted the change. I would wait a while and then check if the original electrical stimulation parameters still induced the original effect on the heart rate and then based on my findings, conduct a second trial.

US stimulation was presented at the following levels:

1. 300 kPa pressure, 500 ms duration, one pulse per second, therefore 50% duty cycle
Mechanical Index (MI) = 0.64
2. 800 kPa pressure, 100 ms duration, one pulse per second, therefore 10% duty cycle
Mechanical Index (MI) = 1.71
3. 1800 kPa pressure, 10 ms duration, one pulse per second, therefore 1% duty cycle
Mechanical Index (MI) = 3.84

Mechanical index can be used as an estimate for the degree of bio-effects a given set of ultrasound parameters will induce. A higher mechanical index means a larger bio-effect. These parameters were chosen to start with since these parameters have been shown to work for suppression of the sciatic nerve in the Lim Lab, and since these parameters span a wide range. If effects are seen with these parameters, lower pressures and pulse durations can be investigated in further detail to identify trends in the results.

5.2 Results from Experiments

5.2.1 Animal 1

5.2.1.1 300 kPa, 500 ms

The following are the results from the first experiment with the setup shown in Figures 5.1-5.3. Initially, electrical stimulation was carried out for ~15-30 seconds (only 15-30 seconds since rapid rise in HR was observed), and the change in heart rate was documented. Then US was turned on and during the 13th minute of US electrical stimulation was carried out again (i.e. paired stimulation) for up to 3 minutes until it reached a stable value, and the maximum change in the heart rate was noted. Thereafter, US was turned off, and then electrical stimulation was carried out again up to 3 minutes until it reached a stable value, and the maximum change in HR was noted. The first trial was carried out at 3 mA, and it was observed that after US, 3 mA no longer excited the nerve as it originally did, so 4 mA was tried, and so on. Sufficient time was

not given for the nerve to recover, and this was remedied in the next experiment. However, the same suppressive effect was observed with a higher current (4 mA) in trial 2 as seen with 3 mA in trial 1, as shown in Figure 5.6 below. The schematic for an individual trial is shown in Figure 5.5 below. Each trial was conducted almost immediately after the previous trial. Trial 4 is shown in Figure 5.6 just to show that 3 mA was able to cause HR increase later on, even though it failed to do so after trial 3.

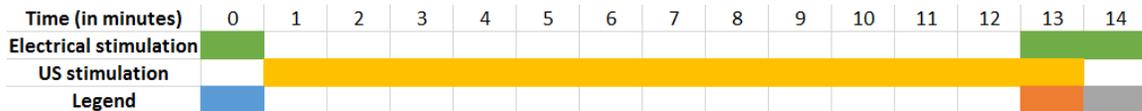


Figure 5.5: Schematic for a single trial corresponding to Figure 5.6

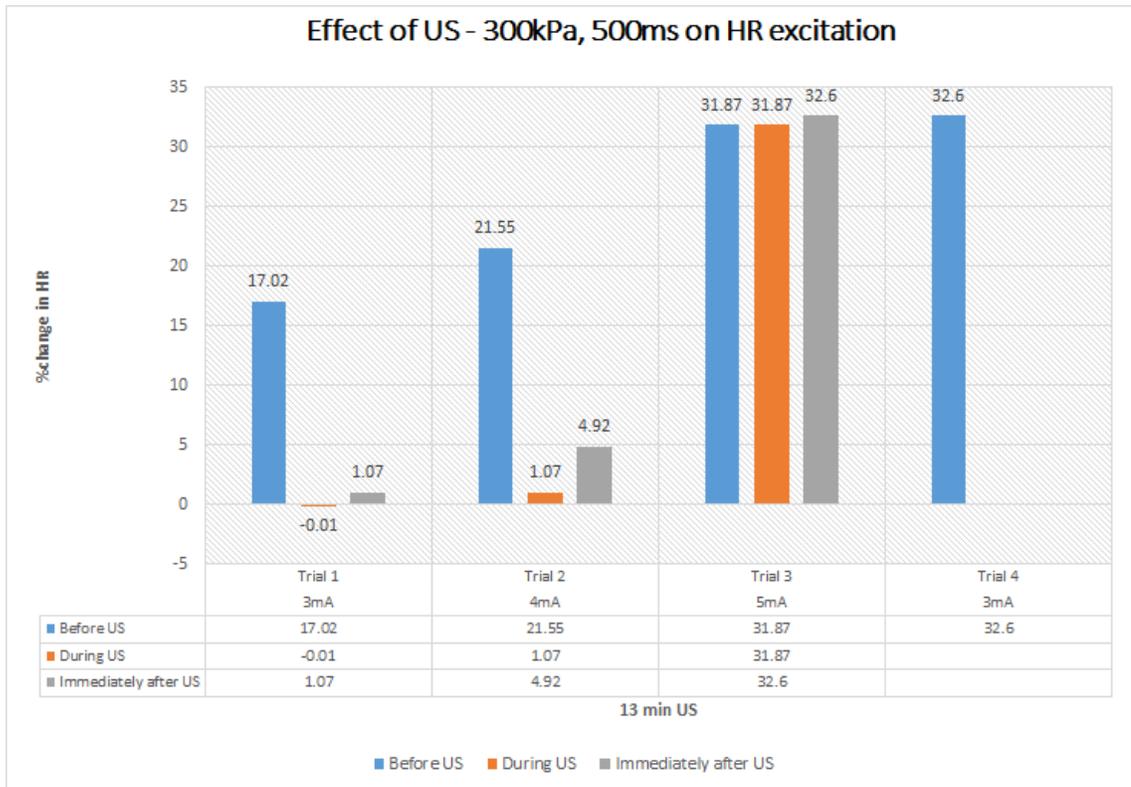


Figure 5.6: Effect of US: 300 kPa, 500 ms for 13 minutes on HR excitation (GP 7)

It can be seen in Figure 5.6 that in Trial 1 and Trial 2, there is suppression of HR increase when US is on. After it has been turned off, there is some recovery of activity, however, it is not up to its original value. In Trial 3, this effect is not observed. However, even 3 mA causes a large increase in HR as seen in Trial 4. I hypothesize that there is a range of currents close to the threshold for activation of the nerve, where the suppression effect of US can be observed. At currents much higher than this threshold, the excitatory effect of the electrical stimulation overpowers the ultrasound inhibitory effect, and thus US causes no change in HR as seen in trial 3 in Figure 5.6.

Below is Figure 5.8 showing an expanded version of trial 2, with more frequent recordings. This figure shows HR change caused by electrical stimulation just before US was turned on (i.e. an increase in HR of 21.55%). Then US was turned on continuously for 13 minutes, and paired stimulation was conducted at 5 minutes, 9.5 minutes, and 13 minutes. Finally, electrical stimulation was performed after US was turned off. The schematic is shown in Figure 5.7 shown below.

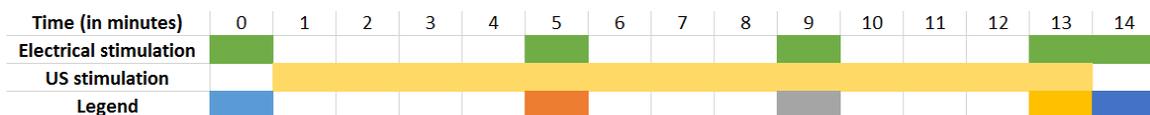


Figure 5.7: Schematic corresponding to Figure 5.8

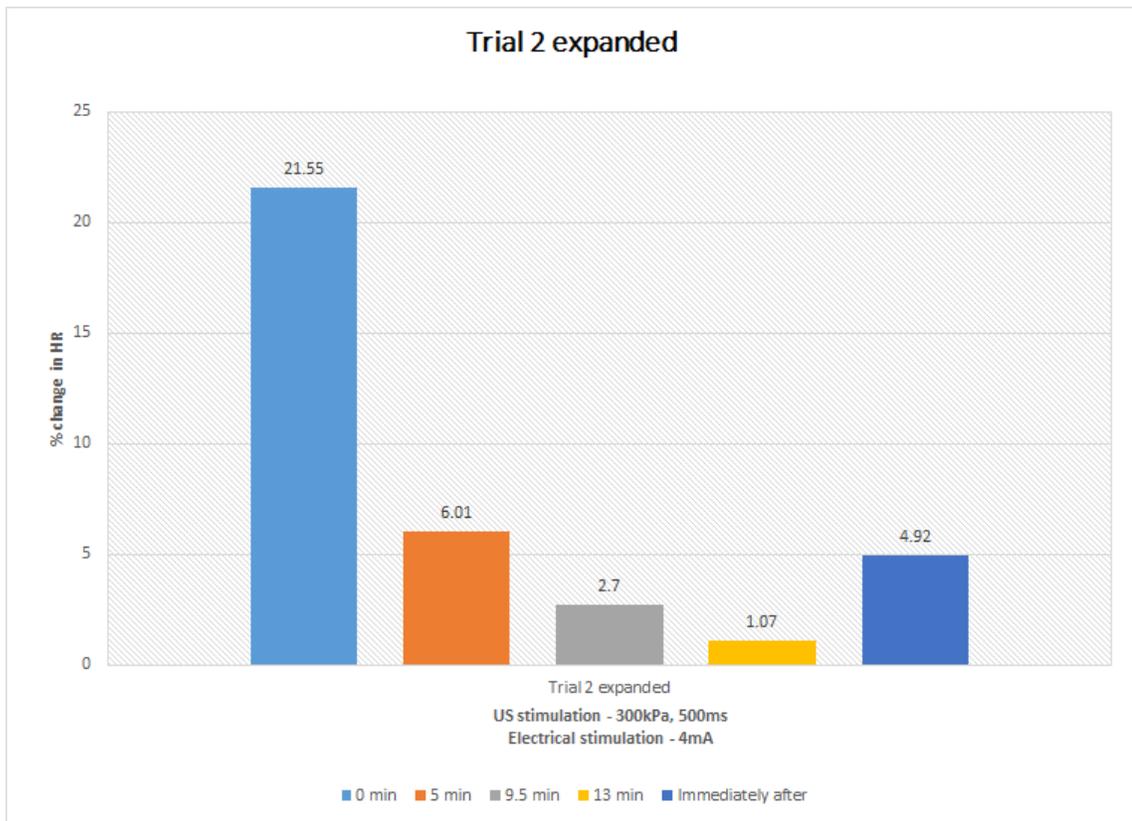


Figure 5.8: Expanded view of Trial 2 of Figure 5.4, showing measurements at various interim periods during the trial.

Gradual suppression can be seen in Figure 5.8 as we advance from 0 minutes to 13 minutes, and the beginning of recovery can be seen immediately after US is turned off. More time was not given for recovery and this was remedied in the next experiment.

5.2.1.2. 800 kPa, 100 ms

In the current experiment, 800 kPa, 100 ms was tried as well, results from which are shown in Figure 5.10 below. Here, US was on for a relatively shorter duration of 8 minutes in Trial 1, and 5 minutes in Trial 2. However, the paired

stimulation measurement (orange) is performed at 2 minutes in both cases (schematic shown in Figure 5.9 below), and a suppressory effect is seen. Also, 2.1 mA no longer excited the nerve after Trial 1 as it did earlier, therefore a higher current of 2.5 mA was used in Trial 2.



Figure 5.9: Schematic for Trial 1 (left panel) and Trial 2 (right panel) corresponding to Figure 5.10

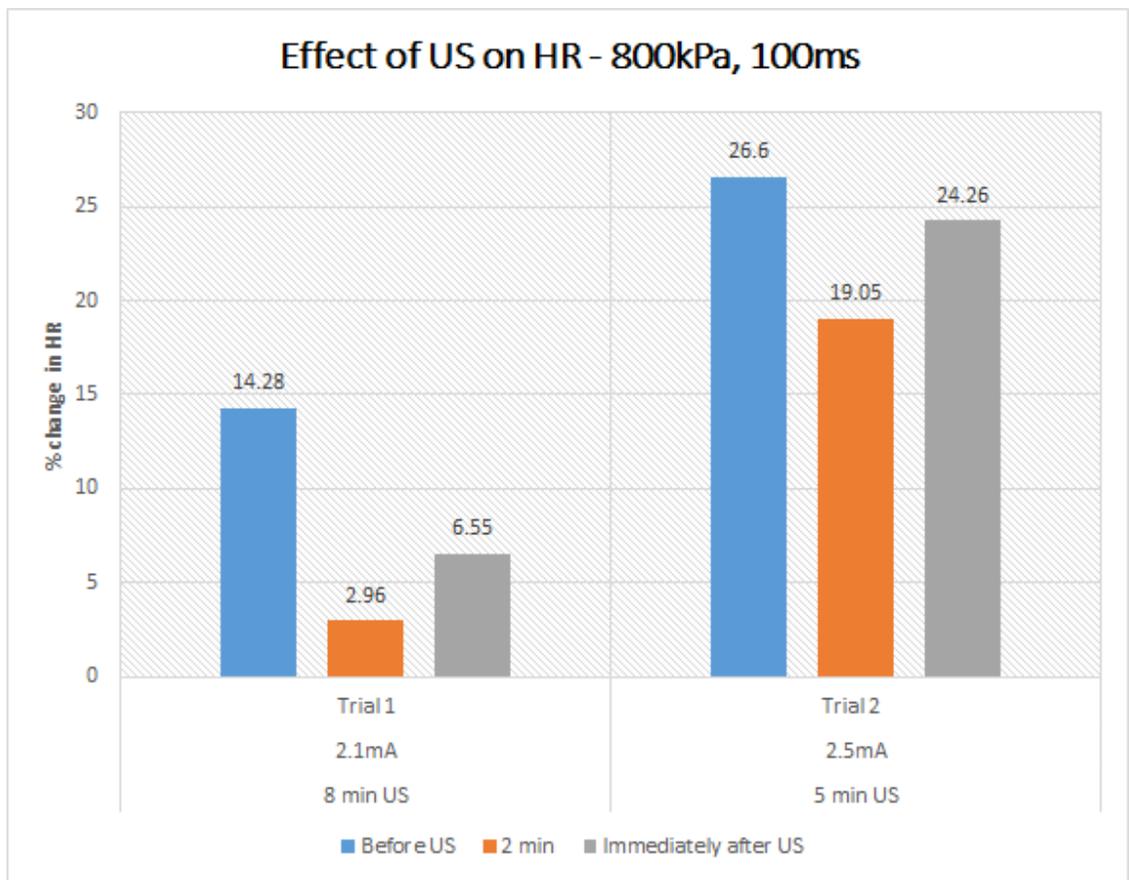


Figure 5.10: Effect of US: 800 kPa, 100 ms on HR excitation (GP 7)

In Figure 5.10, again, the initial measurements (blue) are of electrical stimulation of ~15-30 seconds, since rapid increases were seen. The subsequent measurements show the maximum change in HR, not limiting the duration of electrical stimulation, until the HR settled and stayed stable at a particular value.

5.2.1.3. 1800 kPa, 10 ms

1800kPa, 10ms was tried as well, but this did not cause any change in HR increase, therefore the data is not shown. I hypothesize that the suppression effect of US is a function of pulse duration, and for this case 10 ms pulses were used which is a 1% duty cycle which is much lower than 50% in Section 5.2.1.1 and 10% in Section 5.2.1.2. However, I decided to try the same parameters in my next experiment to see if the results could be reproduced.

5.2.2 Animal 2

5.2.2.1 300 kPa, 500 ms

In this experiment, the protocol was revised to conduct electrical stimulation for one minute, and observe the maximum increase in heart rate in that one minute. Also, a recovery period, same as the duration of US being on, was given after US was turned off, to check if the nerve regains its ability of excitation caused by electrical stimulation (i.e. there is a 7 or 10 minute gap between the orange and grey recordings). There is no paired stimulation in this experiment, the measurements are made immediately after US has turned off. Trial 2 was

conducted immediately after trial 1, and so on. The schematic for each trial is shown in Figure 5.11 below. The results are shown in Figure 5.12 below.

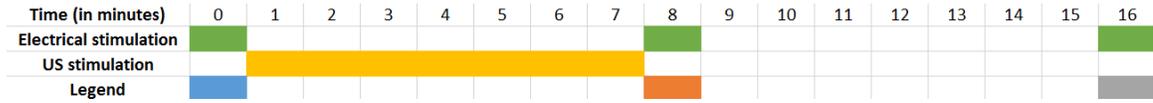


Figure 5.11: Schematic for a single trial corresponding to Figure 5.12

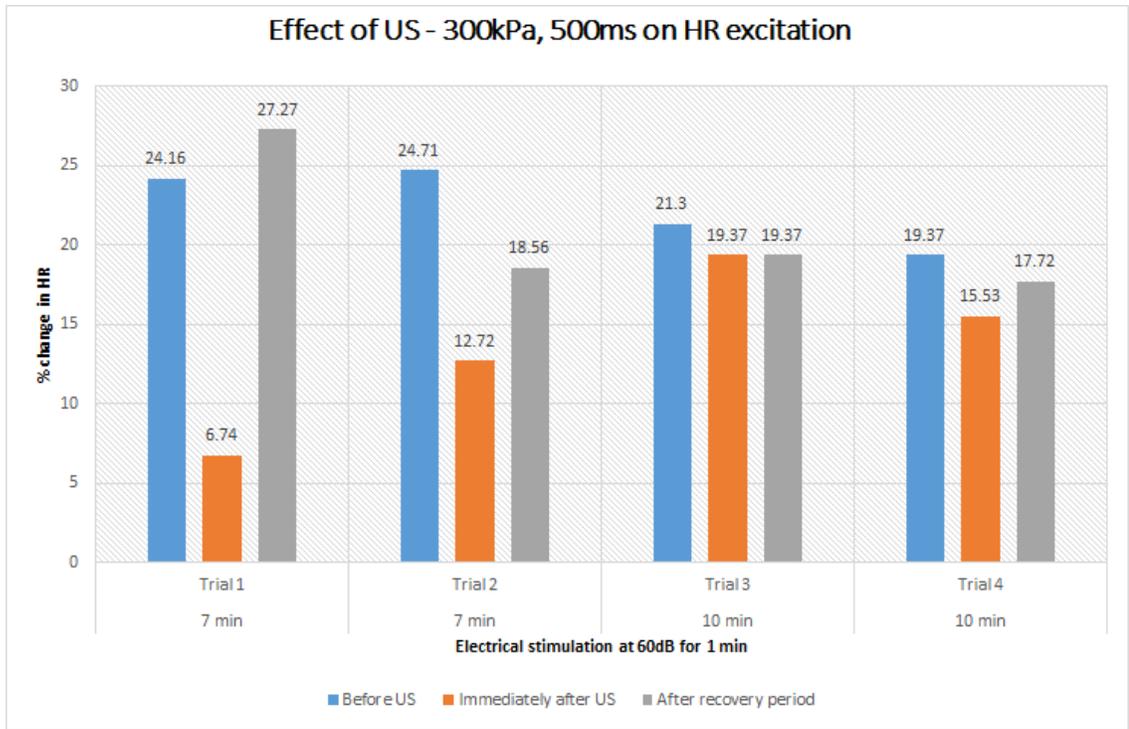


Figure 5.12: Effect of US: 300 kPa, 500 ms for 7 or 10 minutes on HR excitation (GP 8)

Suppression is seen as in Experiment 1. However, these suppression effects are no longer prominent for Trial 3 and 4. It may be that the mechanism of suppression needs some time to recover. Therefore, even though VNS can cause HR increase, it would seem that US acts through a different or partially

different mechanism than VNS does to increase the HR. So repeated US causes itself not to work as efficiently to suppress vagus nerve activity and needs more recovery time. This is merely a hypothesis at this point, and further studies are needed to investigate this result and aim to understand the underlying mechanism.

5.2.2.2 800 kPa, 100 ms

US of 800 kPa, 100 ms was performed as in Experiment 1. Here, 10 or 12.5 minutes of US stimulation was presented. Different current levels were used in each trial since the threshold of activation kept increasing. Everything else is similar to the 300 kPa, 500 ms case. The schematic for a single trial are shown in Figure 5.13 below. The results from this parameter set are shown in Figure 5.14 below.

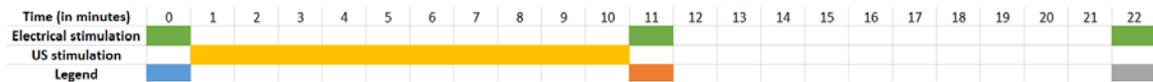


Figure 5.13: Schematic for a single trial corresponding to Figure 5.14

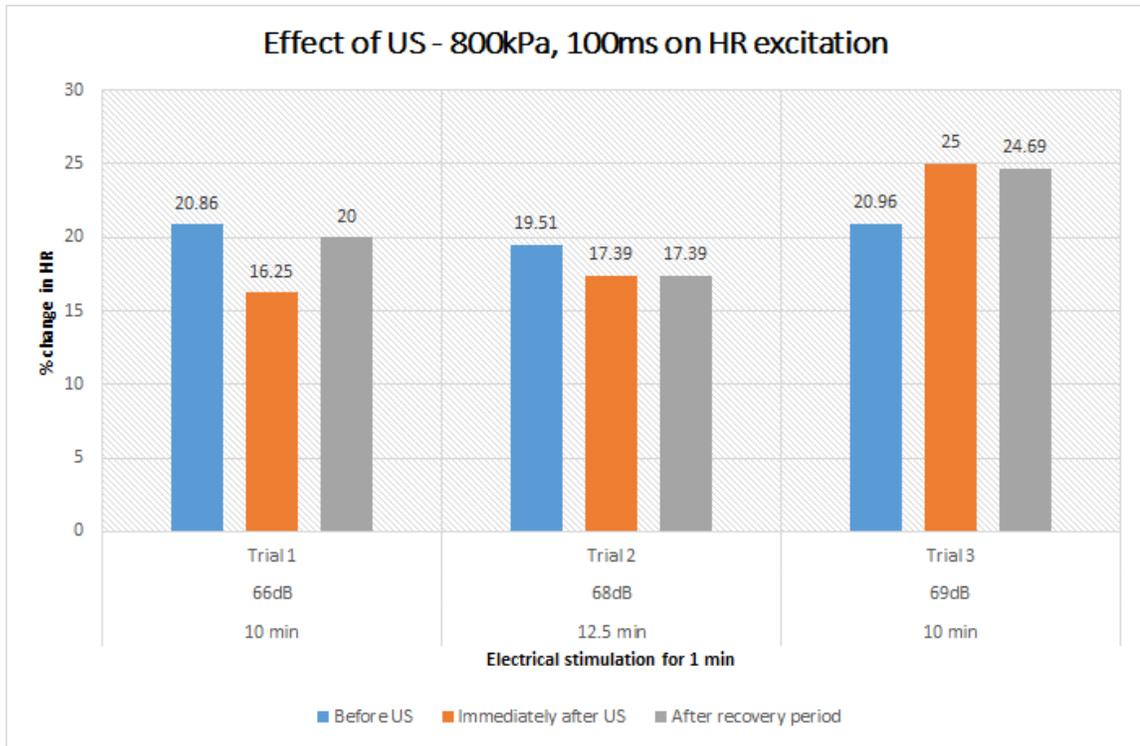


Figure 5.14: Effect of US: 800 kPa, 100 ms on HR excitation (GP 8)

In Figure 5.14, suppression and recovery is observed in Trial 1. 66 dB for electrical stimulation did not cause the same excitation in Trial 2 as in Trial 1, perhaps since the nerve needs a longer time to recover. 68 dB was used for Trial 2, and it did not cause suppression. 69 dB in Trial 3 did not cause suppression as well. This is probably due to the reason I proposed earlier - that stimulation needs to be in an optimal range of the threshold of activation for the nerve, otherwise the electrical stimulation overpowers the inhibitory effect of US.

5.2.2.3 1800 kPa, 10 ms

1800 kPa, 10 ms was attempted as well, but this did not cause any change in HR increase; therefore the data is not shown. This was consistent with

what was observed in Experiment 1, and I am more confident that the suppression effect of US is a function of pulse duration, and 1% duty cycle is too low to cause the suppression effect. Higher durations could not be tried at this pressure level since it is beyond the safety limits for this particular transducer.

5.3 Summary of Results

These experiments and data demonstrate that the right vagus nerve can be suppressed so as to temporarily limit the ability of VNS to cause a drastic increase in HR. These are proof-of-concept experiments to demonstrate that my animal setup works and can be used to further explore a wide range of parameters for suppressing vagus nerve activity in future research in the Lim Lab and to eventually understand the mechanism of US nerve suppression. Temperature can also be monitored during these experiments to assess if the mechanism is a thermal effect. When the setup for CAP recording is better developed (using neuromuscular blocks, etc.), US can also be performed to show suppression of nerve activity that corresponds to the HR effects shown in this chapter. As observed in my experiments with the vagus nerve and in other experiments in the Lim Lab with the sciatic nerve, US does not appear to directly excite nerves. Further research is needed to identify if there are any US parameters that can excite nerves and to identify an effective range of parameters for suppressing and/or enhancing vagus nerve activity, which is now possible with the setups described in this chapter and in Chapter 4.

Chapter 6: Conclusion and Future Work

My results in Chapter 5 demonstrate that US can suppress the right vagus nerve so as to temporarily limit the ability of VNS to cause a drastic increase in heart rate. This was a proof-of-concept finding to show that my animal setup works and can be used to further explore a wide range of parameters for suppressing or modulating vagus nerve activity in future research in the Lim Lab. US has many different parameters that can be varied, which include frequency of the transducer (center frequency), pressure levels, frequency of pulses, pulse duration, pulse shape, etc. Thus, there is a large number of combinations of parameters that can be evaluated for their effects on nerves. To identify an optimal center frequency, pressure and duration that can cause suppression gives a starting point so as to determine which parameters can be tested next. When more parameters have been tested by future research, trends in the results can be identified, and underlying mechanisms can be further investigated. As observed in my experiments with the vagus nerve and in other experiments in the Lim Lab with the sciatic nerve, US does not appear to directly excite nerves. Further research is needed to identify if there are any US parameters that can excite nerves and to identify an effective range of parameters for suppressing and/or enhancing vagus nerve activity.

An ideal readout would be the CAP in the left vagus nerve, and more measures need to be taken to create a better setup in this regard.

Neuromuscular blocks need to be employed to ensure that the activity observed from the recording electrodes is indeed true nerve activity, and not muscle activity artifacts. Most studies investigating the neuromodulatory effects of US have not been performed using *in vivo* preparations, and therefore do not encounter the issue of this artifact. However, *in vivo* preparations are crucial to fully understand the impact of US stimulation as well as electrical stimulation on other physiological conditions, in addition to the nerve in question, especially for assessing the clinical benefits for treatment of a disease or health disorder. The vagus nerve goes to many organs, therefore it is important to assess the effect of VNS on other bodily functions and not just the functions that we desire to be modulated. Many of the other studies which do use an *in vivo* setup do not present controls to ensure true nerve activity, which can be a roadblock for further research carried out in this field investigating mechanisms of VNS and US neuromodulation. Another avenue that can be explored is US modulation of muscle activity, which may have potential for clinical applications. If muscle activation can be blocked by US, this could potentially reduce or eliminate most common side effects of VNS. Muscle blocking can also be of benefit to reduce spasms and muscle pain.

Once US modulation of the vagus nerve has been fully characterized, this work can be translated to the mouse model. The effective US parameters can be tested on mice to observe the effect of US stimulation on the cytokines of the

blood, which control for inflammation. This is the goal of the DARPA UltrRx project. Overall, the main goal of this Master's thesis was to develop a new *in vivo* setup in guinea pigs to be able to electrically activate and record from the vagus nerve while being able to apply ultrasound stimulation to the nerve. In addition to this, I found an alternative readout which can be used to characterize US neuromodulation of vagus nerve activity. I showed proof-of-concept results which demonstrate that US can suppress vagus nerve activity, with quick recovery. I achieved my goal in which my setup can now be used by future researchers in the Lim Lab to systematically characterize a wide range of ultrasound parameters on the ability to modulate the vagus nerve invasively and eventually non-invasively for treating various health disorders, initially focusing on arthritis treatment for the DARPA UltrRx project.

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