ULTRASOUND INDUCED NEUROSTIMULATION

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DOCTOR OF PHILOSOPHY

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Abstract

Ultrasound-induced neurostimulation has recently gained increasing attention. Developments in the use of ultrasound to stimulate and modulate neural activity have raised the possibility of using ultrasound as a new investigative and therapeutic tool in brain research. Little is known about the mechanisms by which it affects neural activity or about the range of acoustic parameters and stimulation protocols that elicit responses. In this thesis, conditions are established for transcranial stimulation of the nervous system in vivo, using the mouse somatomotor response. It is reported that (1) continuous-wave stimuli are as effective as or more effective than pulsed stimuli in eliciting responses, and responses are elicited with stimulus onset rather than stimulus offset; (2) stimulation success increases as a function of both acoustic intensity and acoustic duration; (3) interactions of intensity and duration suggest that successful stimulation results from the integration of stimulus amplitude over a time interval of 50 to 150 ms; (4) the motor response elicited appears to be an all-or-nothing phenomenon, meaning stronger stimulus intensities and durations increase the probability of a motor response without affecting the duration or strength of the response; and (5) motor responses, measured by normalized EMG signals in the neck and tail regions, change significantly when sonicating rostral and caudal regions of the mouse motor cortex. Taken together our findings present good evidence for being able to target selective parts of the motor cortex with ultrasound neurostimulation in the mouse, steps that should provide encouragement for the development of new applications in larger animal models, including humans.
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Chapter 1

Introduction and History

The ability to manipulate the electrical activity in the brain is a valuable tool to map neuronal circuits and elucidate brain function. Brain stimulation has proven to be useful in clinical medicine, and current methods, which include pharmacological, genetic, electrical, optical, and magnetic techniques, have shown efficacy in the treatment of Parkinson’s disease and other neuropsychiatric diseases. These techniques have significant limitations, however, such as being invasive or lacking spatiotemporal control. The use of ultrasound for neurostimulation may be able to overcome these limitations, offering a different approach to understand brain circuitry, and thus may hold substantial promise for new diagnostic and therapeutic applications. It has been known since 1929 that ultrasound can stimulate neurological tissue; however, the ability to localize ultrasound to stimulate specific brain tissues in vivo has only recently been demonstrated.

While it could be stated that the study of ultrasound physics began with Spallanzani in 1794, who deduced that bats use ultrasound to perform echolocation (Graff 1977), it was the discovery of the piezoelectric effect that led to the practical use of ultrasound. Piezoelectricity (piezo is Greek for pressure) was first discovered in 1880 by brothers Pierre and Jacques Curie. They noted that by compressing a crystal (Rochelle salt), they could produce a voltage which could be measured. It was a year later that they confirmed the converse effect: applying a voltage to the same type of crystal caused it to compress (or expand). While a laboratory novelty at first, the
piezoelectric effect would ultimately become very useful, leading to the development of modern medical ultrasound.

Ultrasound is produced by a transducer, a device which converts one type of energy into another. An ultrasound transducer converts electrical energy into motion and vice versa. Modern transducers are made from piezoceramics, and the most common piezoceramic used is called lead zirconate titante (PZT). PZT is a material that consists of dipoles and exhibits the piezoelectric effect, which is shown in Figure 1.1. When an electrical field is applied, the crystal experiences a mechanical deformation and either shrinks or expands, depending on the direction of polarization. If an alternating current is applied to each face of the crystal, it will vibrate, or expand and contract, at the frequency of the applied voltage. This motion causes the propagation of sound waves.

While most attribute the Curies’ discovery of the piezoelectric effect as the discovery that led to modern medical ultrasound, it was not until the 1940s that ultrasound was used as a medical imaging modality. Interestingly, however, ultrasound was first investigated as a therapeutic tool, as early as the 1920s. Wood and Loomis conducted the first bioeffects study with ultrasound in 1926-27, showing that placing bacteria and red blood cells in a planar high intensity field produced destruction and rapid death. They also reported on the deaths of frogs and fish that had been sonicated for up to one minute. This work led directly to ultrasound being used in many biological and industrial applications including the sterilization of solutions and the homogenizing of immiscible fluids (Lynn 1942). In the 1930s, ultrasound was incorporated into the practice of physical therapy to treat bursitis and tendonitis in European soccer players. Later, in 1944, Lynn et al., went on to perform the first transcranial sonications to produce lesions in the brains of dogs, cats and monkeys. Based on these data, in 1955, the Fry brothers, William and Francis, performed neurosurgical procedures on 100 human patients for the treatment of hypokinetic disorders (Nyborg, 2000). While diagnostic and therapeutic ultrasound continued to progress, it was not until the early 1970s when the work of Lele (Nyborg, 2000) would show the importance of temperature and offer a thermal argument for ultrasound induced lesions. Until this seminal work, the predominant school of thought supported the notion that
Figure 1.1: (A) PZT crystal at resting state. (B) Piezoelectric effect demonstrated with PZT crystal expanding and contracting along the direction of polarization when alternating current is applied to the faces. This vibration produces the ultrasound wave which would be propagated through the water or tissue from the front face of the transducer.
ultrasound-induced lesions were formed by a mechanical mechanism (Nyborg, 2000).

The insight that ultrasound induced lesions were mediated by a thermal mechanism broadened the field of therapeutic ultrasound tremendously, resulting in patient treatments ranging from low temperature hyperthermia to high temperature ablative treatments. Along with new treatments came new ultrasound technology, such as transducer array materials and designs and new methods to guide and monitor these treatments. These advancements in ultrasound science and technology have translated to FDA-approved treatments, including the non-invasive ablation of uterine fibroids and the treatment of metastatic bone disease for the alleviation of pain, along with clinical trials using ultrasound to alleviate essential tremor, all under the guidance of MRI. Such advances are now opening the door to new non-thermal uses of therapeutic ultrasound, one of which is neurostimulation, which has been the focus of my doctoral thesis.

In this thesis, I provide a systematic evaluation of the conditions for effective, non-invasive, ultrasound-induced transcranial neurostimulation of the central nervous system using the mouse somatomotor response, and demonstrate localization of this neurostimulation technique \textit{in vivo}.

An overview of ultrasound physics, with a concentration on the theory of particle motion, wave propagation and the interaction of ultrasound with tissue is presented in Chapter 2. The chapter concludes with a section on the applications of therapeutic ultrasound, including neurostimulation.

Chapter 3 establishes conditions for transcranial stimulation of the nervous system \textit{in vivo}. Stimulation success is shown to increase as a function of both acoustic intensity and duration, and the motor response which is elicited is seen as an “all-or-none” phenomenon, meaning stronger stimulus intensities and durations increase the probability of a motor response without affecting the duration or strength of the response. The effect of anesthesia is reported, followed by the discovery that responses are elicited with the stimulus onset, rather than removal of stimulus. The frequency dependence of neurostimulation to the applied ultrasound is reported, along with the observation that continuous wave stimuli are equally or more effective than pulsed stimuli at eliciting responses.
Chapter 4 concentrates on the localization of the neurostimulation phenomenon. Sonicating over different areas of the motor cortex stimulated different areas of the body. While the contractions varied as a result of the location of the transducer in reference to the motor cortex, the success rate of the stimulation did not vary. It is also shown that the latency of the contraction varied between the two positions, but this is a function of anatomical location rather than the position of the transducer. Significantly shorter contraction latencies were seen when sonicating over the caudal region of the motor cortex as opposed to the rostral end.

Chapter 5 summarizes the thesis and proposes future directions and new areas of study based on this work.
Chapter 2

Overview of Ultrasound Physics

2.1 Particle Motion

Sound is a mechanical oscillation or vibration of particles traveling through a medium as a wave. Although there is a transfer of energy through the medium and displacement of the particles, there is no net movement of the medium itself if the medium is non-fluid (tissue). It is the wave that travels from the source, not the particles (Leighton, 2006). As can be seen in Figure 2.1, when the ultrasound is “off”, the particles have a uniform distribution (A, top row). When the ultrasound is turned “on” (B, bottom row), the transducer acts like a piston and produces zones of compression and rarefaction by moving the particles. The average particle motion in the medium is zero.

Figure 2.1 depicts the generation of a longitudinal wave; i.e., a wave in which the particle displacement is parallel to the direction of wave propagation. If the particle motion is perpendicular to the direction of wave propagation, the wave is called transverse. Both types of waves can exist in soft tissue and bone, but liquid media can only support longitudinal waves.

The amplitude of a particle’s displacement, $x(t)$, from its mean position at any time $t$ at a fixed position along the direction of propagation is given by
Figure 2.1: Schematic of ultrasound wave traveling through a medium. (A) Equilibrium state with no disturbance. (B) Formation of longitudinal waves from the piston-like movement of the transducer causing rarefaction and compression zones which results in the particles of the medium oscillating back and forth. Compressions are areas of the wave where particles are close together, and there is high pressure. Rarefactions are areas of the wave where particles are far apart, and there is low pressure.
\[ x(t) = x_0 \sin(\omega t), \quad (2.1) \]

where \( x_0 \) is the maximum displacement amplitude and the angular frequency \( \omega \) is

\[ \omega = 2\pi f. \quad (2.2) \]

The frequency of the wave, \( f \), refers to the number of oscillations per second of the particle in the medium responding to the wave passing through it. Frequency is expressed in hertz (Hz), or cycles per second.

The velocity, \( v(t) \), which is the rate of change of position of that particle, is calculated by differentiating the displacement, \( x(t) \), with respect to time:

\[ v(t) = \frac{dx(t)}{dt} = x_0 \omega \cos(\omega t). \quad (2.3) \]

The acceleration, \( a(t) \), of the particle towards its mean position may be found by differentiating the particle velocity with respect to time. The negative sign indicates that the particle is decelerating as it moves away from its mean position:

\[ a(t) = \frac{dv(t)}{dt} = -x_0 \omega^2 \sin(\omega t). \quad (2.4) \]

As shown in figure 2.2, the particle velocity leads the particle displacement by \( \frac{\pi}{2} \) and acceleration is out of phase by \( \pi \) with the displacement.
Figure 2.2: Representation of particle displacement, acceleration and velocity as a function of phase $\omega$. The velocity is zero at maximum displacement, and the displacement is zero maximum velocity, while the acceleration is out of phase with displacement by $\pi$. 
2.2 Wave Propagation

Ultrasound is defined as sound that has a frequency higher than what can be heard by humans, greater than 20 kHz (20,000 Hz), and it can be divided into two categories, airborne and liquid/solid-borne (EHC 22, 1982). Airborne ultrasound finds use in commercial products such as intrusion alarms, range finders, and dog whistles. Liquid/solid-borne ultrasound has numerous industrial applications such as cleaning, emulsification, welding, and flaw detection. Many medical applications of ultrasound for diagnosis, therapy, and surgery have arisen over the last 50-60 years (EHC 22, 1982). In my thesis, the focus is on the propagation of ultrasonic longitudinal waves, also known as compression waves, with only one frequency component. The basic parameters of a continuous wave are shown in Figure 2.3.

The period \( T \) is the time it takes for one particle, in the medium through which the wave travels, to make one complete oscillation (cycle) about its resting position:

\[
T = \frac{1}{f}. \tag{2.5}
\]

The wavelength, \( \lambda \), is the distance between two consecutive, identical positions in the pressure wave and is defined by the speed of propagation of the sound wave, \( c \) (commonly referred to as the speed of sound), divided by the frequency, \( f \):

\[
\lambda = \frac{c}{f}. \tag{2.6}
\]

The speed of sound \( c \) is primarily determined by the compressibility of the medium and for most practical cases can be considered to be independent of frequency, although a frequency dependence in tissue does exist (termed frequency dispersion). This velocity should not be confused with the particle velocity mentioned earlier. The particle velocity is the speed of individual molecules in a medium displaced by the wave into the compression and rarefaction zones. The wave velocity describes the speed of the wave traveling through the medium. It is determined by the square root
Figure 2.3: Relationship between displacement amplitude and (A) distance \((T)\) and (B) time \((\lambda)\) along the direction of propagation.
of the bulk modulus \( (K) \) divided by the density of the medium \( (\rho) \), through which the sound wave is propagating:

\[
c = \sqrt{\frac{K}{\rho}}. \tag{2.7}
\]

A highly compressible medium such as gas or soft tissue will have a lower speed of sound, as compared to a medium with low compressibility like bone, which will have a higher speed of sound. A less dense medium has a higher speed of sound than a denser medium.

Wave motion also gives rise to a fluctuating particle pressure. This acoustic pressure, \( p(t) \), is the pressure variation about the ambient or undisturbed pressure in the medium. It is equal to:

\[
p(t) = \rho cv, \tag{2.8}
\]

where the product \( \rho c \) (density x speed of sound) is called the specific acoustic impedance, and \( v \) is the particle velocity. This equation is analogous to the similar relationship between electrical voltage, current, and impedance. Acoustic pressure is expressed in units of pascals (Pa), which is equal to one newton per square meter.

Another important quantity in characterizing an ultrasonic wave is acoustic intensity, which is the acoustic energy transmitted per unit time in the direction of acoustic wave propagation per unit area normal to this direction. It is a vector quantity equal to the product of acoustic pressure (a scalar) and particle velocity (a vector), \( I = \rho cv \). However, for many practical cases in which the beam can be considered either essentially a plane or spherical wave (e.g., in the far field, described in the next paragraph, or at the focus), the instantaneous intensity at a given time and position is reasonable and approximated as
\[ I(t) = \frac{p^2(t)}{\rho c}. \]  

(2.9)

Intensity is expressed in units of watts per square meter (W/m\(^2\)), or more commonly, W/cm\(^2\). Several qualified versions of intensity are used in reporting ultrasonic exposures, including temporal-average intensity (\(I_{TA}\)), in which the instantaneous intensity at a point is temporally integrated over a cycle for continuous wave operation, or over a pulse repetition period for pulsed operation, and pulse-average intensity (\(I_{PA}\)), in which the instantaneous intensity at a point is temporally integrated over the pulse duration for pulsed operation. Further, spatial-peak or spatial average may be added to denote either the point in the field having the highest intensity, or the average of the field over a selected area at the plane of interest, e.g. the focal plane. The selected area could be equivalent to the transducer, as would be common when analyzing the near field of a planar transducer, or the full width half maximum of the field, as would be common when analyzing a focused transducer. This is schematically shown in Figure 2.4.

When an ultrasonic wave is generated by a planar transducer as depicted in Fig. 1.1, diffraction effects give rise to constructive and destructive interference patterns, resulting in Fresnel (near field) and Franhauffer (far field) zones. A schematic representation of the field of a plane, circular source can be seen in Figure 2.5. The near field is the area directly in front of the transducer. The field in this area exhibits a series of on-axis maxima and minima. For this planar, circular source, the axial location of the last maximum, \(z_{max}\), is,

\[ z_{max} = \frac{4r^2 - \lambda^2}{4\lambda} \approx \frac{r^2}{\lambda}, \]  

(2.10)

where \(r\) is the radius of the transducer, \(\lambda\) is the wavelength, and the approximate equality applies at high frequencies in which case \(\lambda^2\) is usually small compared to \(r^2\) (Wells, 1969). The plane between the Fresnel and Franhauffer zones is known as the...
Figure 2.4: Schematic representation of the intensities and spatial profiles of the sound field. Panel (A) shows two pulses with the highest intensity denoted by the temporal peak intensity (TP), the average intensity of an individual pulse is denoted by the pulse average (PA) and the average over one period of the pulse repetition is termed the temporal average (TA). For continuous wave operation PA = TA. Panel (B) is a schematic representation of the spatial profile of the ultrasound beam. On the left is an intensity field from the focal plane of a transducer. Depicted on the right is the beam profile through the center of the focus in the intensity field on the left. The point of maximum intensity is termed the spatial peak (SP), while the average across the profile, for a selected width, is termed the spatial average (SA)
natural focus of the transducer. The far field is the region beyond this plane where the sound field gradually drops to zero. This variation in the axial pressure amplitude is demonstrated in Figure 2.5, Panel B.

As an ultrasound wave propagates through a medium, it loses energy due to attenuation. Attenuation is comprised of two parts: scattering and absorption. The amplitude attenuation coefficient ($\alpha$) is defined as:
\[ \alpha = \alpha_a + \alpha_s, \]  
\hspace{1cm} (2.11)

where \( \alpha_a \) is the amplitude absorption coefficient and \( \alpha_s \) is the amplitude scattering coefficient. Both absorption and scattering depend on frequency. Therefore, the attenuation amplitude coefficient is also a function of the ultrasound frequency. The attenuation amplitude coefficient, \( \alpha \), may be expressed as a function of frequency \( (f) \) by:

\[ \alpha = a f^b, \]  
\hspace{1cm} (2.12)

where \( a \) and \( b \) are tissue- or medium- dependent constants (ICRU 1998). The exponent \( b \) has a value of close to 1 for most soft tissue and a value of 2 for water.

### 2.3 Measurement of Ultrasound Field

#### 2.3.1 Hydrophones

The hydrophone is a scientific instrument that measures the acoustic pressure in real time and transforms the pressure into a time-varying voltage. For a hydrophone to be calibrated, the pressure/voltage conversion factor must be known (Leighton, 2007). The hydrophone signal can be analyzed to obtain information such as acoustic pressures, frequency components, and pulse shapes. By scanning the hydrophone through the ultrasound beam, the spatial variation of the sound field can be determined. To provide accurate measurements, the hydrophone must be smaller than the acoustic wavelength of the signal it is measuring. This will help minimize any disturbances to the field produced by the hydrophone itself, as well as limit any spatial averaging that could occur. Spatial averaging can result because the hydrophone signal is proportional to the pressure from the sound wave spatially averaged over the entire active element of the hydrophone. For example, if the hydrophone was larger than the
acoustic focus it was trying to measure, then the hydrophone would not only sense the maximum pressure at the focus, but it would also sense lower pressures outside the focus. Therefore, a pressure maximum can only be obtained if the maximum acoustic pressure is sustained over the entire active element (Leighton, 2007).

There are several standard guidelines regarding spatial averaging and hydrophone size. The International Electrotechnical Commission states that the following criterion may be used as a guide for the maximum effective radius of a hydrophone active element, $a_{\text{max}}$ (IEC, 2007):

$$a_{\text{max}} = \frac{\lambda}{8r} \sqrt{h^2 + r^2},$$

where $h$ is the distance between the transducer and the hydrophone, and $r$ is radius of the transducer, which will minimize errors due to spatial averaging.

There are two commonly used types of hydrophones: needle and membrane (Figure 2.6). Needle hydrophones have a piezopolymer (e.g., polyvinylidene fluoride, PVDF) or piezoceramic sensitive element affixed at the end of a shaft, which is usually 5-10 cm long. The diameter of the shaft usually is made slightly larger than the size of the active element at the tip. A membrane hydrophone is made by stretching PVDF over a frame. Electrodes are evaporated on each side of the membrane and the region of overlap of these leads is the sensitive area (Leighton, 2007).

Needle hydrophones have the advantage of being able to be used in confined spaces and even in vivo. However, the frequency response of needle hydrophones is typically less uniform than that of membrane hydrophones, and their temporal stability can be less reliable. Therefore, membrane hydrophones are often used as a reference measurement device. Once the ultrasound field of a transducer has been characterized and calibrated with a hydrophone, the spatial interactions of the tissue and the sound field can be determined.
Figure 2.6: Examples of hydrophones. The top instrument is a needle hydrophone and the bottom is a membrane hydrophone.
2.4 Ultrasound Tissue Interactions

2.4.1 Thermal Interactions

Diagnostic ultrasound is designed to minimize the interaction of the sound field with the tissue and thus limit the bio-effects. Therapeutic ultrasound, on the other hand, depends on the tissue and sound field interaction to produce the desired effects (Dalecki, 2004). Current ultrasound therapies can be classified into two groups: thermal and nonthermal. In thermal treatments, in which the ultrasound energy is absorbed by the tissue and converted to heat, the goal is to raise the tissue to a final temperature, or reach a thermal dose. In hyperthermia treatments the temperature of tissue is raised to 43-50°C for 30-60 minutes in combination with chemo or radiation therapy. This slight rise in temperature stops cell division and increases blood flow, both of which are thought to aid in cancer treatments (Wu, 2006). In ablative treatments such as high intensity focused ultrasound (HIFU), the goal is to raise the temperature in the tissue to levels at which cell death and coagulative necrosis occur, destroying the tissue. The temperature at the focus is raised above 56°C and maintained for only a few seconds. The rapid deposition of energy produces increases in peak temperature that are unaffected by perfusion. The threshold for cell death depends not only on the type of tissue but also on the duration at which the tissue is held at a certain temperature. The tradeoff between temperature and duration is known as the thermal dose. It was first reported by Sapareto and Dewey (Sapareto, 1984) as an indicator of cell death. Maintaining any tissue at 43°C for 240 minutes will result in necrosis. This time of 240 minutes, denoted \( t_{43} \), is often used as a reference thermal dose. An isodose equation used to define the necrosis resulting from using higher temperatures and shorter exposure times is:

\[
t_{43} = \int_{0}^{t_{\text{end}}} R^{T(t)-43°C} dt,
\]

where \( t_{\text{end}} \) is the sonication duration, \( R = 2 \) when temperature \( T > 43°C \) and \( R = 4 \) when \( T \leq 43°C \). The increase in temperature is based on several factors including...
tissue parameters such as absorption, perfusion, thermal conductivity and diffusivity, and ultrasound parameters such as frequency, sonication intensity and duration. Researchers have applied these principles to deliver ablative treatments in the brain, to treat tumors (McDannold, 2010), creating localized thermal ablations in order to alleviate pain (Jeanmonod, 2012) and treat essential tremors (Elias, 2012). Along with thermal mechanisms, ultrasound also produces nonthermal effects such as cavitation and radiation force.

2.4.2 Non-Thermal Interactions

2.4.2.1 Cavitation

Cavitation refers to the formation, oscillation, growth, and possible collapse of gas-containing cavities. It can occur when a sound wave interacts with a bubble. In this interaction the bubble, which has formed from cavitation nuclei, will oscillate in size around its equilibrium radius in response to compressions and rarefractions caused by the ultrasound wave. When an ultrasound wave travels through tissue it can cause microscopic cavitation nuclei called motes (Leighton, 1994) to form into these bubbles. There are two types of cavitation: noninertial and intertial, referred to, in earlier publications, as stable and transient cavitation, respectively.

Noninertial Cavitation: Noninertial cavitation occurs when the bubble goes through several cycles of growth, expanding under periods of low acoustic pressure and shrinking under periods of high acoustic pressure, never exceeding twice its equilibrium radius. The amplitude of oscillation is highest at the resonant frequency of the bubble and depends mainly on bubble size (Dalecki, 2004). For example, a bubble 3.9 $\mu$m in diameter at 2 MHz is said to be of resonance size (Church, 2008). The bubble loses energy through three mechanisms: viscous damping from the viscosity of the surrounding fluid, radiation damping from the acoustic wave emitted by the bubble, and thermal damping from the transfer of heat out of the bubble into the surrounding liquid (Church, 2008). Under low acoustic pressures, the bubble will oscillate in a linear fashion and the radius versus time plot will be
a simple sinusoid centered on its equilibrium radius. This also causes neighboring liquid particles to vibrate with increased amplitude (Wells, 1969). Regions in tissue with bubble formation have increased attenuation from both increased scattering and absorption. Various bio-effects can be experienced with stable bubble oscillations. The production of heat, microstreaming of the fluid near the bubble, and increased localized shear stresses (Dalecki, 2004) can all occur and subsequently lead to cell damage and increased cell membrane and blood vessel permeability (ter Harr, 1981).

**Inertial Cavitation:** When a bubble is exposed to sufficiently high acoustic pressures, it can expand, doubling its radius, and begin nonlinear oscillations, in which the bubble does not exhibit the same sinusoidal relationship between radius and time as in the noninertial case. Rectified diffusion explains how the bubble grows slowly over several cycles, allowing a net flow of gasses into the bubble. At low ultrasound pressure amplitudes, once the resonant size of the bubble is reached, the pressure inside the bubble counteracts the motion of the fluid outside the bubble and stable oscillations occur (noninertial cavitation). As the pressure amplitudes from the ultrasound rises, the bubble contracts from a radius maximum to a minimum, the motion of the surrounding fluid will obtain such a large momentum that the rising pressure inside the bubble will not withstand the liquid and the surrounding liquid will rush in. The bubble radius will become very small and the bubble will collapse. Since this collapse is dictated by the inertia of the liquid, it is called the inertial collapse. It is this collapse that distinguishes the two types of cavitation: noninertial and inertial. The collapse of the bubble is accompanied by very high temperatures and pressures. It has been estimated that the temperature and pressure inside the bubble reach several thousand K (Leighton, 1994) and several thousand atmospheres (Church, 2008), respectively. Bubble collapse is also associated with the production of shock waves and free radicals, both of which can damage biological tissue. Shock waves can cause severe damage in tissues with high absorption and free radicals can cause double stranded DNA breaks. The tissue where the inertial cavitation takes place is disintegrated and a fluid filled void is left. The thermally significant threshold for cavitation has been reported to be about 5 MPa/MHz in vivo as demonstrated by Hynynen et
al. in the dog thigh (Hynynen, 1991).

2.4.2.2 Radiation Force

A traveling wave possesses momentum; the static force that results from the transfer of that momentum to an object (either reflector or absorber) is termed acoustic radiation force. The pressure on a perfectly absorbing object or target is frequency independent and equal to the energy density, while the pressure on a perfectly reflecting target is twice the energy density. This is because the reversal of field momentum for a reflector requires additional force to be imparted to the target due to conservation of momentum (Wells, 1969). The temporal-average energy density is \( \frac{I}{c} \), where \( I \) is the temporal-average intensity and \( c \) is the speed of sound. Thus, the total radiation force, \( F \), associated with this pressure on a target is

\[ F = \frac{IA}{c} D, \tag{2.15} \]

where \( A \) is the area of the target and \( D \) is a dimensionless factor dependent on the type of target and the direction in which the force produced is measured. (Nyborg, 2006). The product term \( IA \) is acoustic power, \( P \), and, assuming plane wave propagation and normal incidence on a perfectly absorbing target, \( D = 1 \), in which case,

\[ F = \frac{P}{c}. \tag{2.16} \]

2.4.2.3 Acoustic Streaming

As described in the previous section, as a traveling acoustic wave propagates through a medium it exerts a force. If the medium is a fluid, the momentum from the wave is transferred to the fluid and flow of the medium is observed in the direction of the sound propagation, with return flow in the outer regions of the beam. This steady current in the fluid is termed acoustic streaming (Leighton, 1994, Nyborg 2006). There are two types of acoustic streaming; the first is bulk streaming which can be
observed in the body where volumes of fluids are found such as the urine in the bladder, amniotic fluid, and in blood in vessels. Velocity gradients associated with this flow are very high resulting in significant shear stresses which can cause damage in biological materials such as the endothelial layer in blood vessels (Hill, 1986). The second type, microstreaming, is a boundary associated streaming, which results from oscillations of inhomogeneities in the sound field relative to the fluid, or excitation of a small portion of a membrane. Microstreaming forms as eddies of flow adjacent to an oscillating source (Baker, 2001), usually a bubble but vibrating wires have also been shown to cause microstreaming (Nyborg, 2006). The \textit{in vitro} effects of microstreaming has been observed from cells in suspension, and include hemolysis of red blood cells (release of their hemoglobin), release of proteins from \textit{E. coli} bacteria, and DNA fragmentation (Rooney, 1972). While microstreaming is difficult to observe \textit{in vivo}, the shear stresses which occur could distort or tear cellular membranes (Church, 2008).

### 2.5 Applications of Therapeutic Ultrasound

While there are a wide range of applications for therapeutic ultrasound, the most promising aspects of the field of ablative therapies are for treatments of tumors in the prostate (Madersbacher, 1993), breast (Hynynen, 2001), uterine fibroids (Shen, 2009), brain (McDannold, 2010), liver (Kennedy, 2001), and eye (Coleman, 1984) as well as essential tremors cessation (Elias, 2012) and pain alleviation (Jeanmonod, D, 2012). Promising non-ablative treatments include targeted drug delivery (Hynynen, 2008), focal blood brain barrier disruption (Vykhodtseva, 2009), and gene therapy (Hynynen, 2008). Neurostimulation (King, 2013), which is the primary focus is this doctoral work, is yet another area of therapeutic ultrasound. What follows is a systematic study of the parameters involved in ultrasound induced neurostimulation.
3.1 Introduction

Direct modulation of neural activity has substantial relevance for the treatment of neurological and psychiatric disease, as well as for the scientific investigation of the neural mechanisms underlying sensory, motor and cognitive functions of the human brain. For example, deep brain stimulation has been employed effectively for the treatment of advanced Parkinson’s disease and is being applied with increasing frequency to other clinical conditions as well (Lyons, 2011). Transcranial magnetic stimulation (TMS), which has the advantage of being completely noninvasive, is widely used as a laboratory tool among neuroscientists and psychologists and is being employed clinically in treatment for acute depression (Fitzgerald, 2011). Nevertheless, existing techniques for human neuromodulation have substantial drawbacks. The medical risks associated with invasive procedures such as deep brain stimulation are obvious. Furthermore, the applicability of TMS is limited due to poor spatial resolution and its inability to focus on deep brain structures. While optogenetic approaches to neuromodulation have been famously successful in the laboratory, application to human subjects will require doubly invasive procedures: genetic modification to insert light-sensitive proteins into target neural structures, and invasive methods for introducing
light to the target structures (LaLumiere, 2010).

For these reasons, increased attention has recently been devoted to the possible use of focused ultrasound as a tool for noninvasive neuromodulation. Early indications of ultrasound effects on excitable tissue date back many decades. For example, Harvey (1929) studied \textit{ex vivo} frog and turtle ventricular muscle and reported seeing contractions stimulated by high intensity continuous wave ultrasound. Later, more direct evidence of the effects of ultrasound on the nervous system (Wulff, 1951; Fry, 1958) revealed that neural activity could be suppressed and that the effects of ultrasound on neural tissue could be reversible. Eventually, the possibility of excitatory effects became more fully apparent. For example, Gavrilov \textit{et al.} (1996, 2012) used ultrasound to induce a variety of somatosensory sensations in human subjects, including tactile, thermal and pain sensations, which presumably required excitatory activation at the neural level. More recently Khraiche (2008) demonstrated that ultrasound could increase the excitability of neurons in culture, while Tyler \textit{et al.} (2008) demonstrated short-latency, excitatory responses to ultrasound stimulation in a rodent slice preparation. Using low-frequency ultrasound stimulation in the intact mouse, Tufail \textit{et al.} (2010) provided compelling evidence of transcranial ultrasound activation \textit{in vivo}. They elicited reliable motor responses at the behavioral level and demonstrated activation of motor cortex at the neural level, with no apparent damage to the brain after repeated ultrasound stimulation experiments. Following that, Yoo and colleagues (2010) used functional magnetic resonance imaging to show via blood oxygen level dependent contrast signals that ultrasound stimulation could activate as well as suppress activity in the rabbit cortex.

These laboratory advances provide hope for using ultrasound for human brain stimulation. Although the human skull presents a formidable barrier to ultrasound stimuli, ultrasound is already in use for making focused, transcranial ablations in certain clinical applications (Martin 2009; McDannold 2010). In principle, focal neuromodulation should be achievable using the same large phased arrays that make focal ablation possible (Clement 2005, Pernot 2003, Marsac 2012).

While recent laboratory advances are encouraging, much still remains to be learned about how the ultrasound might best be applied to induce neuromodulation efficiently.
We undertook the present study to identify the key parameters required for successful transcranial stimulation in the mouse, using short-latency muscle contractions, measured by electromyography (EMG), as our metric for stimulation success. We confirm Tufail’s (2010) findings that noninvasive neurostimulation can be accomplished reliably using ultrasound frequencies at around 500 kHz, and we establish regimes for ultrasound intensities, durations, and anesthesia levels necessary for successful stimulation. In particular, we have characterized some key properties of how the effectiveness of the ultrasound stimulation depends on an interaction between acoustic intensity and duration including the fact that the motor response appears to be an all-or-none phenomenon. Somewhat surprisingly in a direct comparison we find that short-duration continuous-wave ultrasound is most effective for eliciting motor responses, despite the emphasis of recent studies on pulsed stimulation (Tufail 2010, Yoo 2011). Our findings offer new insights into the mechanisms underlying noninvasive ultrasound stimulation of the intact brain.

3.2 Materials and Methods

3.2.1 Animal Preparation

A total of thirty-three CBL-7 mice were employed in this study. All experiments were performed according to protocols approved by the Stanford Institutional Animal Care and Use Committee, and every precaution was taken to minimize stress and the number of animals used. At the beginning of each experiment, the a mouse was anesthetized in an induction box using 2% isoflurane (Butler Animal Health Supply, Dublin OH) delivered with oxygen at a rate of 2L/min. Once anesthetized, the animal was removed from the induction chamber and fitted with a facemask through which 2% isoflurane and oxygen were continuously delivered at 2L/min. Ophthalmic ointment was applied to keep the eyes moist, and depilatory cream was applied to the head or other body surfaces where the ultrasound transducer would be placed. After hair removal, the animal was placed, unrestrained, in a cylindrical acrylic holder so that the limbs and the tail were suspended as illustrated in Figure 3.1. Using a 23
gauge catheter for each lead, two EMG electrodes were inserted approximately 3-5 mm apart, into the triceps area of one limb in order to record the bioelectric potential difference across the muscle tissue. Each EMG electrode consisted of a 28 gauge enamel coated copper wire with the end stripped by approximately two millimeters, and bent into the shape of a small hook. After insertion of each EMG lead, the associated catheter was pulled out of the tissue, leaving the lead implanted. An EMG common ground wire was attached to the tail of the animal.

With the EMG leads connected and the animal in the holder, the anesthetic was reduced to 0.1% isoflurane, 2L/min oxygen, the level at which most of the experiments were performed. This was achieved using a vaporizer (Fluotec MKIII, VetEquip Inc, Pleasanton CA) specifically calibrated for this low level of anesthesia. Once set, fine adjustments were made in response to observations of the animals physiological state including respiration rate and responsiveness to the ultrasound stimulation. At the lowest anesthetic levels, the animals were semi-alert but calm. To avoid unnecessary stimulation, we performed the experiments in a quiet room with no activity in the animals line of sight. Throughout the experiments, the animals tail rested on a heating pad to assist maintenance of a normal body temperature. The average of the few spot checks of the rectal temperature that were recorded was 35°C which was within a few degrees of normal. In all respects (eating, drinking, locomotion), the animals appeared normal after recovering from anesthesia.

3.2.2 Ultrasound Stimulation

Ultrasound stimuli were generated by a single element, planar transducer (V301, Olympus, Waltham, MA) with a diameter of 25.4 mm, a center frequency of 500 kHz, and a bandwidth from 340-650 kHz at -6dB. A plastic coupling column was fitted to the outside of the transducer. The total length of the coupling column was approximately 27.8 mm from the face of the transducer to the end of the column, with an upper internal diameter of 25.4 mm. At 21.5 mm from the face of the transducer there is a step in the column and the inner diameter changes to 12.7 mm as illustrated in Figure 3.1. The end of the coupling column was sealed with 2 mil
Figure 3.1: Diagram of experimental setup. The mouse was placed in an acrylic holder in the prone position but was otherwise unrestrained. The transducer was fixed to a three axis positioning system and coupled to the mouse using ultrasound gel. The end of the transducer coupling column sat approximately 2 mm from the animals head. EMG leads were placed in the forelimb in the approximate area of the triceps muscle. The common ground lead was attached to the tail.
polyethylene and filled with deionized, degassed water. Ultrasound gel (Aquasonic, Parker Laboratories, Fairfield, NJ) coupled the end of the transducer column to the head (or other body surface) of the animal. Ultrasound gel was also placed on the surface of the transducer to prevent bubbles from adhering during assembly of the setup. The transducer setup was fixed to a three-axis positioning system that allowed free movement of the transducer over the head of the animal. For all experiments the transducer was positioned approximately 2 mm from the surface of the animals head.

All experiments were conducted using continuous wave ultrasound sonications at the center frequency of the transducer (500 kHz), unless otherwise noted. The driving signal for the transducer was a sine wave produced by a function generator (Agilent 33220A, Santa Clara, CA) and amplified with a 50 dB RF amplifier (Amplifier Research, 150A100B, Bothell WA). In the experiments where we compared the effectiveness of pulsed ultrasound with continuous wave ultrasound, a second function generator (Agilent 33250A, Santa Clara, CA) was deployed to control the pulse repetition frequency and the number of cycles per pulse. In our experiments, ultrasound intensities ranged from 0.01-79.02 W/cm$^2$ (0.03-1.11 MPa). Intensities were calculated using the peak to peak voltages, as measured with a calibrated hydrophone (HNR 0500, Onda, Sunnyvale CA) placed in the X-Y coordinates at the position of maximum pressure and in the Z direction approximately 2 mm from the end of the coupling column using an acoustic instrument measuring systems (AIMS) scanning tank system (model AST503, Onda, Sunnyvale, CA). Peak to peak voltages were first converted to pressure based on calibration data provided by the hydrophone manufacturer. The pressure was then squared and divided by the product of the density of the medium taken to be 1028 kg/m$^3$ and the speed of sound in the medium taken to be 1515 m/s (Tufail 2010) to give intensity. Sonication durations varied from 20 - 480 ms.

### 3.2.3 Data Aquisition

The function generators were controlled by a computer running custom software written in Matlab (Mathworks, Natick, MA). The software allowed the user to specify
ultrasound stimulation parameters for a given experiment, including sonication durations, amplitudes, pulse repetition frequencies, and the option to change between continuous wave and pulsed sonications. The specified sonication conditions were then generated in a pseudo-random sequence, with a minimum interval between sonications of 5 seconds. The desired indication of a physiological response to sonication was a collective contraction, or “twitch” of the musculature (generally involving all four limbs) as described by Tufail et al. (2010). At the light anesthesia levels used in these experiments, collective muscle contractions occasionally occurred spontaneously and could be confused with sonication-evoked twitches. To ameliorate this difficulty, we monitored EMG activity continuously in real time, and delivered sonication stimuli only during moments of quiescence in muscle activity. Average activity was measured over a window of 250 ms immediately before sonication, and if it exceeded a manually adjusted threshold, the software would wait one second before looking again to see whether the spontaneous muscle noise was below the specified threshold. Only when the spontaneous activity was below this threshold would a sonication ensue.

3.2.4 Electromyography

The EMG leads from the animal were attached to a differential amplifier with a gain of 1000x (WPIU, DAM 50, Sarasota FL), and the signals were bandpass filtered from 300 Hz to 3 kHz. The animal was grounded by a lead from the differential amplifier that was placed on the tail. The output signal from the differential amplifier was then fed to a data acquisition device (Labjack U3, LabjackCorp., Lakewood, CO) that was connected to the computer. EMG activity was digitized at 1 kHz and stored to disk along with the sync signals from the function generators via the same data acquisition device. Prior to analysis, the stored EMG signal was rectified about its mean and further smoothed by convolving it with a symmetrically truncated Gaussian kernel of overall width 40 ms and full width at half maximum 23 ms.
3.2.5 Analysis

In the course of acquiring data from many experiments, we developed a number of heuristics and metrics to help automate the detection of ultrasound evoked muscle activity. The biggest challenge in formulating these measures was to find a way to differentiate ultrasound evoked responses from spontaneous activity, as mentioned above (Data Acquisition).

The first heuristic was to define a contraction as having occurred when the filtered, rectified signal reached three standard deviations above the average noise level for at least 100 ms. To estimate the noise level we averaged the rectified EMG signal activity during a 50 ms window directly before the onset of the ultrasound stimulus. We could reasonably assume that spontaneous activity just before the stimulus would be low because of our use of online monitoring to delay sonication when activity was too high (see Data Acquisition). Using this procedure we calculated the contraction duration to be the amount of time between rising above and falling below this three standard deviation threshold. Between these two threshold crossings, the integrated area under the contraction curve was used as a measure of the strength of the contraction.

The second heuristic was to define the contraction latency as the time from the onset of the sonication, as measured from the onset of the sync signal, to the point where the EMG signal rose above the three standard deviation noise threshold. Figure 3.2 illustrates these metrics in relation to a sample EMG signal.

A summary metric, the “success rate”, was defined as the ratio of the number of contractions identified using these rules, divided by the total number of sonications attempted, expressed as a percentage. The success rate metric clearly included occasional spontaneous twitches that were unrelated to the sonication stimulus, which we attempted to account for posthoc (see below, and Results).

A primary goal of this study was to identify effective parameters for ultrasound stimulation of the brain, but the full parameter space was potentially very large. Initially, therefore, we explored specific regions of the space to gain familiarity with the key variables for successful stimulation, before mapping the space more extensively. The following sections describe the types of experiments we performed.
Figure 3.2: (A) An example sync pulse 80 ms in duration that governed the duration of the sonication. (B) The red trace, resulting from a 16.8 W/cm² sonication, is a sample EMG signal following amplification (gain 1000x, high pass filter 300 Hz, low pass filter 1 kHz). The blue trace is the rectified, smoothed EMG signal. A muscle contraction was defined to begin when the filtered signal rose above the third standard deviation of the noise level, represented in the figure by the horizontal dashed line, for at least 100 ms. The time from the beginning of the ultrasound pulse to the beginning of the contraction was defined as the latency.
3.2.6 Effect of the Skull

Although in larger animals the skull is known to present a significant barrier to the propagation of ultrasound, the same does not appear to be true in the mouse as phase correction has been shown to be unnecessary to produce focused lesions in the brain through the intact skull (Hynynen, 2006). To ascertain the effect the mouse skull would have on our ultrasound field, we performed hydrophone scans with a calibrated hydrophone (Onda, Sunnyvale, CA) in a degassed water tank. An *ex vivo* mouse skull was placed at the end of the transducer coupling column and a 20 mm x 20 mm X-Y hydrophone scan with step size 0.2 mm was performed in a rectangular grid. The hydrophone was placed as close to the skull as possible, about 1-2 mm away from the inner surface. Figure 3.3 A and B show the results of the hydrophone scans with and without the mouse skull. Both scans resulted in peak amplitudes of 0.12 MPa demonstrating no measurable loss of signal due to the skull. We also observed no shift in the beam location. Therefore, we have chosen to ignore the effect of the mouse skull in our calculations of ultrasound intensity. Figure 3.3 C shows the beam plot of our ultrasound field with the coupling column as used in our experiments. The plot is a 20 mm x 100 mm scan through the center of the transducer along the z axis. The zero point on the z axis is as about 1mm away from the membrane at the end of the coupling column which would place the surface of the brain at approximately 3 mm on the same axis. The field would encompass the basal ganglia and the motor cortex in the mouse brain.

3.2.7 Anesthesia

To examine the effects of different levels of anesthesia on neurostimulation efficacy, EMG signals from eleven animals were recorded following sonications at an acoustic intensity of 16.8 W/cm² for a duration of 80ms, parameters that elicited reliable responses in preliminary experiments. Responsiveness at each anesthesia level was examined in blocks, each consisting of 20 sonications. Each anesthesia level was visited at least twice for each animal. As described above (Animal Preparation), the animals were initially prepped using 2% isoflurane at 2L/min, which kept them fully sedated.
Figure 3.3: Hydrophone scans of the ultrasound field of the transducer used in the experiments. Panel A shows the scan of the ultrasound field with no skull present. Panel B shows the scan with an \textit{ex vivo} mouse skull placed in the field at the end of the transducer coupling column. We detected no shift in the ultrasound focus and no drop in the peak ultrasound signal. Based on this we were able to ignore the effect of the mouse skull in our calculations of ultrasound intensity. Panel C shows the beam plot of our ultrasound field with the coupling column as used in our experiments. The plot is a 20 mm x 100 mm scan through the center of the transducer along the z axis. The zero point on the z axis is about 1 mm away from the membrane of the coupling column.
during implantation of the EMG leads and during the setup of the experiment. The isoflurane was then turned down to 0.02% and the mouse was allowed to acclimatize until some spontaneous movements of the limbs were observed, which indicated that a light anesthesia level had been achieved. At this point, the experimental protocol began. We measured ultrasound stimulation efficacy at three different anesthesia levels: 0.02, 0.1, and 0.5% isoflurane, in the order 0.5, 0.02, 0.5, and 0.1%. To permit equilibration of anesthesia between blocks, we waited 10 minutes after each increase in isoflurane concentration and 15 minutes after each decrease before sonicating and recording the EMG signals.

3.2.8 Coupling Control

Mice can hear in the ultrasonic range, though not at the carrier frequency used for these experiments (500 kHz). Nevertheless, to be certain that the behavioral responses we observed were not a “startle” response to auditory stimuli, we removed the coupling gel from the 2 mm space between the transducer and the head while holding all other conditions constant. Specifically, three animals were sonicated at 16.8 W/cm² for 80 ms for a total of forty times under each condition: coupled, uncoupled and recoupled. First, sonication stimuli were delivered and the resulting EMG signals were recorded and analyzed using our standard experimental setup described before. Next the coupling gel was dislodged without moving the transducer or the animal by sliding a piece of paper between the animals head and the end of the column, leaving a visible air gap between the column and the head. After further sonications in the uncoupled state, the gel was reintroduced to the space between the head and the transducer using a syringe, while making sure no air was trapped in between. The same stimulation protocol was then repeated. As a further control, sonication stimuli were applied (with coupling gel) to skin over the upper neck, in close proximity to the brain stem and upper spinal cord.
3.2.9 Acoustic Intensity

To explore the effect of acoustic intensity on success rate, we performed sonications using a range of intensities that spanned a broad range of success rates (Table 3.1) while keeping the sonication duration fixed at 80 ms. Over seven hundred sonications per condition spread over 15 animals were performed while the intensities were varied according to a pseudo-random schedule. There was a minimum of 30 sonications per animal for each condition. The use of randomized schedules controlled for possible system non-stationarities, such as varying anesthesia levels or gradual changes in physiological responsiveness of the mouse. The resulting latencies, muscle contraction durations, and contraction strengths were computed from the recorded EMG signals.

The primary difficulty in establishing the acoustic intensity threshold for effective stimulation arose from spontaneous twitches that occurred during our measurement interval, as indicated above. Estimates of stimulation success rate, as well as quantitative measures of response latency, duration, and contraction intensity, could be skewed by these spontaneous twitches. We therefore took several steps to correct for the effects of spontaneous twitches. First, our preliminary observations indicated that muscle contractions typically occurred 68 - 80 ms following successful sonications. The frequency of contractions after 200 - 300 ms was typically indistinguishable from the spontaneous rate. When detecting sonication induced twitches, therefore, we ignored responses that occurred more than 300 ms following sonication onset.

Second, we corrected our estimates of latency, duration and contraction intensity using measurements of false positive responses during a 300 ms interval following sham sonications, which were interleaved with real sonications (Table 3.1, intensity 0 W/cm²). Table 3.1 presents the raw success rates and mean latencies that we measured during the 300 ms analysis interval, as well as “adjusted” success rates and latencies obtained by subtracting the estimated effect of the spontaneous twitches. In essence, we subtracted the observed distribution of spontaneous twitches over the 300 ms interval from the observed distribution of successes at each sonication condition. Arithmetically, this reduces to the following formula for adjusted mean latency:
\[
\frac{(L_t \times S_t) - (L_s \times S_s)}{S_t - S_s},
\]

where \(L_t\) is the mean latency of responses detected for a specific sonication condition, \(S_t\) is the total number of responses (successes) detected for that condition, \(L_s\) is the mean latency of spontaneous responses observed during sham sonication, and \(S_s\) is the total number of responses observed during sham sonication. This metric, like any ratio metric, is unreliable if the denominator is near 0 (when the observed rate is roughly equal to the spontaneous rate), and Table 3.1 therefore reports “NA” for adjusted mean latency in the sham condition itself as well as for the two lowest sonication intensities at which the number of responses increased minimally, if at all, over the spontaneous rate.

Finally, our quantitative criteria failed to reject a small number of spontaneous contractions that grew very gradually in amplitude over several tens of ms. We therefore discarded by hand twenty contractions where it was evident posthoc that the response had in fact begun before the leading edge of the sync pulse.

### 3.2.10 Sonication Duration

As with acoustic intensity, we explored the effect of sonication duration by performing sonications over a range of durations that spanned a broad range of success rates (Table 3.2). In this case we held intensity fixed at 4.2 W/cm² and performed a total of just under seven hundred sonications per condition in randomized order on 14 animals. There was a minimum of 29 sonications per animals at each condition. Again, we computed the resulting latencies, contraction durations, and contraction force from the recorded EMG signals, ignoring any responses that occurred more than 300 ms after sonication onset. As described above for acoustic intensity, estimated success rates and contraction durations were adjusted for spontaneous contraction rates measured during sham sonications. We discarded by hand thirteen contractions where it was evident, posthoc, that the response had begun before the leading edge of the sync pulse.
3.2.11 Onset vs. Offset Response

Although the exact mechanism by which ultrasound affects neural activity remains unknown, it is at least plausible that physiological responses could occur following the onset of stimulation, the offset, or both (Menz 2010). To address this question, we used a particular combination of ultrasound intensity (4.2W/cm$^2$) and duration (320 ms) that had elicited high success rates in previous experiments. Because the response latencies were typically on the order of 60-70 ms (Tables 3.1 and 3.2), trials with 320 ms duration sonications provided an opportunity to disambiguate responses associated with the onset and offset of sonication. EMG signals from six hundred sixty-three sonications across fourteen animals were obtained for this analysis. In our previous analyses we only counted the first contraction event that occurred after the initiation of an ultrasound pulse, assuming additional later contractions to be spontaneous events. However, if both sonication onset and offset could elicit responses, we might have expected to see pairs of twitches occur in succession, or responses occurring only to the offsets. To examine these possibilities we measured responses during two epochs during and following each 320 ms sonication. First, the latency and the success rate were examined from the sonication onset until the end of the sonication, 320 ms later.

Second, the signal was analyzed from the sonication offset (in this case 320ms after the onset) until 640 ms after sonication initiation. Within each window at most only one contraction event was scored. The point was to see whether the distribution within the second window revealed any kind of response peak after the offset similar to the peak seen after the onset in the first window. If so, this might suggest that the offset also played an important role in triggering a contraction.

3.2.12 Ultrasound Frequency

To examine the effect of the fundamental ultrasound frequency on success rate, three animals were anesthetized and placed in the holder as previously described, using a minimum of 16 sonications per animal for each condition. The transducer was driven at several frequencies within its bandwidth: 250, 300, 350, 400, 450, 500, 550 and
600 kHz. Using 40,000 cycles, sonications were applied at the same three driving voltages for each frequency. The highest voltage 300 mVpp was determined by the need to avoid causing damage to the transducer. The lower two voltages 75 and 150 mVpp were chosen to span the response curves of the animals at 500 kHz. Since the transducer response was very different at each frequency, the acoustic intensity was calculated for each driving voltage and frequency from data collected with a calibrated hydrophone. These experiments were performed in a series of blocks. Within a block, sonications were performed at the same frequency but using a randomized sequence of intensities. When moving to a new block a new frequency was picked at random from the above list and a new randomized sequence of intensities was applied.

3.2.13 Continuous vs. Pulsed

To compare continuous with pulsed ultrasound, we ran randomly interleaved sequences of these two types of sonications using fixed durations of 80 ms and with varying intensities. For pulsed ultrasound, we used the same fundamental frequency of 500 kHz with a pulse repetition frequency held constant at 1.5 kHz. Each pulse consisted of 100 cycles representing a duty cycle of 30%. The resulting pulse average and temporal average intensities were calculated for each condition using a calibrated hydrophone. Each set of conditions was repeated twice for each run. There was a minimum of 40 sonications per animal for each condition tested. All combinations were chosen in a pseudo-random sequence.

3.2.14 Pulse Repetition Frequency (PRF)

Having compared the effects of pulsed ultrasound with continuous wave stimulation, we next examined the effect of varying the PRF. Each pulse was kept at 100 cycles of 500 kHz, with 120 pulses in the pulse train, but we varied the pulse repetition frequency over a range between 11-3000 Hz. Sonications were performed on five animals at three spatial peak pulse average intensities (4.2, 16.8, and 29.9 W/cm²). For each animal, the PRFs and intensities were varied in random order, and each set of conditions was repeated three times using a minimum of 10 sonications per animal.
at each condition.

### 3.2.15 Full Parametric Space for Continuous Wave Stimulation

To examine the full span of continuous wave ultrasound parameters, sonications were performed on two animals. As usual, parameters were varied in a random order. For each animal, the sonication intensities were 0, 0.26, 1.1, 2.4, 4.2, 9.4, 12.8 and 16.8 W/cm$^2$, and the sonication durations were 0, 20, 40, 80, 160, 240, 320 and 480 ms. Every combination of intensity and duration was tested, with ten repetitions per condition. The success rate was calculated for each condition.

### 3.3 Results

#### 3.3.1 Anesthesia

Figure 3.4 illustrates the effect of isoflurane anesthesia on the twitch response to sonication. The panels depict sample runs of four sonications at anesthesia levels of 0.5% (A), 0.1% (B), and 0.02% (C) isoflurane. The black traces show the ultrasound sync pulses, while the red traces illustrate typical EMG responses to the sonication stimuli; spontaneous contractions are identified with black arrows. The illustrated anesthesia levels corresponded to stimulation success rates (see Methods) of 10%, 98.7%, and 94.6%, respectively, averaged across all experiments. As is evident from the data, both spontaneous and sonication-evoked contractions were rare at 0.5% isoflurane, but became more frequent as the anesthesia level was lowered. Apart from these anesthesia experiments the data reported in this paper were obtained at 0.1% isoflurane, since this provided robust sonication-evoked contractions with relatively few spontaneous contractions.
Figure 3.4: Sample EMG traces taken from experimental runs that show the effect of anesthesia levels on stimulation. Arrows represent spontaneous contractions, i.e., events not elicited by the ultrasound. Stimulus intensity set to 16.8 W/cm$^2$ for all sonications, while the oxygen level in all experiments was set to a delivery rate of 2L/min, and the sonication duration was kept constant at 80 ms. The black spikes represent the sync pulses recorded from the ultrasound signal generator and the red traces represent the raw EMG signals. Panel A shows a typical run of sonications performed at an anesthesia level of 0.5% isoflurane in which all effects, including spontaneous contractions, were eliminated. Panel B shows a typical run of sonications performed at an anesthesia level of 0.1%, the level at which most experiments were conducted. This level enabled the effects of ultrasound stimulation to be observed without too much contamination from spontaneous movements. Panel C represents a typical run of sonications performed at 0.02% isoflurane, a level of anesthesia we found too low to be able to conduct experiments reliably.
3.3.2 Coupling

As a control, the transducer was uncoupled and recoupled from the heads of three animals by removing and replacing the coupling gel while holding the position of the transducer constant with respect to the head. Uncoupling reduced the success rate from 97%, to 11.5%, which closely matches the spontaneous contraction rate of 13% observed during sham sonications (see below). After recoupling the transducer, the success rate returned to 95%. Thus we can rule out suprasonic auditory responses as a cause of sonication-evoked contractions. As a second control, sonications were performed with the transducer positioned on the cervical region of the neck near the brainstem. In these experiments the success rate was 16%, again indistinguishable from the sham control rate. We therefore infer that the effect results from ultrasound stimulation of the brain itself, and not from stimulation of superficial peripheral nerves or spinal circuits.

3.3.3 Effect of Acoustic Intensity

Figure 3.5 illustrates individual intensity-response functions obtained from 15 mice. Success rates increased systematically with sonication intensity, with a 50% success rate being reached, on average, at around 2.5 W/cm$^2$. For most mice the intensity-response curves are sigmoidal, with a threshold near 0.25 W/cm$^2$ and saturation beginning around 4.25 W/cm$^2$. For these animals, complete saturation would likely have occurred at higher intensities. A few animals were less sensitive to the sonication (right-most curves in Fig. 3.5). These animals produced curves with thresholds near 1 W/cm$^2$ and little to no saturation at the highest sonication intensities.

Figure 3.6 depicts frequency histograms for twitch latency, duration and strength for all six stimulus intensities across the same 15 mice. As expected from the curves in Figure 3.5, the frequency histograms become more densely populated as success rate improves at higher stimulus intensities (top to bottom). There is little difference in the frequency of twitches between the sham condition (0 W/cm$^2$) and the two lowest intensity levels (left column, top three panels). At 1.1 W/cm$^2$, however, the frequency of twitches exceeds the baseline expectation, with an average response latency of
Figure 3.5: The relationship of the success rate versus intensity for 15 mice. Each red line represents the data from one animal. The solid black line represents the average of those animals. The dashed horizontal line marks the 50% success rate which occurs on average at approximately 2.5 W/cm². All sonications were continuous wave with durations of 80 ms performed at 500 kHz.
around 70ms. For higher sonication intensities, additional responses accumulated in this early epoch, shortening the mean latency to approximately 55-60 ms (see also Table 3.1).

As discussed in Methods, the measured success rates at all sonication intensities inevitably contained some false positive (spontaneous) responses. Such responses occurred in 15.4% of the sham trials with zero acoustic intensity level (top row of histograms, Figure 3.6). Assuming this rate to have been roughly constant regardless of the sonication condition, we calculated “adjusted” mean latencies and success rates (Tables 3.1 & 3.2) by subtracting the weighted effects of the spontaneous responses from the raw data (see Methods). For sonication intensities that elicited a reliable increase in twitch responses above the spontaneous rate, the adjusted mean latencies were 55-72 ms, with a slight trend toward shorter latencies at higher intensities.

Apart from this possible decrease in latency, the histograms in Figure 3.6 reveal little or no evidence for changes in twitch durations (middle column) and strengths (right column) as sonication intensity increases. Thus, although the probability of a twitch response increased substantially with sonication intensity, the duration and strength of the twitches were unaffected.

### 3.3.4 Effect of Sonication Duration

Figure 3.7 shows that stimulation success rate increased systematically with sonication duration for a constant intensity of 4.2 W/cm$^2$. Success rates of 50% occurred, on average, for a sonication duration of 29 ms. In contrast to the intensity-response functions in Figure 3.5, we did not observe a hard threshold at the lower end of the range of durations employed (i.e. the curves for most mice do not exhibit an inflection at the lower end), although shorter duration stimuli may have revealed a threshold. The duration-response functions typically saturated at a success rate of just over 80% for durations exceeding 80 ms. These features of the data are also evident in the numbers in Table 3.2. As in Figure 3.6, the histograms in Figure 3.8 become more densely populated as success rate increases with sonication duration (top to bottom
Figure 3.6: Frequency histograms of latency, contraction duration and the contraction force. Sonication intensity increases down each column while sonication duration was kept constant at 80 ms. The data represent 15 mice and a total of over seven hundred sonications per condition. For the two lowest intensities there was little evidence of stimulation above the sham condition (top row), but at 1.1 W/cm$^2$, we saw an appreciable build up in activity centered upon an unadjusted latency of around 60 ms.
## Success Rate, Latencies and Contraction Duration of Evoked Responses

Sonication Duration: 80 ms

<table>
<thead>
<tr>
<th>Sonication Intensity (W/cm²)</th>
<th>Success Rate (%)</th>
<th>Adjusted Success Rate (%)</th>
<th>Mean Latency (ms)</th>
<th>Latency Standard Deviation (ms)</th>
<th>Adjusted Mean Latency (ms)</th>
<th>Mean/Median Contraction Duration (ms)</th>
<th>Contraction Duration 10th/90th percentiles (ms)</th>
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<tr>
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<td>15.4</td>
<td>0</td>
<td>96</td>
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<td>NA</td>
<td>622/444</td>
<td>154/1103</td>
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<td>87</td>
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<td>609/500</td>
<td>130/1208</td>
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<td>170</td>
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<td>134/1170</td>
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<td>28.7</td>
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<td>50</td>
<td>72</td>
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<td>153/1235</td>
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<td>68</td>
<td>37</td>
<td>59</td>
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<td>200/1159</td>
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<tr>
<td>16.8</td>
<td>79.8</td>
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<td>63</td>
<td>33</td>
<td>55.0</td>
<td>754/547</td>
<td>220/1463</td>
</tr>
</tbody>
</table>

Table 3.1: Success rate, latencies and muscle contraction durations and the adjusted weighted versions for the ultrasound evoked responses in 15 mice. While increasing the acoustic intensity (left column), the sonication duration was held fixed at 80 ms. Adjusted success rates and mean latencies were calculated by subtracting a constant spontaneous rate of contractions based on rates observed for the zero intensity sonications as described in Methods. For the first two rows the adjusted mean latencies have no meaningful values because all of the events were likely to have been spontaneous.
in the figure), and the sham condition reveals a low but constant stream of spontaneous contractions (13.4 \% false positive rate across sham conditions in all 14 mice). Again, we assumed the frequency of spontaneous contractions to be constant for the other sonication durations as well, and computed adjusted mean latencies (Methods) which appear in Table 3.2. But like the histograms for acoustic intensity (Fig. 3.6), the shape of the distributions for contraction duration and strength appear not to change in any systematic way with sonication duration.

### 3.3.5 Sonication Onset and Offset

In principle, neural activation might occur at sonication onset, offset, or both. We tested these possibilities by examining more closely the responses to 320 ms duration sonications. Figure 3.9A shows the distribution of responses detected following onset of the 320 ms sonications (intensity = 4.2 W/cm$^2$), while Figure 3.9B depicts the distribution of responses detected following offset of the same sonications. Clearly, responses occurred in response to the onset of the ultrasonic stimulus with latencies of 30-100 ms. Running the detection algorithm afresh beginning at sonication offset revealed no responses above the spontaneous contraction rate. These data demonstrate that sonication onset is the critical stimulus for neurostimulation in our experiments, although it is also apparent that the ultrasonic stimulus needs to be integrated over a finite duration to become effective (Figure 3.7).

### 3.3.6 Ultrasound Frequency

We examined the effect of sonication frequency by testing three intensity levels at eight frequencies from 250 kHz to 600 kHz that spanned the approximate bandwidth of our transducer. As illustrated in Figure 3.10, the primary effect was that substantially higher intensities were required to achieve the same success rates as the frequency increased. We were unable to test even higher intensities because of possible damage to the transducer. Our frequency response data follow the same trend as those of Tufail et al. (2010) who observed larger responses (normalized EMG amplitudes) for lower fundamental frequencies.
Figure 3.7: The relationship of the success rate versus sonication duration for 14 mice. Each red line represents the data from one of the fourteen animals. The black line represents the average of those animals. The dashed horizontal line marks the 50% success rate which occurred on average at approximately 29 ms. All sonications were continuous wave, performed at an intensity of 4.2 W/cm$^2$ at 500 kHz.
Figure 3.8: Frequency histograms of latencies, contraction durations, and the contraction strengths. Sonication duration increases down each column while sonication intensity was kept constant at 4.2 W/cm$^2$. The data represent 14 mice and just fewer than seven hundred sonications per condition. Above 20 ms there appears to be little qualitative change in the histograms with increasing sonication durations, suggesting that the most important feature at this intensity was what happened in the first 40 ms or so.
Table 3.2: Success rate, latencies and muscle contraction durations and the adjusted weighted versions for the ultrasound evoked responses in 14 mice. While increasing the sonication duration (left column), the acoustic intensity was held fixed at 4.2 W/cm$^2$. Adjusted success rates and mean latencies were calculated by subtracting a constant spontaneous rate of contractions based on rates observed for the zero duration sonications, as described in Methods. The first row of the adjusted mean latencies has no meaningful values because all of the events were likely to have been spontaneous.

<table>
<thead>
<tr>
<th>Sonication Duration (ms)</th>
<th>Success Rate (%)</th>
<th>Adjusted Success Rate (%)</th>
<th>Mean Latency (ms)</th>
<th>Latency Standard Deviation (ms)</th>
<th>Adjusted Mean Latency (ms)</th>
<th>Mean/Median Contraction Duration (ms)</th>
<th>Contraction Duration 10th/90th percentiles (ms)</th>
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<td>13.4</td>
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<td>20</td>
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<td>28.1</td>
<td>77</td>
<td>53</td>
<td>64</td>
<td>802 / 585</td>
<td>176 / 1559</td>
</tr>
<tr>
<td>40</td>
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<td>47.6</td>
<td>68</td>
<td>43</td>
<td>58</td>
<td>682 / 527</td>
<td>179 / 1360</td>
</tr>
<tr>
<td>80</td>
<td>77.4</td>
<td>64.0</td>
<td>67</td>
<td>37</td>
<td>60</td>
<td>789 / 555</td>
<td>206 / 1559</td>
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<tr>
<td>160</td>
<td>83.4</td>
<td>70.0</td>
<td>72</td>
<td>41</td>
<td>66</td>
<td>771 / 583</td>
<td>220 / 1590</td>
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<tr>
<td>320</td>
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<td>68.8</td>
<td>72</td>
<td>41</td>
<td>66</td>
<td>831 / 599</td>
<td>250 / 1700</td>
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</tbody>
</table>
Figure 3.9: Responses to sonication onset versus offset. The histogram in panel A was collected from 14 animals representing the distribution of latencies measured for 531 sonications within the first 320 ms after sonication. The histogram in panel B is based on the same sonications as A, but the analysis window began at the end of the sonication (320 ms) and extended until an additional 320 ms later. The bulk of responses occur in A with a latency peak at an average of 68.6 ms. No such peak is seen in B, demonstrating that neurostimulation with ultrasound is tied to the onset of sonications rather than the offset. All sonications used continuous wave ultrasound with intensity 4.2 W/cm$^2$ at 500 kHz.
Figure 3.10: Continuous wave sonications of varying intensity and 40,000 cycles were used to examine the success rates at different fundamental frequencies. These plots were obtained in three animals in blocks of sonications where the frequency was held constant in each block but intensities were presented in pseudo-random order. All conditions were tested in each animal. Each curve represents the average of all sonications performed at that frequency. Error bars represent the standard error of the mean. The same three driving voltages were used at all frequencies and the intensity at each condition was measured using a calibrated hydrophone. The intensities required to achieve comparable success rates increase rapidly with increasing frequency.
3.3.7 Continuous Wave vs. Pulsed Ultrasound

All experiments described thus far employed continuous wave ultrasound, but because earlier studies of in vivo neuromodulation (Tufail, 2010, 2011; Yoo, 2010) used pulsed ultrasound, we directly compared the efficacy of continuous wave with pulsed ultrasound. Figure 3.11 reveals that when calculating the spatial peak, pulse average intensity (panel A), continuous wave ultrasound was more effective at intensities above about 10 W/cm$^2$, but when calculating the spatial peak, temporal average intensity (panel B) there was no significant difference until about 30 W/cm$^2$.

3.3.8 Pulse Repetition Frequency

The results from the pulse repetition frequency experiment are shown in Figure 3.12. There was little response at PRFs less than about 100 Hz, which corresponded to a duty cycle of 2%. Above this point, the success rate increased with pulse repetition rate, up until 3000 Hz, the maximum repetition rate tested, which represented a 60% duty cycle.

3.3.9 Sonication Parameter Matrix

Figures 3.5 and 3.7 illustrate success rates for two “slices” through the full intensity-duration space of sonication stimuli, but they do not provided any insight concerning possible interactions between intensity and duration. Therefore, in two animals we sampled an 8x8 matrix of intensities and durations (including sham conditions), accumulating 10 sonications for each condition in each animal. Figures 3.13 A and B depict the full matrix of data for each animal color coded for success rate.

Note that instead of plotting the data against intensity, we chose for one of the axes the normalized amplitude of the ultrasound stimulus. The two physical quantities are directly related in that amplitude is proportional to the ultrasound pressure whereas intensity is proportional the square of the pressure. The motivation for this change of variable was that amplitude provided a better fit than intensity in our attempts to model the data (Figure 3.13 panels C and D). Using logistic regression, we modeled the data obtained from the two mice in several different ways (see Appendix 1.) The
Figure 3.11: Success rates for continuous wave and pulsed wave sonications for various amplitudes in six mice. The pulsed ultrasound parameters consisted of a pulse repetition frequency held constant at 1.5 kHz, while using 100 cycles per pulse. The spatial peak pulse average (A) and the spatial peak temporal average (B) intensity were calculated at each data point and used to compare the efficacy of pulsed and continuous wave ultrasound. Continuous wave sonications resulted in a higher success rate than pulsed ultrasound for the same intensity levels when comparing the pulse averages. The same held true at the highest intensity when comparing the temporal average. Asterisks denote points where the continuous wave data are significantly different (p < 0.05) from the pulsed data using the two tailed t-test. Error bars represent the standard error of the mean.
Figure 3.12: The effect of modulating the PRF of the driving signal for pulsed sonifications compared in 5 animals. Using 100 cycles per pulse with a pulse train of 120 pulses, the success rate was measured at different PRFs. The pulse repetition frequency (duty cycle) did not seem to affect the success rate until the PRF reached approximately 100 Hz, corresponding to a duty cycle of 2%, at which point the success rate rose with the increasing PRF up to 3000 Hz, which represented a 60% duty cycle. Error bars represent the standard error of the mean.
model that gave the best fits (model 2) included an interaction term based on the product of amplitude and duration, which proved to be the most important factor by far in determining the success rate. In contrast, a model consisting only of terms representing amplitude and duration independently (model 1) was unable to provide a good fit and even in model 2 these independent terms made little contribution. In fact, the coefficients ($\beta_1$ and $\beta_2$ – see Appendix Table) of the independent terms in model 2 were all slightly negative, while the coefficients for the interaction term were large and positive. ($\beta_3 \sim 36$ for mouse I, $\sim 20$ for mouse II). Another of the models (model 3) employed an interaction term based on intensity and duration, but the resulting higher deviance values compared with model 2 show this was not as successful in fitting the data.

The nature of the interaction between amplitude and duration can be seen in the lines of constant success plotted in Figure 3.13 panels E and F. These reveal in particular how amplitude and duration can trade off against one another so that as stimulus amplitude is reduced, success rates can be maintained in some cases by extending the sonication duration. For mouse 1, the effect of extending sonication duration is substantial for intervals up to 50-100 ms. For mouse 2, the period of effective temporal integration is somewhat longer, extending up to 100-150 ms. Thus the key variable governing successful neurostimulation appears to be the integral of amplitude over a time interval of 50-150 ms.

3.4 Discussion

Ultrasound stimulation provides a promising new approach for noninvasive modulation of brain activity, with numerous potential applications in the treatment of neurological and psychiatric disease (for recent reviews, see Jagannathan, 2009, Bystritsky, 2011). Critical to realizing this promise is identification of ultrasound stimulation parameters and protocols that are most effective for neural stimulation in vivo. We undertook the current study to gather needed parametric information, using quantitative measurement of motor responses in the mouse to assess the efficacy of stimulation (Tufail, 2010, 2011). In addition, our data provide new constraints on the possible
Figure 3.13: Three dimensional plots showing stimulation success rates versus normalized amplitude and duration. The plots in the two left panels (A, B) represent data collected from two mice, one panel per mouse. Each colored square represents the success rate from ten sonications performed on a single animal using the corresponding sonication parameters. The highest normalized amplitude value 1 corresponds to an intensity of 16.8 W/cm$^2$. The blue edges at the bottom of these plots represent areas with low stimulation success rates. The dark red areas, representing the highest success rates, are generally clustered in the far corner where both intensity and duration are at the highest. Each middle plot (C, D) represents the corresponding output of a sigmoid model fitted to the mouse data using logistic regression (model 2, appendix 1). The two panels (E, F) represent lines of constant success (%) derived from the sigmoid model plotted as a function of normalized amplitude and duration.
mechanisms of ultrasound-based stimulation of the brain.

### 3.4.1 Conditions for Effective Stimulation

We confirm the basic finding of Tufail and colleagues (2010) that low frequency ultrasound activates the mouse motor system robustly, resulting in transient, short latency contractions of the skeletal musculature (Fig. 3.4, Table 3.1). We also confirm their finding that stimulation efficacy increases for a given intensity as the ultrasound carrier frequency is lowered. In our data, the intensity required to achieve the same efficacy decreased by two orders of magnitude as the frequency went from 500 to 250 kHz. Finally, we confirm the observations of several groups that successful stimulation of the motor system depends critically on anesthesia level (Fig. 3.4). Stimulation efficacy is reduced substantially as anesthesia deepens, whether the stimulating technique is ultrasound (Tufail, 2010), electrical microstimulation (Brecht, 2004), or optogenetic (Aravanis, 2007, Kahn, 2011).

Our data reveal several new aspects of ultrasound stimulation that have not been addressed quantitatively in prior studies. First, the somatomotor “twitches” elicited by ultrasound stimulation appear to be ballistic, “all-or-none” responses. Increasing the intensity and/or strength of stimulation increased the probability of a response dramatically (Figs. 3.5 and 3.7), but the duration and strength of the twitch were not correlated with the stimulus parameters we manipulated. Thus the distributions of twitch duration and strength elicited by weaker stimuli appear simply to be scaled versions of the distributions elicited by the strongest stimulus (Figs. 3.6 and 3.8, middle and right columns). Even the spontaneous twitches (top row, Figs. 3.6 and 3.8) exhibit the same distributions of duration and strength when scaled for detection probability.

In contrast to the all-or-none motor responses that we have observed, the clinical potential of ultrasound stimulation depends on achieving a graded neural response so that the desired “dose” of neural activation can be controlled via the physical parameters of the stimulus. We are optimistic that this will be achievable. Both the somatomotor and oculomotor systems are well known to incorporate threshold
nonlinearities such that “subthreshold” electrical stimulation yields no overt motor response whereas “suprathreshold” stimulation yields an all-or-none motor response over a broad range of electrical stimulation parameters (Glimcher, 1993). However, more graded behavioral responses have been observed to electrical and optogenetic stimulation of sensory areas of the brain (Salzman, 1990; Murasugi, 1993; Huber, 2008). Thus, the all-or-none motor responses in our study probably reflect the intrinsic organization of motor circuitry, rather than a fundamental feature of ultrasound activation of neural tissue. This issue must be examined carefully in future studies, perhaps using physiological (as opposed to behavioral) metrics to monitor the response to ultrasound stimulation (Yoo, 2011).

Second, we found that continuous-wave stimulation is as effective as pulsed-burst stimulation when the stimuli are equated for spatial peak temporal average intensity (Fig. 3.11B). At the highest intensity tested, in fact, continuous-wave stimulation was significantly more effective than pulsed-burst. So far as we know this is the first published comparison of the effect of continuous-wave versus pulsed ultrasound on neuromodulation. The effectiveness of continuous-wave stimulation was somewhat surprising to us given the emphasis on pulsed-burst stimulation in prior studies of ultrasound neuromodulation. Pulsed-bursts may ultimately be advantageous for decreasing the risk of temperature build-up in the tissue (see below), but in terms of stimulation efficacy, pulsed-bursts have no inherent advantage over continuous-wave stimuli.

Interestingly, we found that success rates for pulsed-burst stimulation increased steadily up to 3 kHz, the maximum PRF tested (Fig. 3.12). This finding contrasts strikingly with the results of electrical microstimulation of neural tissue, for which behavioral responses typically plateau at pulse frequencies in the range of 300-600 Hz, a frequency band that corresponds to typical maximum firing rates of neurons. In the primate superior colliculus, for example, Stanford et al. (1996) showed that as the frequency of electrical pulses was varied between 100 and 700 Hz, eye movements exhibited a peak amplitude at around 500 Hz, eye movement velocities peaked at around 300 Hz, while movement durations peaked at around 200 Hz, thereafter showing a significant decline with increasing frequency. Electrical microstimulation
is thus often performed using pulses in the sweet spot between 200–300 Hz. In our experiments there appeared to be no such sweet spot for pulsed ultrasound. Such qualitatively different phenomenology suggests that the underlying mechanism for ultrasound-induced neurostimulation is fundamentally different from that of electrical stimulation.

Finally, we explored the shape of the stimulus-response functions for both the intensity and duration of ultrasonic stimuli. The efficacy of ultrasound stimulation increased as a function of both parameters (Figs. 3.5 and 3.7) over the ranges we tested. Although the precise results varied from animal to animal, 50% success rates occurred on average for intensities of about 2.5 W/cm$^2$ and durations of about 50 msec. The “matrix” analysis of Figure 13 shows that these parameters directly interact: lower intensities are more effective if the duration of the stimulus is increased, and vice versa. Over intervals of 50-150 ms, therefore, stimulation efficacy appears to depend more on the integral of stimulus intensity over time than on either variable alone.

Because the overall efficacy of ultrasound stimulation depends so dramatically on the carrier frequency (Fig. 3.10), we do not place particular emphasis on the exact values of stimulus intensity and duration that generate 50% success rates - these intensity values, and perhaps the duration values as well, will certainly change with carrier frequency. Rather, we emphasize that efficacy increases with both parameters, and that the shape of the stimulus-response functions is generally sigmoidal, exhibiting thresholds at low stimulus intensities and durations, and saturation at high intensities and durations.

Our finding, that efficacy increases with stimulus intensity comprises the most serious discrepancy between our results and those of Tufail et al. (2010), who reported that efficacy decreases with increasing stimulus intensity (their Fig. 4c, vertical axis). The discrepancy may be partly resolved by the different metrics of efficacy employed in the two studies - “success rate” in ours and “normalized EMG amplitude” in theirs. It is not possible to extract success rates from the data in Tufail et al., 2010, so we recalculated portions of our own data in terms of “normalized EMG amplitude” as illustrated for 15 animals in Figure 3.14. As is evident from the figure, normalized
EMG amplitude remains constant, or increases weakly, as stimulus intensity increases. The data in Figure 3.14 were all obtained using a carrier frequency of 500 kHz, whereas those of Tufail et al., were obtained using data collected at various frequencies ranging from 250 to 500 kHz. To see whether lowering the frequency might affect these results, we conducted a set of experiments on one animal using a carrier frequency of 250 kHz (Fig. 3.15). Weak intensities generated higher success rates at the lower carrier frequency as expected (compare the x-axes in the two panels, and recall Fig. 3.10). However, even in these data there was no evidence of a decrease in response with increasing stimulus intensity: normalized EMG amplitude remained constant or increased slightly. A full explanation of this discrepancy between the two studies must await further experiments.

3.4.2 Mechanism

The mechanism underlying ultrasound stimulation of the brain is completely unknown at present. Two broad classes of mechanism - thermal and mechanical - have been considered. Ultrasound stimulation is known to heat soft tissue (OBrien 2007; AIUM, 1988), and increases in temperature are capable of activating neurons whose membranes contain temperature-sensitive ion channels (Benham, 2003, Patapoutian, 2003). However, temperature measurements during ultrasound activation of neural tissue have revealed negligible temperature increases for pulsed-burst stimulation protocols (Tufail, 2010, Yoo, 2011). Although we did not make temperature measurements, we calculated using standard equations of ultrasound physics (OBrien, 2007; Tufail, 2010) that the temperature change generated in our experiments would have been about 0.01°C (Appendix 2), an amount that would produce negligible changes in neuron excitability (Colucci, 2009). Furthermore, the relationship we observed between stimulation efficacy and carrier frequency also argues against a thermal explanation. We achieved high success rates for all carrier frequencies tested (250-500 kHz), but the necessary intensities were two orders of magnitude higher at 500 kHz than at 250 kHz (Fig. 10). According to Wells (1975) the efficiency with which ultrasound is absorbed and thereby transformed into heat is proportional to frequency. If the mechanism
Figure 3.14: Comparison of normalized EMG amplitude with success rate as a function of increasing sonication intensity. The normalized EMG amplitude remains almost flat (the non-zero slope of the linear regression fit did not reach statistical significance as measured by a two tailed t-test $p=0.08$) while the success rate builds gradually at lower intensities and more rapidly at higher intensities. Because it increased monotonically with increasing sonication intensity, we chose success rate as our primary metric of stimulation success. Error bars represent the standard error of the mean.
Figure 3.15: Comparison of normalized EMG amplitude as a function of increasing sonication intensity. The normalized EMG amplitude remains almost flat for N=1 animal.
of neurostimulation were thermal, we would have expected success rates to increase with frequency because of increased absorption, but we saw instead a strong trend in the opposite direction. This trend cannot be explained by increased skull absorption of sound at the higher frequency, which is negligible for the thin skull of the mouse (Fig. 3). For several reasons, therefore, thermal mechanisms can be ruled out as the primary mechanism of ultrasound stimulation in the mouse motor system.

The main alternative to a thermal explanation is that ultrasound stimulation is mediated by one (or a combination) of several mechanical processes that fall into two broad categories (Khraiche, 2008; Krasovitski, 2011; Tyler, 2011, 2010): phenomena associated with cavitation, which entails the formation and collapse of gaseous cavities, or microbubbles, created by ultrasound in the absorbing medium, and non-cavitational effects which include acoustic radiation force, a phenomenon associated with the propagation of acoustic waves through an attenuating medium (Wood, 1927). The basic notion underlying this family of explanations is that mechanical deformation of neuronal cell membranes, or proteins embedded in the membranes, could affect ion channel kinetics or membrane capacitance in a manner that promotes depolarization, and thus increased action potential discharge (Johns, 2002; Tyler, 2011).

The strong dependence of our stimulation effects on carrier frequency (Fig. 10) that argues against a thermal explanation is nearly fatal for the radiation force hypothesis as well. With increasing acoustic frequencies we would expect greater tissue absorption, leading to larger displacements and higher stimulation success rates. Again, our data reveal a strong trend in the opposite direction.

The mechanism that is best supported by our data is cavitation. In contrast to the thermal and radiation force hypotheses, cavitation is consistent with inverse dependence of success rate on carrier frequency since microbubbles form more readily at lower ultrasound frequencies (Hynynen, 1991; Sponer, 1990). The exact relationship we observed did not exhibit either a linear (Hynynen, 1991) or near linear (Sponer, 1990) dependence on frequency suggested by previous cavitation studies, but revealed instead a more rapid reduction in success rate with frequency. This quantitative discrepancy may arise from nonlinearities elsewhere in the chain of events linking cavitation to neural activity. The intensity required to achieve the same efficacy at 250 kHz
was two orders of magnitude smaller than that required at 500 kHz. This difference cannot be accounted for simply by the doubled pulse width (with a fixed number of cycles), as increasing the duration of sonication at 500 kHz also did not increase efficacy appreciably beyond 80ms (Fig. 3.7). By pointing to stimulus amplitude rather than intensity, the models that provided the best fits to our data in Figure 3.13 are also consistent with a mechanism such as cavitation that depends directly on pressure (in contrast with radiation force which is proportional to intensity, or pressure squared).

Cavitation is a complex phenomenon that comes in two main forms, inertial and non-inertial. Inertial cavitation requites higher energies and is known to be associated with tissue damage. The mechanical index identifies an inertial cavitation pressure threshold for diagnostic ultrasound scanners (AIUM, 2000). While the mechanical index of our system never exceeded the FDA limit of 1.9, it has been reported that acoustic cavitation of the non-inertial kind leading to stable bubble production occurs in vivo in mammalian tissue at intensities ranging from about 100 mW/cm$^2$ - 3 W/cm$^2$ (ter Harr, 1981, 1982, 1986, Santos, 2009) The intensities used in this paper are above this level and the frequencies we used are lower, leaving open the possibility that cavitation could be playing a role in neuromodulation, and while it is true that ter Harrs first observation point for cavitation is at one minute, Farny et al.(2009) observe cavitation occurring immediately. In summary, our primary point is that cavitation is the one of the three major candidate mechanisms that is qualitatively consistent with a number of key features of our data.

3.5 Summary

While it has been known for decades that ultrasound can modulate activity within the central nervous system, systematic evaluation of the conditions for effective neurostimulation has been lacking. Our study provides such an analysis. By using acoustic stimulation sequences in which the parameters of interest are randomly and systematically varied, we have been able to identify some of the most important regimes for eliciting neurostimulation events.
Nevertheless, for ultrasound neurostimulation to become a useful tool in neuroscience, further work is required to localize ultrasound stimulation to specific target areas of the brain. Thus far all of the motor responses that we have observed have been bilateral (typically involving all four limbs), implying bilateral activation of motor cortex. An important goal for future experiments is to demonstrate the ability to evoke lateralized responses, though this may prove difficult in the mouse model because of the small size of the animals head compared to the size of focused ultrasound fields. Larger animal models may help alleviate this problem. In the rabbit, for example, Yoo et al. (2011) have observed lateralized fMRI activation of motor cortex using a focused transducer.

Higher carrier frequencies and focused transducers should also provide greater stimulation resolution although higher frequencies may require higher intensities and hence exacerbate the problems in transmitting ultrasound across the skull with sufficient power. Until recently the problem of transmitting ultrasound energy across the human skull has presented the biggest obstacle to achieving therapeutic ultrasound treatments in the brain. However, using multiple element phased array transducers, Clement (2005), Pernot (2003) and Marsac (2012) have shown it is possible to reconstruct a tightly focused region of ultrasound energy through the skull by applying appropriate amplitude and phase corrections. In light of this, it may be that using intensities and durations comparable to those employed in our mouse model can be scaled up to larger animals, perhaps including humans. If so, this study will help to lay the foundation for the next steps towards making in vivo ultrasound neurostimulation an exciting and promising new technology.

### 3.6 Appendix 1

The model used in the matrix plots (Figure 13 panels C and D) consists of a sigmoidal relationship based on the logistic function. The exact equation is:

\[
\text{probability of response} = \frac{1}{1 + e^{z}},
\]

(3.2)
where the exponent $z$ can take different forms depending on which factors we include. In the simplest case (model 1) using only normalized stimulus amplitudes and durations as inputs we have:

$$z = \beta_0 + \beta_1.\text{amp} + \beta_2.\text{dur}. \quad (3.3)$$

The $\beta$ coefficients, which we determined using logistic regression, represent the strength of the contribution the various factors make within the logistic equation. We compared this model in its ability to fit the data obtained from two mice with another (model 2) that also included the interaction term $\text{amplitude} \times \text{duration}$:

$$z = \beta_0 + \beta_1.\text{amp} + \beta_2.\text{dur} + \beta_3.\text{dur}. \quad (3.4)$$

The $\beta$ coefficients and the deviance of the two models are shown in Appendix Table 3.1. The results show that the interaction term substantially improves the fit of the model and makes the largest contribution to its success ($\beta_3$, in rows 2 and 5.) We also tried models using normalized stimulus intensity (model 3) instead of normalized stimulus amplitude (where intensity $\alpha \text{amplitude}^2$) but these were less successful in fitting the data.

### 3.7 Appendix 2

We can estimate the maximum likely temperature change $\Delta T$ (Tufail, 2010; OBrien, 2007) in brain tissue from,

$$\Delta T = \frac{Q\Delta t}{C_v}, \quad (3.5)$$

where $Q$ is the rate at which heat is produced, $\Delta t$ s the pulse exposure time (0.08 s), and $C_v$ is the heat capacity per unit volume for brain tissue. $C_v$ can be calculated
from

\[ C_v = C \times \rho, \quad (3.6) \]

where \( C \) is the specific heat capacity (\( \approx 3.6 \text{ J/g/K} \)) for brain tissue, and \( \rho \) is the density of the medium (1028 Kg/m\(^3\)). \( Q \) can be calculated from

\[ Q = \frac{\alpha P^2}{\rho c}, \quad (3.7) \]

where \( \rho \) is the density of the medium (1028 Kg/m\(^3\)), \( c \) is the speed of sound in the medium (1515 m/s), \( \alpha \) is the absorption coefficient of brain (\( \approx 0.03 \text{ Np/cm for 0.5 MHz} \)) and \( P \) is the pressure amplitude of the ultrasound (0.51 x 10\(^6\) Pa) which at 500 kHz corresponded to 16.8W/cm\(^2\), the highest intensity we used in obtaining the data for Table 3.1.

3.8 Appendix Table

Coefficients and deviance values for two different animals (I and II) using four different models based on logistic regression. Models 1 and 2 both include independent terms for amplitude and duration, while model 2 alone also includes the interaction term amplitude x duration. Model 3 instead includes independent terms for intensity, duration, and the interaction term intensity x duration. Model 4 was similar to models 2 and 3, but instead of employing amplitude or intensity as an input, it used instead amplitude\(^n\) where \( n \) was allowed to vary. Model 4 produced the best fits as seen from the lowest deviances for values of \( n=0.56 \) for mouse I and 0.63 for mouse II.
Table 3.3: The $\beta$ coefficients and the deviance of the two models.

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<th>$\beta_1$</th>
<th>$\beta_2$</th>
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<th>Deviance</th>
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Chapter 4

Localization of Neurostimulation

4.1 Introduction

In recent years the idea of directly modulating neural activity in brain using ultrasound has triggered considerable scientific interest. Such direct modulation if applied with sufficiently high temporal and spatial resolution could make possible new treatments for neurological and psychiatric diseases, and could provide powerful new tools for investigating mechanisms of the human brain from sensory processing to the mysteries of cognitive function. Current techniques for inducing neural modulation, which include pharmacological treatments, electrical stimulation techniques such as deep brain stimulation (DBS), and transcranial magnetic stimulation (TMS), generally have significant limitations. Pharmacological agents usually need to be applied directly to the site of interest which may require surgical intervention. Similarly, while DBS has already been employed for the effective treatment of clinical conditions including Parkinsons disease (Lyons, 2011), it also requires surgery. TMS has the advantage of being noninvasive and is being employed clinically in the treatment of acute depression (Fitzgerald, 2011), though it has relatively poor spatial resolution and is unable to focus on structures deep in the brain. This puts ultrasound neuro-modulation in a tantalizing position because, while being noninvasive, it could, with current array technology, reach deep brain structures with good spatial resolution (Pernot 2003, Clement 2005, Marsac 2012). With recent developments in the use of
MRI guided focused ultrasound (MRIgFUS) researchers have shown that it is possible to perform brain surgery in humans non-invasively using ultrasound (Martin 2009, McDannold 2010). By capitalizing on these techniques, ultrasound neuromodulation therefore has the potential to become a powerful tool for localized neurostimulation.

However, at the moment ultrasound neuromodulation has yet to be used clinically, and its limitations and capabilities remain under scientific investigation. While evidence for the stimulating effects of ultrasound on neural tissues has accumulated over many decades (e.g., Harvey 1929, Fry 1958, Gavrilov 1996, Tufail 2010, Bystritsky 2011, Yoo 2011, King 2013), little is known about how it actually works and it is only in the last few years that the possibility of using it to stimulate brain structures in living organisms has even begun to look feasible. Tufail et al. (2010) provided compelling evidence of transcranial ultrasound activation in vivo using low-frequency ultrasound stimulation in the intact mouse demonstrating activation of motor cortex with no apparent damage to the brain after repeated ultrasound stimulation experiments. More recently King et al. (2013) established effective driving parameters and conditions for achieving transcranial stimulation of the nervous system in vivo, using the mouse somatomotor response.

These developments alone have been encouraging, but for ultrasound neuromodulation to become a truly useful technique, compelling evidence for localized effects in the brain are needed. So far such localization has proved difficult to establish although there have been some hints. Using functional magnetic resonance imaging (fMRI), Yoo et al. (2011) measured blood oxygen level dependent contrast (BOLD) signals while sonicating rabbit motor cortex and showed a response that was localized to the sonicated hemisphere. To confirm that the effect was neurostimulatory they performed additional experiments while the animal was outside the scanner and obtained forepaw movements contralateral to the sonication site though no quantifiable details were provided on the magnitude of any differences between contralateral and ipsilateral activity.

In a follow up study, the same lab (Kim, 2012) used ultrasound to stimulate the abducens nerve of a rabbit. The localized effect was confirmed by the occurrence of ipsilateral eye movements while the contralateral eye remained unaffected as would
be expected by stimulating the nerves directly rather than the motor cortex. The researchers did not directly measure action potentials from the sonicated nerve and no attempt was made to quantify the effect, but the ensuing lateralized abductive eye movements were, at least, indicative of nerve stimulation.

From published maps of the topographic organization of the motor cortex in mice, distinctive areas are seen to control different muscle groups. Specifically, the areas controlling the neck and tail regions are located at the rostral and caudal regions of the motor cortex (Tennant, 2010). Based on this, we were able to position the ultrasound transducer at the two different positions (rostral and caudal) along the midline of the brain and provide evidence for a localized effect of ultrasound stimulation in the motor cortex by demonstrating robust differences in response which resulted in overt behavioral differences seen in twitches elicited in the tail and neck regions. While it was difficult to discern by eye differences in the twitches elicited in the neck muscle, measurements of the associated EMG responses in both regions revealed distinctly different patterns of responses that changed according to the position of the transducer. This rostral/caudal localization closely matched published maps of the motor cortex made using electrical stimulation (Tenant, 2010). We show how the observed patterns provide good evidence for localized activations occurring within different parts of the motor cortex. By quantifying the phenomenon we have also established relationships between the ultrasound stimulation location, success rate, peak EMG amplitude, and latency. In particular, we show that while success rates did not vary significantly while sonicating over two different areas of the motor cortex, the EMG signals associated with the resulting contractions were quantitatively different. We have also characterized how the latency of the ultrasound stimulation depends on anatomical position. Interestingly, in contrast to the marked rostral/caudal localization, we were unable to demonstrate any right/left localization effects, a consequence possibly of the small size of the mouse brain and/or the cortical connections between brain hemispheres.
4.2 Materials and Methods

4.2.1 Animal Preparation

A total of ten CBL-7 mice were employed in this study. All experiments were performed according to protocols approved by the Stanford Institutional Animal Care and Use Committee, and every precaution was taken to minimize stress and the number of animals used. To begin each experiment, a mouse was anesthetized in an induction box using 2% isoflurane (Butler Animal Health Supply, Dublin OH) delivered with oxygen at a rate of 2L/min. After the animal was completely anesthetized, it was removed from the induction chamber and fitted with a facemask through which 2% isoflurane and oxygen were continuously delivered at 2L/min. Ophthalmic ointment was applied to protect the eyes, and depilatory cream was applied to the area of the head used for the experiment. After the hair on the head was removed, the animal was placed, unrestrained, in a cylindrical acrylic holder so that the limbs and the tail were suspended as illustrated in Figure 4.1. Using a 23 gauge catheter for each lead, two EMG electrodes were placed approximately 2-3 mm apart, into the base of the tail and two more leads were placed in the neck region, in order to record the bioelectric potential difference across the muscle tissue. When performing lateralization experiments, two EMG electrodes were inserted into the triceps area of both forelimbs. The EMG electrodes consisted of a 28 gauge enamel coated copper wire with the end stripped approximately two millimeters, and bent into the shape of a small hook. The EMG lead was inserted into the muscle through the catheter and then the associated catheter was pulled out of the tissue, leaving the lead implanted. A common ground wire was attached to copper tape that was wrapped around one of the animals hind feet.

Once the EMG leads were connected and the animal was placed in the holder, the anesthetic level was reduced to 0.02% isoflurane, 2L/min oxygen, the level at which most of the experiments were performed. The anesthesia was administered through a vaporizer (Fluotec MKIII, VetEquip Inc, Pleasanton CA) specifically calibrated for this low level of anesthesia. Once set, fine adjustments were made in response to
Figure 4.1: Diagram of the experimental set up. The mouse was placed in an acrylic holder in the prone position but was otherwise unrestrained. The transducer was fixed to a three axis positioning system and coupled to the mouse using ultrasound gel. The end of the transducer focusing apparatus was located approximately 2 mm from the animals head. EMG leads were placed in the neck muscles and at the base of the tail on the dorsal side. The common ground lead was attached to the hind foot.
changes in the animals physiological state which included respiration rate, responsiveness to the ultrasound stimulation and spontaneous movement. At this low anesthetic level, the animals were sedated, but semi-alert. While there was no evidence for any noxious effect of the stimulation at the intensities used, the animals were unrestrained in the holder so they were free to crawl out. To avoid unnecessary disturbance to the animal, we performed the experiments in a quiet room with no activity in the animals line of sight. Throughout the experiments, the animals tail rested on top of a heating pad to assist maintenance of a normal body temperature. In all respects (eating, drinking, locomotion), the animals appeared normal after recovering from anesthesia.

4.2.2 Ultrasound Stimulation

The ultrasound was generated by a single element, focused transducer (V301, Olympus, Waltham, MA) with a diameter of 25.4 mm, a focal length of 31.75 mm and a center frequency of 500 kHz. A plastic coupling column was fitted to the transducer (Fig. 1). The total length of the coupling column was approximately 27.8 mm from the edge of the transducer to the end of the column, with an upper internal diameter of 25.4 mm. At 21.5 mm from the edge of the transducer there is a step in the column and the inner diameter changes to 12.7 mm as illustrated in Figure 4.1. The end of the coupling column was sealed with 2 mil polyethylene and filled with deionized, degassed water. The transducer and coupling column were assembled under water to assure no air was trapped inside. Ultrasound gel (Aquasonic, Parker Laboratories, Fairfield, NJ) was used to couple the end of the transducer column to the head of the animal. The transducer setup was fixed to a three-axis positioning system. For all experiments the transducer was positioned approximately 2 mm from the surface of the animals head which allowed free movement of the transducer over the head of the animal. Beam plots of the ultrasound field at the end of the coupling column are displayed in Figure 4.2.

All experiments were conducted using 80 ms continuous wave ultrasound sonifications with an intensity of $3 \text{ W/cm}^2$ (0.2 MPa) at the center frequency of the transducer (500 kHz), settings we had found to be particularly efficacious in previous explorations.
of the parameter space for ultrasound neurostimulation (King 2013). The driving sig-
nal for the transducer was a sine wave produced by a function generator (Agilent
33220A, Santa Clara, CA) and amplified with a 50 dB RF amplifier (Amplifier Re-
search, 150A100B, Bothell WA). Intensities were calculated using RMS voltages, as
measured with a calibrated hydrophone (HNR 0500, Onda, Sunnyvale CA) placed in
the X-Y coordinates at the position of maximum pressure and in the Z direction ap-
proximately 2mm from the end of the coupling column using an AIMS scanning tank
system (model AST503, Onda, Sunnyvale, CA). RMS voltages were first converted
to pressure based on calibration data provided by the hydrophone manufacturer. The
pressure was then squared and divided by the product of the density of the medium
taken to be 1028 kg/m$^3$ and the speed of sound in the medium taken to be 1515 m/s
(Tufail 2010) to give intensity.

4.2.3 Data Acquisition

The function generator was controlled by a computer running custom software written
in Matlab (Mathworks, Natick, MA). The software allowed the user to specify ultra-
sound stimulation parameters for a given experiment which it would then apply in a
pseudo-random sequence. The minimum interval between sonications was 5 seconds.
The indication of a physiological response to sonication was a collective contraction,
of the musculature (movement of the neck muscles or a flip of the tail). At the light
anesthesia levels used in these experiments, muscle contractions occasionally occurred
spontaneously and could be confused with sonication-evoked twitches. To ameliorate
this difficulty, the EMG activity was continuously monitored in real time; sonications
were then delivered only during moments of quiescence in muscle activity. Average
activity was measured over a window of 250 ms immediately before sonication, and if
it exceeded a manually adjusted threshold, the software would wait one second before
looking again to see whether the spontaneous muscle noise was below the specified
threshold. Only when the spontaneous activity was below this threshold would the
software allow a sonication to ensue. This procedure has previously been reported in
King et al. (2013).
Figure 4.2: Hydrophone scans of the ultrasound field of the focused 500 kHz transducer used in the experiments. The upper panel (A) shows the scan of the ultrasound field along the XZ plane defined by our positioning system starting approximately 3 mm from the end of the cone. The lower panel (B) shows the scan in the XY axis approximately 3 mm from the end of the cone.
4.2.4 Electromyography

The EMG leads from the animal were attached to a differential amplifier with a gain of 1000x (WPI, DAM 50, Sarasota FL), and the signals were bandpass filtered from 300 Hz to 3 kHz. The animal was grounded by a lead from the differential amplifier that was attached to copper tape affixed to one of the hind limbs. The output signal from the differential amplifier was then fed to a data acquisition device (Labjack U3, LabjackCorp., Lakewood, CO) that was connected to the computer. EMG activity was digitized at 1 kHz and stored to disk along with the sync signals from the function generator via the data acquisition device. Prior to analysis, the stored EMG signal was rectified about its mean and further smoothed by convolving it with a symmetrically truncated Gaussian kernel of overall width 40 ms and full width at half maximum 23 ms.

4.2.5 Analysis

In previous experiments (King 2013) we developed a number of metrics to provide a reliable framework for comparing the effects of sonications under different conditions and on different mice. These metrics also help to overcome the problem of spontaneous activity, which as mentioned above (Data Acquisition) can confound identification of genuine stimulation events.

We started by defining a contraction as having occurred when the filtered, rectified EMG signal reaches three standard deviations above the average noise level for at least 100 ms. To estimate the noise level we averaged the rectified EMG signal activity during a 50 ms window directly before the onset of the ultrasound stimulus. We ensured that the spontaneous activity just before the stimulus was sufficiently low by monitoring, in real-time, the EMG signals and postponing any sonications when the activity exceeded a manually defined threshold (see Data Acquisition). Using this procedure we calculated the contraction duration to be the interval of time between rising above and falling below this three standard deviation threshold. For contraction latency, we measured the time from the onset of the sonication to the point where the EMG signal exceeded the three standard deviation noise threshold. The onset of the
sonication was determined by the onset of the sync signal. Figure 3.2 again illustrates these parameters in relation to a sample EMG signal.

For the first metric of interest, the success rate, we took the ratio of the number of contractions divided by the total number of sonications attempted, expressed as a percentage. Although the success rate is generally a useful parameter in assessing the overall effectiveness of the ultrasound stimulation, it was not sufficient to distinguish between the different types of responses we saw in searching for localized effects. We therefore used an additional metric based on the normalized peak EMG amplitude. Rectified EMG signals were normalized for each recording location in each animal by dividing by the maximum amplitude of the rectified signal observed in the EMG lead regardless of the position of the transducer. Normalizing the data in this way helps ensure that the results from multiple animals can be averaged meaningfully without the data being dominated by one or two animals that happened to have large EMG signals, because of multiple factors such as differences in lead placement and potential differences in anesthesia levels. Averages were calculated for each EMG lead location (neck and tail) and sonication location (rostral and caudal) by taking the weighted mean of the normalized peak EMG amplitudes obtained for each of the four possible combinations of lead location and transducer position (neck/rostral, neck/caudal, tail/rostral, and tail/caudal). The averages were weighted according to the number of sonications using the formula \[ \frac{\sum w_i x_i}{\sum w_i} \], summed over all animals \( i=1 \) to \( n \), where \( w_i \) represents the number of successful sonications from the \( i \)th animal, and \( x_i \) represents the averaged normalized EMG signal from the same animal.

### 4.2.6 Experimental Design

To begin the experiment, an initial procedure to identify the most effective stimulation site prior was performed. Visually the animals head was aligned to the x axis (see Figure 4.1) of the positioning system. The transducer was then centered over the head and lowered until it was 2mm above the surface of the animals head thus fixing the position along the z-axis. The transducer was then moved in the rostral and caudal directions (i.e., along the x axis) in approximately 0.5mm steps. Each time
the animal was sonicated we used continuous wave ultrasound for a duration of 80 ms at intensities varying between 0.7-3 W/cm$^2$ while adjusting the transducer position until we reached the point at which the tail movement was judged by eye to have reached a maximum. At this location, the x coordinate was temporarily fixed and test sonications were performed while varying the position along the left-right axis (i.e., the y axis) again until the point of maximum tail movement was located. The y coordinate was then fixed and further adjustments were made along the x axis. This procedure was repeated until we arrived at the position of maximum tail movement.

Once the optimal x and y coordinates for eliciting tail movement had been located, the transducer was moved along the x axis in the rostral direction until a maximum signal was observed from the EMG implanted in the neck region of the mouse. This maximum always occurred in the rostral direction along the x-axis. We used the EMG signal to optimize the effect of the sonications on the neck region because contractions of the neck muscle were much more difficult to observe visually than tail movements. Once these two optimal locations, the position of maximum tail movement, and the position of maximum neck muscle EMG, were found, they were noted. With the y and z axes now fixed, we performed sonications at these two positions on the x-axis, selectively stimulating these two distinct areas of the motor cortex. At each location, eight sonications were scheduled in a pseudo-random order by the software: four at 3 W/cm$^2$, a moderate intensity which still yielded a high success rate, and four sham sonications at 0 W/cm$^2$. These eight sonications constituted a single run, after which the transducer was moved along the x axis to the opposite position. Ten runs were attempted in each location for each animal which resulted in 40 sonications at each power and at each of the two locations along the x-axis.

When performing the lateralization experiments, the transducer was positioned initially over the midline and bregma of the mouse's head. Slight adjustments in position were made until bilateral forelimb movement was observed in response to the sonications. Once optimized we secured the x coordinate (rostral/caudal), and then the transducer was moved along the y axis (left/right) to search for variations in lateral response. The experimental protocol including anesthesia level was exactly the same as the rostral/caudal experiments.
4.2.7 Effect of the Skull

In larger animals the skull is known to present a significant barrier to the propagation of ultrasound (Clement 2005, OReilly 2011). However, the same does not appear to be true in the mouse as phase correction has been shown to be unnecessary to produce focused lesions in the brain through the intact skull (Hynynen, 2006). We have also previously shown (King, 2013) that the mouse skull, being very thin, offers little to no impediment to neurostimulation by ultrasound. Therefore, we ignored the effect of the mouse skull in our calculations of ultrasound intensity. Figure 4.2 shows the beam plots of the ultrasound field emerging from the combination of the transducer and the coupling column used in our experiments. The plot in Figure 4.2A represents a scan that runs along the z-axis, passing through the center of the transducer. The scan begins about 2-3mm away from the membrane at the end of the coupling column, close to where the ultrasound beam would come into contact with the surface of the brain. Figure 4.2B is a plot in the x y plane orthogonal to the z-axis and approximately 3mm away from the end of the coupling column. Using a published map of the mouse motor cortex derived from intracortical microstimulation (Tennant, 2010) and the full width half maximum of our ultrasound intensity field map, we determined that the field is large enough to cover 30% of mouse motor cortex but small enough to be able to stimulate different regions selectively.

4.3 Results

4.3.1 Rostral / Caudal Success Rates

Figure 4.3 shows the success rates from the implanted EMG leads, neck and tail, while sonicating at the rostral and caudal positions on the head of the mouse. The first set of blue bars in the graph shows the difference in the neck EMG signals when sonicating over the two areas (rostral/caudal) of the head.

While sonicating at an intensity of 3 W/cm², there was no significant difference in the success rates (See Methods Analysis) in eliciting contractions in the neck muscle at the two locations. The average success rate with the average standard error was
Figure 4.3: Success rates as measured from EMG signals obtained in the neck and at the base of the tail with the transducer in the rostral and caudal positions. Sonications were performed at an intensity level of 3W/cm² and 0 W/cm². The sham condition of 0 W/cm² measures the spontaneous contraction rate. Despite visually observable differences in the stimulation induced at 3W/cm² there was no significant difference, confirmed with the students t-test, in the success rates with respect to the location of the transducer. The error bars represent the standard error.
81% (+/- 5%). Similarly, there was no significant difference in the success rates as measured by stimulation at the tail where the average rate with the average standard error was 82% (+/- 5%). The spontaneous contraction rate, represented by the 0 W/cm² sham sonications produced a success rate of about 12%, similar to previously reported results for spontaneous movement (King, 2012).

### 4.3.2 Rostral / Caudal Normalized Peak EMG Amplitudes

While there were no obvious differences in the success rates at different sonication locations, the normalized peak EMG amplitudes (See Methods Analysis), clearly did change. As seen from the blue bars on the graph in Figure 4.4, the weighted mean of the normalized EMG amplitudes in the neck muscles was significantly higher (0.68 +/- 0.03 standard error of the mean (S.E.M.)) when sonicating over the rostral area of the head compared with the caudal area (0.50 +/- 0.03 S.E.M.) (p < 0.001). The opposite was true for the tail EMGs amplitudes (the green bars in Figure 4.4), where the two values 0.43 +/- 0.08 S.E.M. (rostral sonication) and 0.6 +/- 0.04 S.E.M. (caudal sonications) were also significantly different (p < 0.001).

### 4.3.3 Rostral / Caudal Latencies

In addition to the normalized peak EMG amplitudes changing with respect to the sonication position, latency, the time taken between onset of sonication and the EMG signal rising three standard deviations above the noise, also varied as shown in Figure 4.5. For EMG signals at either the neck or tail, the latency was much shorter when sonicating in the caudal region of the brain. On average there was a 53 ms difference between the two locations. When examining the EMG signals from just the neck region, there was a statistical significant difference between the average latencies observed at rostral and caudal regions (68 ms and 38 ms respectively p < 0.001). An even larger difference was seen when examining the signals from the EMG leads implanted in the tail region. The latency dropped from an average of 112 ms when sonicating over the rostral region to 36 ms when sonicating over the caudal region. The distribution of these latencies is shown in the histograms in Figure 4.6.
Figure 4.4: Weighted mean of the normalized peak amplitude from the two EMG locations, neck and tail, when the transducer is at the rostral and caudal positions. When the transducer was at the rostral region of the head, corresponding to the neck area of the motor cortex, the neck EMG demonstrated a higher average normalized peak amplitude than when the transducer was at the caudal region of the head. When sonicating in the caudal position the weighted mean of the normalized peak EMG amplitude in the tail was larger. This was consistent with the pattern of behavior we saw where tail contractions were more vigorous when sonicating over the caudal region of the head rather than the rostral end. The difference is statistically significant and the error bars represent the standard error. (*** p-value < 0.01).
Figure 4.5: EMG latencies in neck and tail for sonications applied at each region of the brain. Latencies were larger when the transducer was in the rostral position compared to the caudal posterior. This was true for both signals from the neck and tail EMGs. The difference is statistically significant and the error bars represent the standard error. (*** p-value < 0.01)
Figure 4.6: Latency histograms from the neck (top) and tail (bottom) EMGs recorded when the transducer was located at each of two positions. The left (or right) panels display the latency data when the transducer was located over the rostral (or caudal) region of the motor cortex.
4.3.4 Right Left Lateralization

In initial exploratory attempts to achieve localized effects from ultrasound neurostimulation, visually, we only observed bilateral movement of the forelimbs regardless of the location of the transducer. To confirm this we recorded EMG signals in three animals from each forelimb. The success rates as defined by our metric based on EMG signals (See Methods - Analysis) were within the range 75% to 90% for both right and left forelimbs and varied little when the transducer was moved from the right side of the brain to the left. Furthermore, we observed little difference between the normalized EMG amplitudes or the latencies of the EMG responses (Table 4.1).

4.4 Discussion

We undertook the current study to investigate the feasibility of localizing ultrasound neurostimulation to different parts of the motor cortex. By positioning the ultrasound transducer at rostral or caudal positions over the mouse's head, we were able to observe by eye obvious variations in muscle contractions in the tail but were unable to induce any lateralized variations in muscle contractions by comparable movements of the transducer along the from right to left (y axis). To quantify the localized effects, we measured normalized EMG responses in both neck and tail regions and confirmed that in both areas there were significant variations in muscle contractions depending on the position of the ultrasound transducer along a rostral/caudal axis. These findings provide good evidence for localized activations within the mouse brain.

When the transducer was over the caudal region of the motor cortex, we were able to induce a vigorous tail movement in response to a sonication as opposed to a small twitch of the tail we observed when we sonicated over the rostral region. These variations were only seen at a very low level of anesthesia (0.02% isoflurane). Although we also observed changes in the normalized EMG amplitude in the neck region, the behavioral manifestation of these variations was not visibly obvious. However, changes in the amplitude of responses in the tail region might be expected to be more prominent than elsewhere because of the length and leverage of the tail.
**Table 4.1:** Only bilateral movement was observed visually when sonicating over the left and right hemispheres. This is confirmed by the EMG signal from both forelimbs in three animals. Little difference was observed in the latency, success rate, or normalized mean peak EMG amplitude when the transducer moved from one side of the brain to the other. S.E.M represents the standard error of the mean.
The use of normalized EMG amplitude to discern less overt changes in response was necessary as we did not observe significant changes in the sonication success rates (as defined by our metric based on EMG signals see Methods). When the transducer was moved between the two positions along the rostral/caudal axis that maximized the neck response at one end and the tail response at the other (see Figure 4.3), the success rate in eliciting twitches in each region remained high at about 80%. However, differences were apparent in the normalized peak EMG signals (see Figure 4.4). In each case, the weighted means were statistically higher in the neck region when sonicating rostrally and higher in the tail region when sonicating caudally. The pattern was consistent with the known topographic representation (Tennant, 2010) of the mouse motor cortex obtained using electrical intracortical microstimulation (ICMS) where circuitry controlling musculature in the neck regions is represented rostrally in motor cortex while the tail region is represented caudally (see Figure 4.7). This shows that activation of different parts of the motor cortex could be occurring when sonicating over different positions of the brain (rostral/caudal). The same topographic maps also shows the representation of forelimb to be intermingled with the representation of the hindlimb which might explain the lack of any obvious localization effect on the forepaws. Using our transducer, it would be difficult to independently activate areas of the motor cortex which control the forelimbs without activating activating the areas which controls the hindlimb.

The response variations we saw in moving the transducer along the rostral/caudal axis of the motor cortex stand in contrast to the lack of any obvious lateral effect we saw moving from right to left. While it is recognized that the motor cortex generally controls muscular activity on the contralateral side in all mammals, ipsilateral responses to electrical stimulation in the motor cortex have also been widely reported in a variety of animals including monkeys (Bucy, 1933, Lauer, 1952), cats (Garol, 1942), rats (Kartje-Tillotson, 1985) and mice (Brus-Ramer 2009, Tennant, 2010). Generally ipsilateral responses are sparser and weaker, and appear to depend on supporting activity in the opposite hemisphere. Nevertheless, their presence suggests the bilateral responses we saw when sonicating the right or left hemisphere could arise from the
Figure 4.7: Diagram of the mouse motor cortex. The top panel shows the approximate area of the different regions of the motor cortex and the color map for the lower panel. The lower panel shows the spatial function of the mouse motor cortex. Translucent circles indicated the approximate size of the ultrasound focal spot. Note the neck region at the rostral end (yellow squares) and the tail region at the caudal location (orange squares).
simultaneous excitation of both contralateral and ipsilateral pathways. In their microstimulation study on mice, Tennant et al. (2010), reported an absence of ipsilateral movements at the movement thresholds that elicited contralateral responses with the exception of sites representing the vibrissae where stimulation caused near simultaneous bilateral effects. In our experiments it could have been that we were working too far above the thresholds necessary to distinguish between the different sensitivities of the contralateral and ipsilateral pathways. Another possibility is that the small size of the mouse brain made it simply too difficult to focus the ultrasound cleanly on just one area controlling contralateral movement, or even on one hemisphere at a time. ICMS is, undoubtedly, a more precise technique, particularly in the smaller animal model, than ultrasound in inducing neurostimulation and given the intermingling of cortical mappings (Li 1991, Pronichev 1998) and the presence of inter-hemispherical connections including the corpus callosum, obtaining a clear lateralized effect in the mouse using ultrasound may be particularly difficult. Studies on larger animal models should help to resolve the question of how feasible lateralized effects are in motor cortex using ultrasound. It has also been shown that lateral effects of cortical stimulation can be strongly influenced by the depth and type of anesthesia. In monkeys, when using barbiturates, or deeper levels of anesthesia, Bucy et al. (1933) and Lauer (1951) reported seeing only contralateral responses. This could offer an alternative explanation for the contralateral only responses reported by Yoo et al. (2011) when sonicating the motor cortex of rabbits anesthetized with ketamine / xylazine.

The changing pattern of latencies (see figure 4.5) when sonicating different areas of the motor cortex present something of a puzzle. In both neck and tail regions, latencies were on average substantially longer when sonicating at the rostral position (70 ms neck, 110 ms tail) compared with the caudal position (38 ms neck, 36 ms tail). One possible complication in interpreting the latency effects arises from the close connections between somatosensory cortex and motor cortex. Matyas et al. (2010) showed that whisker retraction in mice is not driven by motor cortex directly but instead via primary somatosensory cortex S1, a region that lies behind the motor cortex M1. Surprisingly, their comparison using ICMS in motor cortex and somatosensory cortex showed the latter to have shorter latencies by a few milliseconds. It is not
known whether S1 contributes to other motor responses besides those of the vibrissae and even if it does the observed timing difference would not be enough to explain the larger difference in latencies we observed. However, the mere fact that S1 can trigger some motor responses directly raises the question of what effects ultrasound stimulation might have on this region. Given its proximity to and relative position with M1, S1 would inevitably receive more ultrasound exposure as the transducer was moved caudally. A better understanding of the varying latencies will require further experiments to elucidate which motor circuits are being excited by the ultrasound.

Nevertheless, our current findings may cast light on the variable nature of latencies observed in previous studies. Tufail et al. (2012) reported average latencies of 22 ms when stimulating skeletal muscle groups and an average latency of 123 ms when sonicating the hippocampus. King et al. (2013) report latencies when sonicating over the entire motor cortex ranging from 55 ms–70 ms, with a trend of slightly decreasing latency with increasing sonication intensity. While there could be some methodological differences that explain at least part of these different response timings, in this current study we see that latency is strongly influenced by the location of the sonication, consistent with the idea that different circuits of the brain are activated in the different locations.

4.5 Summary

For ultrasound neurostimulation to become a useful tool in neuroscience, demonstrating that ultrasound stimulation can be localized to specific target areas of the brain is a critical objective. In our experiments we have taken an important step in this regard by demonstrating the ability to evoke variable responses by differentially sonicating rostral and caudal areas of the motor cortex. In particular, by using acoustic stimulation sequences in which the parameters of interest are randomly and systematically varied, we have quantified the phenomenon and established relationships between the ultrasound stimulation location, success rate, peak EMG amplitude, and latency. We did not see lateralized responses for reasons that may be due either to the mosaic-like nature of the cortical motor maps, or to the small size of the animals head compared
to the size of focused ultrasound fields, or a combination of both. Larger animal models may help clarify this issue as well as using shorter wavelength/higher frequency ultrasound. Such techniques should make it possible to target brain structures with more precision using ultrasound although higher frequencies typically require higher intensities to elicit neurostimulatory effects (King, 2012). The tradeoff between higher frequency and better resolution on the one hand and neurostimulation efficiency and higher intensities on the other will be particularly important when trying to overcome the problems in transmitting ultrasound across skulls of larger animal models with sufficient power. In addition, the defocussing effects of the skull will need to be examined in more detail but are likely to be surmountable using phased ultrasound arrays. Scaling up current neurostimulatory methods and applying MRIgFUS techniques used for treatment brain disorders to achieve localized stimulation in the human brain thus remains a promising objective, and one that looks more feasible in the light of our localized effects in the mouse.
Chapter 5

Conclusion

The development of ultrasound induced neurostimulation is highly relevant to understanding brain function. While it has been known for decades that ultrasound can modulate activity within the central nervous system, systematic evaluation of the conditions for effective neurostimulation has been lacking. In this doctoral work, I have provided a study of the parameters involved for successful ultrasound induced neurostimulation and localized the effect to specific areas of the motor cortex in a mouse model. These findings will help contribute to an understanding of the phenomenon of noninvasive neurostimulation and lead to the development of patient treatments. Scaling up current neurostimulatory methods to achieve localized stimulation in the human brain is a promising objective, and one that looks more feasible in the light of our studies in the mouse.

5.1 Summary of Ultrasound Induced Neurostimulation

Examining the success rates under different conditions and identifying the parameters involved in ultrasound induced neurostimulation are necessary to further understand and develop this technology/modality. In this work the parameters used to define a muscle contraction (signal amplitude, latency, duration) were established, and the
success rate of attempted stimulations was used to quantify the effect. This metric was used to understand and test ultrasound parameters and key properties. Stimulation of the brain resulted in muscle contractions which were recorded with EMG leads placed in different locations. While examining the parameters involved it was found that the effectiveness of ultrasound induced neurostimulation depends on an interaction between acoustic intensity and duration. It was also noted, based on our observations under the conditions studied, that the motor response is an all-or-none phenomenon. The parameters that lead to successful ultrasound induced neurostimulation are described in Chapter 3 of this thesis.

Ultrasound induced neurostimulation was only achievable under light anesthesia conditions; therefore, the animals were maintained in a semi-conscious state. With too high a level of anesthesia, no stimulation was observed, too low and the animal would exhibit too much movement to perform the experiment. In light of these observations, the anesthesia levels which allowed for the optimal performance of the experiments were quantified.

The effect of the ultrasound carrier frequency on the success rate was also reported. The intensity needed to maintain a constant success rate across all the frequencies tested, increased as the frequency increased; from 250 kHz to 600 kHz. The intensity required to match the 50% success rate at the two frequencies increased by two orders of magnitude demonstrating that, in our experiments, there is a frequency component to ultrasound induced neurostimulation in the mouse, a phenomenon which may help lead to a better understanding of the mechanism involved.

The mouse model can be difficult to work with due its size, but it was advantageous because the effect of the skull could be ignored for our intensity calculations. Balancing the tradeoff between higher frequency and better resolution on the one hand and neurostimulation efficiency and lower intensities on the other will be particularly important when trying to overcome the problems in transmitting ultrasound across skulls of larger animal models with sufficient power. The contribution of the skull of larger animals will have to be taken into account and compensated for, problems we have been able to avoid in the mouse.

Contrary to other reports, we found that continuous wave ultrasound was just as
effective at performing stimulation, and in some cases, even more effective than pulsed ultrasound. When examining increasing PRFs, the success rate continued to increase to the longest PRF tested. This may help to identify an underlying mechanism for ultrasound induced stimulation, and at the very least allows for a less complicated experiment.

5.2 Summary of Localization of Stimulation

Therapeutic use of ultrasound induced neurostimulation will only be viable if precise localization to specific areas of the brain is achieved. It was shown in Chapter 4 that when sonicating over different areas of the brain, that while the success rate did not change, the normalized weighted peak EMG did. The change in the EMG signal as a function of transducer position coincided with what was seen visually. When the transducer was over the caudal region of the motor cortex, we were able to induce a vigorous tail contraction, as opposed to a small twitch of the tail, which was observed when we sonicated over the rostral region. The opposite was seen in the neck muscles: a vigorous twitch over the rostral region of the motor cortex and a slight twitch when sonicating over the caudal area of the motor cortex. These findings demonstrate the ability to preferentially stimulate different areas of the mouse motor cortex using ultrasound; findings which match published topographic maps of the mouse motor cortex obtained in electrical microstimulation studies (Tennant, 2010).

Interestingly, the change in the latency did not follow the same pattern as the normalized weighted EMG. Sonications performed at the rostral end of the motor cortex produced longer latencies, as compared to sonications performed over the caudal area of the motor cortex. The change in latency was independent of the location of the EMG lead (tail or neck). These observations demonstrate that latency is strongly influenced by the location of the sonication, which is consistent with the idea that different circuits of the brain are activated in the different locations.
5.3 Future Work

This doctoral work has provided a systematic evaluation of the parameters used for successful ultrasound induced neurostimulation, and demonstrated localization of the effect with a high rate of success in a mouse model. Translation of this experimental technique into the clinical setting will present several limitations and challenges. With the ultrasound transducer array technologies available at this time, it will be possible to correct any phase aberrations which occur when sonicating through the skull, compensate for the increased attenuation, and target a large portion of the brain with a relatively small focal spot. Ultrasound is noninvasive, an advantage over DBS, and offers better depth abilities and comparable spatial resolution to TMS, but because of the current array geometry used, some areas such as superficial structures may be a challenge to target. The development of new array designs and sonication techniques could offer solutions to overcome this limitation.

There is also a tradeoff in the ultrasound frequency in terms of penetration and resolution. It will need to be low enough to propagate through the skull, keeping the attenuation low, but high enough to create a small focal spot. The current clinical trials and approved treatments which use HIFU for brain disorders will provide useful data on the optimal conditions for achieving the ideal focal spot with low attenuation.

Combining ultrasound neurostimulation with MRI will allow precision targeting, as well as the ability to perform functional studies, which could elucidate new neural pathways. Functional MRI could be a more sensitive and exhaustive method for monitoring the effects of ultrasound induced neurostimulation in the whole brain, rather than neuromuscular changes localized only to the primary activation site. High temporal resolution could be obtained with electroencephalography; however, integrating such technology may be more feasible in a larger animal model. The findings of this doctoral dissertation in combination with previously published techniques, will serve as the foundation for future work with ultrasound induced neurostimulation in larger animals, using MRI for precise localization.

The field of therapeutic ultrasound is vast and expanding rapidly. Although the roots of therapeutic ultrasound were firmly planted decades ago, novel ideas and
technologies are driving the field forward. The combination of MRI with ultrasound has propelled the use of therapeutic ultrasound into clinical practice, but much work is needed before noninvasive ultrasound induced neurostimulation enters the clinical setting. However, this is an exciting technology with promising clinical applications. This thesis has provided the foundation for the next steps towards understanding the phenomenon of \textit{in vivo} ultrasound induced neurostimulation, and will propel this technique forward as a potential therapeutic treatment for a wide variety of human diseases and conditions.
Bibliography


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