To the Faculty of Washington State University:

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Few things in my life have been accomplished with such determination and perseverance. This project represents the determination of many and the perseverance of even more. To say that it takes a village to produce a doctoral project is not an understatement. The scientists, professors, mentors, colleagues, classmates, family and friends who have supported me, inspired me, encouraged me, and guided me will forever be appreciated.

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Stimulation of the rodent cerebral cortex is used to investigate the underlying biological basis for the restorative effects of slow wave sleep. Neuronal activation by optogenetic and ultrasound stimulation elicits changes in action potentials across the cerebral cortex that are recorded as electroencephalograms. Optogenetic stimulation requires an invasive implantation procedure limiting its application in human studies. We sought to determine whether ultrasound stimulation could be as effective as optogenetic techniques currently used, in an effort to further understand the physiological and metabolic requirements of sleep. We successfully recorded electroencephalograms in response to transcranial ultrasound stimulation of the barrel cortex at 1 and 7 Hz frequencies, comparing them to those recorded in response to optogenetic stimuli applied at the same frequencies. Our results showed application of a 473 nm blue LED positioned 6 cm above the skull and ultrasound stimulation at an output voltage of 1000 mV_{pp} produced electroencephalograms with physiological responses of similar amplitude. We concluded that there exists an intensity-proportionate response in the optogenetic stimulation, but not with ultrasound stimulation at the frequencies we surveyed.

Activation of neuronal cells in response to optogenetic stimulation in a Thy1-ChR2 transgenic mouse line is specifically targeted to pyramidal cells in the cerebral cortex. ChR2 responses to optogenetic stimulation are mediated by a focal activation of neuronal ion channels.
We measured electrophysiological responses to ultrasound stimulation, comparing them to those recorded from optogenetic stimuli. Our results show striking similarities between ultrasound-induced responses and optogenetically-induced responses, which may indicate that transcranial ultrasound stimulation is also mediated by ion channel dependent processes in cerebral cortical neurons.

The biophysical substrates for electrical excitability of neurons impose temporal constraints on their response to stimulation. If ultrasound-mediated responses are, in fact, ion channel mediated responses, ultrasound-induced responses should exhibit time-dependence characteristics similar to those of optogenetically-triggered responses. Minimal stimulus duration thresholds and the temporal limits of paired pulse facilitation for ultrasound stimulation were identical to those of optogenetic stimulation. Collectively, these experiments demonstrate an electrophysiological basis for low-frequency transcranial ultrasound stimulation of cerebral cortical neuronal activity.
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Dedication

This dissertation is dedicated to women of all ages in pursuit of any scientific endeavor,

and to those who find joy in the science of our universe.
CHAPTER 1: INTRODUCTION

1.1 Sleep

Sleep is essential for physical and mental well-being. Performance of mentally challenging tasks becomes increasingly suboptimal when one is deprived of sleep. The only way to overcome the negative effects of sleep deprivation is to recover the lost sleep. Sleep is therefore a restorative process. The restorative aspects are demonstrated by improved performance on the psychomotor vigilance task as described by Belenky et al. in the Journal of Sleep Research, 2003\(^1\). Liu et al. chronicled homeostatic changes in neuronal excitability and suggested that sleep promotes synaptic homeostasis\(^2\). In addition, Dash et al. describe changes in neurotransmitter release resulting from sleep subsequent to enforced wakefulness in the Journal of Neuroscience in 2009\(^3\). However, the biological basis for the restorative effects of sleep remains a critical unanswered question in biomedical science.

1.2 Sleep Classification

Sleep is classified as one of two stages: non rapid eye movement (NREM) sleep or rapid eye movement (REM) sleep. A sleep stage is defined based on the electroencephalogram (EEG) generated by measuring the electrical potential between two sites on the scalp in humans or within the skull in mice. This electrical potential is driven by changes in the activity of neurons, which are the electrically excitable nerve cells of the cerebral cortex. Electrical excitation of neurons is a function of the opening and closing of ion channels located within the neuronal membrane. When a cation channel is opened, cations – with a concentration higher outside the cell, rush into the neuron. The channel then closes and the neurons quickly pump the cations out, again creating a higher concentration of cations in the extracellular environment. This cumulative voltage fluctuation of large numbers of neurons creates field potentials within the
skull. Electroencephalograms undergo very predictable changes throughout the wake/sleep process enabling researchers to ‘visualize’ sleep.

NREM sleep is characterized by oscillations in the EEG at a frequency of 1 – 4 Hz, known as slow wave activity (SWA). The production of SWA is a self-organizing property of the cerebral cortex and is critical to the restorative function of sleep. Since most of the time spent asleep is in NREM sleep, the production of slow wave activity in the cerebral cortex is likely to be the single most important function of sleep. It is known that although only 5% of body mass is occupied by the brain, the brain is responsible for utilizing approximately one quarter of the glucose used by the body when at rest. We have seen significant correlations between the amount of slow wave activity and the rate of reduction of cerebral glucose utilization relative to wakeful periods in mouse models. A long-term goal of the Wisor lab is to manipulate sleep slow wave activity as a means to understand and therapeutically improve the restorative process of sleep.

1.3 Manipulation of the Cerebral Cortex

The Wisor lab designed a rodent optogenetic stimulation system allowing for the manipulation of the electrical activity of pyramidal neurons, the most common class of neuron in the cerebral cortex and the primary source of EEG field potentials, with millisecond precision. We have successfully used the optogenetic technique to routinely manipulate local activity of the cerebral cortex without disrupting the animal’s global sleep state, and to study specific relationships between the rate at which lactate concentrations decline during slow wave sleep and the magnitude of the slow wave activity recorded with an electroencephalogram.
1.4 Optogenetics

Optogenetics is a technique developed by Karl Deisseroth and his colleagues at Stanford University in 2004. The work was published as Millisecond-timescale, genetically targeted optical control of neural activity in Nature Neuroscience in 2005 and the technique was named “Method of the Year” in December 2010 in Nature Methods. Deisseroth et al. describe the process which combines genetics, virology and optics allowing scientists to instantaneously activate or silence specifically targeted groups of neurons within a particular circuit in the brain with precision not matched by other standard techniques.

The optogenetic technique requires the use of genetically modified animals, in addition to an invasive procedure in which fiber optic cannulas are surgically implanted into the animal’s skull. We use the Thy1-Channelrhodopsin2 (Thy1-ChR2) transgenic mouse line (JAX strain #7612). ChR2 is a retinylidene protein with the ability to act as a sensory photoreceptor controlling phototaxis when expressed in its native form (in unicellular green algae). This protein is easily expressed in neurons via transfection techniques and allows depolarization of excitable cells in transgenic animals. Current laser technology allows neuroscientists to focus a 473 nm light source directly onto the targeted cerebral cortex coordinates through a fiber optic cable with the aid of a transistor-transistor logic (TTL) triggering device. The blue light shining on the neurons of the cerebral cortex causes voltage fluctuations, which can be recorded via an EEG.

1.5 Lactate Concentration Declines with Slow Wave Activity

We believe that we are the first group to utilize a unique combination of optic and genetic techniques to induce slow waves in the electroencephalogram of awake, fully alert, freely moving animals. We have been able to manipulate local activity of the cerebral cortex without disrupting the animals’ global sleep states. Given that lactate is the key glucose metabolite in the
brain, we worked to determine whether our optogenetic manipulations of slow wave activity would have the same effects as those of spontaneous sleep. Using statistics to quantify the magnitude of the relationship between lactate concentrations and slow wave activity, we showed that lactate concentration declines with slow wave activity in slow wave sleep periods and increases in the absence of slow wave activity during wake.6

The protocol we have developed for manipulations of slow wave activity will allow comparison of induced effects on neuronal electrical and metabolic changes to those produced by spontaneous cortical slow wave activity. Furthermore, it allows for investigation into the actual mechanisms for sleep and may lead to therapies for disrupted sleep. Demonstrating that a particular population of cells is a regulator of slow wave sleep allows us to use this population to manipulate brain activity in cases where slow wave sleep generation is disrupted. Ultimately, this work may be particularly enlightening in the study of human medical conditions, such as epilepsy or Parkinson’s disease, in which abnormal cortical activity may be corrected with similar techniques. However, while the optogenetic method allows scientific investigation in an animal model, these techniques are not applicable in human studies. It is therefore necessary to develop and implement new techniques for manipulating slow wave activity within the cerebral cortex.

1.6 Non-invasive Techniques for Neuronal Stimulation

Neural activity modulation has proved beneficial in the treatment of psychiatric and neurologic diseases and has been useful for investigating the underlying mechanisms of sensory, motor, and cognitive functions. Noninvasive techniques currently employed include electrical stimulation via transcranial direct current manipulation and transcranial magnetic stimulation. Unfortunately, these techniques do not allow for precise spatial or temporal resolution that may
be necessary to target specific regions of the cerebral cortex. Tufail et al. showed that transcranial pulsed ultrasound can offer noninvasive stimulation of brain circuits with a spatial resolution of approximately 3.0 mm.\textsuperscript{11}

1.7 Ultrasound Stimulation

Used in medical diagnostic imaging applications since the mid-1950s, ultrasound is simply defined as sound waves of greater than 20,000 cycles per second, well above the human hearing range. Typical medical diagnostic frequencies range from 2 to 15 MHz. Ultrasound transducers exploit the piezoelectric effect and the pulse echo principle to convert electrical energy into sound energy and back again. Ultrasound vibrations travel as sound pressure waves and require an elastic medium in order to permeate.

The Food and Drug Administration states that the use of ultrasound imaging retains an excellent safety record over the past twenty years, without the risks commonly assigned to x-ray radiation.\textsuperscript{12} However, ultrasound is not limited to medical imaging. Changing power, intensity and duration of pulses allows for the use of ultrasound as a therapeutic technique for destruction of cancerous cells.\textsuperscript{13} High intensity, high frequency ultrasound has been successfully used as a noninvasive alternative to endoscopic third ventriculostomy to alleviate hydrocephalus.\textsuperscript{14} While the long term effects of tissue heating and cavitation may not be fully understood, the use of transcranial focused ultrasound is generally thought to be minimally invasive in the treatment of a variety of intracranial pathologies;\textsuperscript{12, 14} and for treating somatic conditions such as kidney stone breakup, or tumor removal. This makes the use of ultrasound particularly interesting in the stimulation of the cerebral cortex as a noninvasive approach to manipulate slow wave activity.

The use of ultrasound stimulation to elicit action potentials in neurons in the cerebral cortex is a relatively new undertaking; however scientists have known that ultrasound has the
ability to influence nerve activity for almost one hundred years. Direct stimulation of action potentials with ultrasound was initially done with brain tissue in vitro. Great strides have been made in the application of ultrasound stimulation to intact brain tissue in animal models. William Tyler, assistant professor at Virginia Tech’s School of Biomedical Engineering and Sciences, and his students developed a method of ultrasonic neuromodulation by brain stimulation with transcranial ultrasound in 2010. Following the methods and procedures of this published work, a fully functioning ultrasound stimulation system was established in the Wisor lab and we have successfully applied transcranial ultrasound stimulation to the rodent cerebral cortex to induce whisker twitching in anesthetized mice.

An immediate goal of this dissertation project was to test the hypothesis that electrophysiological parameters of the electroencephalogram associated with sleep slow wave activity are responsive to non-invasive low frequency transcranial ultrasound stimulation. Doing so might allow one to exert potentially restorative effects akin to those of sleep slow waves in human subjects.

1.8 Technical Challenges

1.8.1 Electroencephalogram recording during ultrasound stimulation

One challenge presented by this goal stemmed from the fact that, while ultrasound has previously been shown to produce action potentials in the cerebral cortex, successful recording of an electroencephalogram in response to this stimulation had been elusive. The Tyler group reported success in measuring muscle contractions with electromyogram in response to stimulation of a cerebral cortex region known to regulate whisker movement. In order to investigate whether the useful information gained in the invasive optogenetic studies can be reproduced with noninvasive ultrasound, EEG recording is essential.
With the use of custom-made tungsten electrodes and a carefully designed surgical protocol, we successfully recorded EEG responses to two types of stimuli – optogenetic and ultrasound. Our typical recording of optogenetic responses involves freely moving mice fitted with surgically implanted cannula to allow for post-surgical stimulations. Given that a goal of this project was to reproduce the stimulation of the cerebral cortex by optogenetics with ultrasound for the purpose of manipulating slow wave activity, it was essential to conduct head-to-head comparisons of the electrophysiological response to both types of stimuli recorded in the same fashion. Therefore, it was necessary to design a surgical protocol in which the animal remains anesthetized for the duration of the experiment, as the ultrasound transducer is too large to be fitted onto a mouse’s head for post-surgical stimulation. The use of the genetically modified mice was imperative given that optogenetic stimulation requires the use of the transgenic line. Nonetheless, ultrasound stimulation does not require the use of transgenic lines – in fact, the Tyler lab did not use these genetically modified strains in their original applications of ultrasound stimuli.\textsuperscript{11}

1.8.2 Electrical noise elimination during ultrasound stimulation

Special consideration was taken to remove extraneous electrical noise in the surgical suite while recording electroencephalograms. The use of a powerful radio frequency (RF) amplifier, necessary for the production of ultrasound waveforms, created a disruption to the recorded signals. The RF amplifier was housed in its own Faraday cage to eliminate this interference. The use of grounding EEGs implanted during surgery, grounding wires attached to the stereotaxic equipment and surgical table, along with an enclosed Faraday cage designed to block unwanted electrical noise during recording made it possible to collect unambiguous signals in the
electroencephalogram. The successful recording of physiological response to ultrasound stimulation paradigms was the first essential step toward making this project a success.

1.8.3 Noninvasive Stimulation

It was also important to establish a method of stimulation that could be interpreted as noninvasive. The optogenetic technique required the use of a craniotomy for application of the surgical model. We successfully tested the hypothesis that ultrasound could be applied transcranially, as reported by Tufail et al.\textsuperscript{11} Our preliminary investigations showed that application of both optogenetic and ultrasound stimulation via a thinned section of skull over the somatosensory ‘whisker barrel’ cortex, a region that regulates whisker movements, produced whisker twitching. To test the effectiveness of a noninvasive procedure, we showed that ultrasound neuromodulation resulted in visually confirmed whisker twitching when targeting the whisker barrel cortex transcranially. It was necessary for comparison purposes to apply stimuli through an opened craniotomy for the final experimental setup, but confirmation of effective transcranial application of ultrasound stimulation of the cerebral cortex was essential to the immediate goals of the project.

In order to proceed with this investigation, it was important to understand how ultrasound energy affects the material medium through which it travels. Of interest would be determining whether the bone density of the skull would make a difference in the effective use of ultrasound transcranially, given that larger animals possess thicker skull material. In 2012, Stanford University Department of Bioengineering professor Randy King published results of transcranial ultrasound experiments conducted by applying continuous-wave sonication using a transducer with a 500 kHz center frequency in order to ascertain some basic parameters for application of ultrasound-induced neurostimulation. This group recorded only electromyogram information, not
electroencephalogram responses. With the use of hydrophone scans of the ultrasound field of a transducer, King et al. showed that there was no significant shift in ultrasound focus and no drop in the peak ultrasound signal in a field with no skull when compared to an ex vivo mouse skull held at the end of a coupling column filled with ultrasound gel.\textsuperscript{17} This allowed the investigators to conclude that it would be safe to ignore the effects of the mouse skull in any ultrasound intensity calculation. Initial calculations of ultrasound intensity reported in the literature range from 0.01 to 79.02 W/cm\textsuperscript{2}.\textsuperscript{17}

Younan et al. sought to describe the specific acoustic pressure distribution of ultrasound stimulation inside the brain by reproducing the experiments of the Tyler group.\textsuperscript{18} It is important to note that, to date, there is not a great deal of published material with regard to ultrasonic neuromodulation and any stride in solidifying scientific understanding of this procedure will be valuable to the scientific community at large – particularly impacting the field of neuroscience. Much of Younan’s study was based on numerical simulations of ultrasound propagation in the head cavity, as acoustic pressure distributions in the mouse brain cannot be measured in vivo. Younan’s work describes that an average acoustic pressure threshold was 0.68 $\pm$ 0.1 MPa at the focus of the transducer with a corresponding spatial-peak pulse average intensity $I_{\text{sspa}} = 7.5$ W/cm\textsuperscript{2}. This group did not set out to determine whether these pressures would create tissue damage, only to determine what the average values might be inside the brain.

It has been established that types of anesthetic and anesthetic application levels produce significant changes in the shape of evoked responses in rodents compared to those of sleep and wake states.\textsuperscript{19} King’s group did a comparison study to optimize the effective ultrasound parameters using isoflurane anesthesia. While the group compared 0.5\%, 0.1\% and 0.02\% isoflurane, they report that 0.1\% provided the most reliable parameter value.\textsuperscript{17} Younan et al.
reported slight pressure variations when comparing anesthetic stages. Deep anesthesia resulted in 0.77 ± 0.04 MPa, while lighter anesthetic stages resulted in 0.61 ± 0.03 MPa. Interestingly, this group relied on visual motor response to determine these thresholds, as even EMG recording proved to be difficult due to electrical cross talk with the transducer driving signals. We found that light anesthesia stages, approximately 0.75%-1.5% isoflurane, provide the most reliable condition under which to record electrophysiological responses to ultrasound with EEG.

1.9 Experimental Overview

The work described in this doctoral project was conducted in three stages. The first experiment established evidence of motor response to stimulation of the sensory cortex via transcranial ultrasound. The physiological evidence of ultrasonic stimulation recorded via an electroencephalogram established that the techniques previously used in this lab, with respect to optogenetics, could be reproduced noninvasively. This experiment relied on a head-to-head comparison of the response to optogenetic stimulation via a light-emitting diode (LED) placed directly on the dura mater, the brain’s translucent protective covering, and ultrasound - both applied at 1 and 7 Hz frequencies. The second experiment resulted in an intensity-response curve identifying response-equivalent stimulus intensities for optogenetic and ultrasound stimuli. This experiment was necessary in order to further our understanding of slow wave activity, as well as investigate the relative shapes of the resulting voltage versus time graphs. Insight into the type of cortical neuronal response – hyperpolarization versus depolarization may provide foundational evidence for the underlying mechanisms of ultrasound stimulation in the cerebral cortex. The final experiment compared varying pulse durations and interstimulus intervals for both types of stimuli. Our goal was to ascertain the minimal pulse duration of ultrasound stimulation that would produce a reliable physiological effect. Future experiments in this lab will aim to
determine the refractory period in response to the minimally effective duration of ultrasound stimulation.

1.10 Conclusion

Ultrasound and optogenetic manipulations use distinct physical mechanisms to mediate ion fluxes in the cerebral cortex. Ultrasound waves generate ion fluxes by disrupting the integrity of otherwise ion-impermeant lipid bilayers in the brain. In optogenetic stimulation, absorption of photon energy induces a transient change in the 3-dimensional structure of ChR2 proteins, temporarily rendering them capable of generating ion fluxes. Given the distinct mechanisms of energy transfer that characterize ultrasound and optogenetic manipulation, it is important to determine whether these two manipulations have divergent effects on the excitability of brain activity. This project tests the overarching hypothesis that neuronal activation by low frequency transcranial ultrasound stimulation can be used to manipulate sleep slow wave activity as a means to understand and therapeutically improve the restorative process of sleep.
CHAPTER 2: RESEARCH DESIGN AND METHODOLOGY

2.1 Electroencephalogram (EEG) and electromyogram (EMG) Construction

In order to proceed with this investigation, it was necessary to design and construct electroencephalogram and electromyogram electrodes, along with an accompanying pre-amplifier headpiece to be used with our physiological recording equipment. While our preliminary investigations included exploration of several different types of electrodes, ultimately, we chose specifically constructed tungsten electrodes.

Tungsten electrodes were constructed from perfluoroalkoxy alkane (PFA) coated tungsten wire purchased from A-M Systems in Sequim, WA. (Catalog No. 797000, 0.008” bare diameter, AWG 32). PFAs are fluorocarbon based and provide high resistance to solvents. The wire was cut into 3 cm segments, one end of which was stripped to remove the PFA coating using a Micro-Strip precision stripper from Micro Electronics, Inc. in Seekonk, MA (Catalog No. MS1-05S-__FS). Next, a 4-inch section of Teflon coated wire (Alpha Wire, 2840/7, 32 AWG purchased from DigiKey) was soldered to the exposed end of the tungsten electrode. Approximately 3.0 mm of wire on each end of the Teflon coated wire was stripped and pre-tinned using a flux formulated from 96 g of ZnCl₂, 9.6 g of NH₄Cl, 25 mL H₂O, 10 mL of concentrated HCl and 3.0 g of boric acid. Flux was heated to 100 degrees Celsius in an H₂O bath at the time of use. High temperature solder (Sn/Ag/Cu #66/331) was used to tin the wires. After soldering, the soldered wires were rinsed with sodium bicarbonate solution to neutralize the acid. Solder joints were coated with bio-compatible epoxy (EPO-TEK 354-T, 2-part epoxy with a 10-minute cure time at 150 degrees Celsius) to prevent unwanted electrical noise due to exposed wires. The epoxy was cured by placing the covered electrodes into a small toaster oven and heating for ten minutes at 150 degrees Celsius.
Enamel-coated forceps were used to bend a 90-degree angle just past the epoxy ball. It was essential to use enamel-coated forceps because uncoated forceps damage the polyimide wire insulation, which would cause a break in electrical continuity of the electrode. Under a dissecting microscope, we placed the electrode on a micrometer. The electrode was carefully measured and cut to a length of 2.0 mm with a pair of wire snips. Continuity from the exposed tip of the tungsten electrode to the stripped end of the Teflon-coated wire was verified with a multimeter by placing one lead directly to the tip of electrode, while the other lead was placed to the prepared, tinned end.

![Figure 2.1 - Tungsten electrodes were constructed to record EEG response to LED optogenetic and ultrasound stimulation in the cerebral cortex. Each electrode was tested for continuity before surgical implantation.](image)

The EMGs were constructed using a smaller diameter PFA-coated tungsten wire. Approximately 10 cm of wire was cut and both ends of the wire were stripped using a micro wire-stripping tool. Following the full procedure for the soldering with tungsten, one end of the
tungsten wire was tinned and soldered to a 40 mm length of pre-tinned Teflon coated wire. Bio epoxy was applied to the solder joint and each EMG was temperature cured as previously described. Next, the un-tinned end of the tungsten was carefully slipped into the syringe end of a one-inch 25 gauge sterile needle. It was important that the tungsten’s coating remain intact while sliding through the needle to preserve continuity, so caution was used. The sharp part of the needle was necessary for the implantation of the EMG into the whisker pad. The stripped tip was trimmed to approximately 1.0 mm and bent with a pair of sharp forceps to create a barbed hook at the end of the wire. Since the tip was already stripped of the protective jacketing, the wire was not harmed by using the sharps. The ‘hook’ was allowed to hang over the lumen opening of the needle. The protective cap of the needle was replaced until use during surgery in order to prevent damage to the EMG tip.

Figure 2.2 – EMGs were constructed with the use of tungsten wire and 25 gauge needles. The tungsten barb protruded from the sharp tip of the needle to allow for implantation into the whisker pad during the surgical procedure.

2.2 Electrode Montage Construction

The electrode montage was constructed with a piece of single pin, connector strip header (Mill-Max Manufacturing Corp. from Digi-Key, part #ED8250-ND). An eleven-pin segment (corresponding to the 11-input connector of the MultiChannel Systems preamplifier) was cut
with a pair of snips. Eleven pieces of Alpha Wire were cut to lengths of approximately 6.0 cm. On each wire, 2-3 mm tips were stripped and tinned on each end as described above. Pin 1 and pin 11 (the ground pins) were soldered together in a 3-way joint with a third wire. The other end of this third wire was tinned for later use during surgery. Only pins 3 – 5 were used for recording channels, and pin 2 for reference; it was necessary to ground the remaining, unused pins together with pins 1 and 11 – the ground positions. The eleven wires were attached to the eleven pins by applying a small amount of flux to each pin and then soldering one piece of the Alpha wire to the corresponding pins on the strip. All eleven wires were soldered to one side of the strip, leaving the other side of the strip (eleven exposed pins) to be placed directly into the 8-channel miniature preamplifier from Multichannel Systems (MPA81 – see Figure 2.4). The solder joints were rinsed in sodium bicarbonate mixture to neutralize the acid in the flux. Canned air was used to dry the montage after rinsing. Next, the solder joints were covered with a 2-part biocompatible epoxy (Epoxy Technology, 354-T, a medium cure thixotropic paste). The epoxy was cured by placing the headpiece into the small toaster oven and heating for ten minutes at 150 degrees C. The continuity was tested using a multimeter with each pin.

![Figure 2.3- MPA81, Multichannel Systems schematic drawing for an 8-channel miniature preamplifier. For these experiments, the following channel designation was used:](image)

Pin 1 – GND (ground)
Pin 2 – Reference Input
Pin 3 to 5 – Recording Channels 1 to 3: EEG1, EEG2, EMG
Pin 6 to 10 – Grounded Channels
Pin 11 – GND (ground)
2.3 Programming MC_Rack Electrophysiological Recording Software and MC_Stimulus II Stimulus Generator Software

2.3.1 MC_Rack Electrophysiological Recording Software

We used the Multichannel Systems software, MC_Rack, to collect data for these optogenetic/ultrasound experiments ([www.multichannelsystems.com](http://www.multichannelsystems.com), Germany). We used the MC_Rack version 4.4.8 to build a custom recording module utilizing the MC_Card hardware menu options. The hardware rack was configured to record a digital track for the transistor-transistor logic (TTL) trigger from a stimulus generator via a BNC connection on the back of the computer and three electrophysiological recording channels (two EEG channels and one EMG channel; labeled Recording Channels). Recording settings were as follows: Amplifier Gain = 1000.00, Input Voltage Range = +/- 819.2 mV, and a Sampling Frequency of 5000 Hz. The filtering option chosen was a Butterworth 2nd Order with a high pass of 0.5 Hz. A band pass filter with a high pass of 0.5 Hz and a low pass of 30.0 Hz was applied to the real-time display of

![Figure 2.4 – A completed MPA81 miniature preamplifier and corresponding headpiece. The first and last pins were soldered together with pins 4 through 8 for the ground, the orange wire was soldered to the reference pin, and the three blue wires were soldered to the recording channels 3, 4 and 5.](image-url)
electrophysiological data display. Data files were recorded in the custom format of Multichannel Systems. MC_DataTool software was used to convert this file to a raw binary file for data analysis in the MATLAB programming environment.

2.3.2 MC_Stimulus II

The Multichannel Systems STG4002 TTL generator was used to control the timing of stimulation and the parallel TTL channel in the recording. The stimulus generator software used for programming this stimulus generator was MC_Stimulus II. This stimulus generator software allows the user to control pulse types, voltage steps, stimulation frequencies and pulse duration intervals. These parameters varied across experiments, as specified in subsequent chapters of this dissertation.

2.4 Surgical/Experimental Protocol

2.4.1 Experimental Animals

Experiments were performed on B6.Cg-Tg(Thy1-COP4/eYFP)18Gfng/J transgenic mice (JAX strain #7612)\textsuperscript{10}. The mouse thymus cell antigen 1 (Thy1) promoter drives the expression of both the blue-light-sensitive cation channel, Channelrhodopsin2 and enhanced yellow fluorescent protein. Channelrhodopsin2 is expressed in cerebral cortical pyramidal neurons,\textsuperscript{10} the majority population of cerebral cortex neurons. While ultrasound stimulation has been applied in non-transgenic mice, it is necessary to use the genetically-engineered ChR2-expressing mouse for optogenetic stimulation. Optogenetic stimulation serves as a control stimulus, as it has unambiguous EEG effects, as documented in our previous work\textsuperscript{6}. Founder mice were bred to CD1 mice at Washington State University. The mice used in experiments, hereafter referred to as Thy1-ChR2 mice, were B6.Cg-Tg(Thy1-COP4/eYFP)18Gfng/J X CD1 F1 males weighing 25.0
to 34.0 grams. Experimentation was approved by the Institutional Animal Care and Use Committee of Washington State University.

Surgery was performed under sterile conditions in adherence to regulatory guidelines. Surgeons wore clean lab coats and sterile surgical gloves. A sterile surgical field was placed over the animal. All surgical tools were appropriately sterilized. Electrodes, fiber optic cables and plastic connectors were subjected to 60 seconds of immersion in ethanol and dried with an application of canned air.

2.4.2 Surgical supply list

1. Scalpel
2. Scissors (straight)
3. Sharps
4. Small screwdriver
5. Straight forceps
6. Enamel coated forceps
7. Stereotaxic probe holder and probe
8. Cotton swabs (both types, wide and narrow tips)
9. Sterile gauze pads (large)
10. Straight hemostat
11. Wire nippers
12. 0.5mm and 0.7 mm ball burr bits (new)
13. Prepared fiber optic cable
14. Ultrasound transducer and cap with snipped syringe tip
15. Ultrasound gel
16. Sterile saline solution
17. Betadine
18. 2% Hydrogen peroxide
19. 70% Ethanol
20. Ortho-Jet dental resin (2 parts, pink liquid and white powder)
21. Fine tipped paint brush
22. Tungsten EEGs
23. Three screw EEGs, one of them attached to a 2 ft. section of Alpha wire with an alligator clip on the opposite end
24. Tungsten EMG
25. MC Systems preamplifier-connector montage
26. Shrink tubing (5 pieces, cut to ~ 5.0 mm lengths)
27. Stereotaxic grounding wire with alligator clip
28. Soldering iron and high temperature solder
29. Soldering flux
2.4.3 Anesthetic Protocol

Anesthesia was administered using an IMPAC Multi Patient system from Vet Equip Inc. Typically, a 5% isoflurane/95% oxygen was used for induction and 2.5% - 3% isoflurane/97% oxygen was used to maintain the animal during surgery. It was necessary to adjust the isoflurane levels often during these experiments – during the craniotomy portion, the levels were high; but during the application of the stimuli, the dosage was lowered appropriately. Positive results were obtained by observation of whisker twitches in response to stimulation under 0.75% to 1.5% isoflurane. Careful monitoring of the animal was required, as temporary increases in the amount of anesthesia delivered to avoid movement during surgery were necessary. It was necessary to maintain 1.5% isoflurane level in order to minimize the animal’s wakefulness during stimulation.

2.4.4 Surgical Site Preparation

Upon initial administration of isoflurane/oxygen, the animal’s head was shaved to remove hair from the surgical field (the skull area from between the nose to the back of the neck). Use of a heating pad, a clean absorbent sheet and an extra isoflurane tube were necessary for this process. The mouse’s weight and surgical start time were recorded. A 5% isoflurane dose was administered to induce anesthesia. The mouse was placed belly down on the heating pad, the isoflurane tube was placed over the snout, and a reduced dose of 2% isoflurane was applied while clippers were used to shave the animal’s head. The animal’s breathing was monitored to ensure that it was not receiving too much anesthetic. After shaving, the mouse was securely placed into a stereotaxic device with appropriate use of ear bars. Once the mouse’s snout was in the nose cone, the screws that securely fasten the baseboard into place were tightened. Whiskers were trimmed on both sides of the animal’s snout in order to ensure that they did not touch the stereotaxic device.
Ophthalmic ointment was used for corneal protection during surgery, with care taken not to get the ointment on the whiskers, as this may have inhibited the movement of the stimulated whiskers. With the animal securely placed in the stereotaxic device, the EMG was implanted in the whisker pad contralateral to the barrel stimulation site.

The top of the head was cleaned using three applications of a scrub/rinse cycle of Betadine scrub followed by an ethanol rinse. Each application of Betadine and ethanol was applied with a new sterile cotton swab. A front to back pattern was used to scrub from the top of the proposed incision to the neck area. The swab was discarded after each use.

Next, a medial incision was made on the top of the mouse’s head, beginning between the eyes and following to the back of the head. The skin was retracted on either side with forceps.

![Figure 2.5 - The EMG is inserted directly into the whisker pad of the mouse’s snout on the side contralateral to the craniotomy over the whisker barrel cortex.](image-url)
allowing for visualization of the skull. A 3\% hydrogen peroxide solution followed by sterile saline (0.9\% NaCl) was applied cyclically three times, just as with the Betadine/ethanol treatment, using a new sterile swab for each application to clean the skull. Excess connective tissue was removed by lifting the skin with forceps and clipping with scissors. A 3 mm incision in the skin at the center of skull (~10 mm from the start of the incision between the eyes) was made in order to retract a piece of the skin on the side where the craniotomy would be performed. Careful use of a cotton swab with hydrogen peroxide was necessary to expose the suture lines and remove remaining tissue. Once the suture lines were clearly visible, a small amount of ethanol was applied with a sterile swab to dry out the area.

Figure 2.6 – Carefully make a 3 mm incision in the skin on the side ipsilateral to the barrel cortex site.
Next, bregma, the site where the four skull plates meet, was located and marked using a cauterizing tool. The stereotaxic coordinates of bregma serve as landmarks for electrode placement and optogenetic/ultrasound stimulus configurations. An electrode is placed at 0.86 mm anterior/1.5 mm lateral relative to bregma to target the motor cortex. Optogenetic and ultrasound stimulations are placed at 1.7 mm posterior/2.5 mm lateral relative to bregma to target the somatosensory barrel cortex (a source of direct neuronal connections to motor cortex) for stimulation. These positions were also marked using the cauterizing tool. The stereotaxic stylus and stereotaxic arm were then removed to allow ample room for the craniotomy, drilling and EEG placement.

2.4.5 Craniotomy Procedure

A craniotomy was performed by first scoring an even square (approximately 3 x 3 mm) around the marked stereotaxic coordinates for the barrel cortex. A high-speed dental drill and a new 0.5 mm ball burr bit were used to establish a uniform perimeter around the barrel cortex - approximately 3 x 3 mm square, by etching the skull. Slow, light passes were made with the drill carefully piercing the perimeter of the craniotomy site. When enough of the bone had been removed, a pair of sharp forceps was used to lift away that section of the skull. The protective layer of the dura mater over the surface of the brain was not punctured with this process, as the piece was peeled away, leaving the dura intact.

2.4.6 Electrode Placement

A 0.5 mm ball burr bit was used to drill an initial hole through the skull for placement of the EEG electrode at the site marked for the motor cortex. The same bit was used to drill four more holes in the skull – one for a reference electrode, one for a ground electrode, one for the second EEG electrode (contralateral frontal), and one for a body ground. (See Figures 2.7-2.8)
The body ground allowed the animal’s body to be grounded to the surgical table via a 0.5 m wire (Alpha Wire, 2840/7, 32 AWG purchased from DigiKey) and an alligator clip. Extensive electrical grounding was essential in this experiment, as the recording was done in an open room subject to a great deal of electrical noise.

It was necessary to use a 0.7 mm ball burr bit to enlarge the three holes used for standard stainless steel screw-type reference and ground electrodes. These electrodes require 20 mm of 30 AWG tinned copper wire (available from AlphaWire, Elizabeth, NJ), soldered to 1/16 inch diameter stainless steel screws. The screw electrodes were secured into place and the wires were positioned such that the wires radiated outward from the center of the animal’s skull.

A pair of enamel forceps was used to insert the tip of a tungsten electrode into the drilled opening for the motor and frontal positions. Ortho-Jet Acrylic Resin, a fast curing, 2-part orthodontic acrylic resin, was used to secure the tungsten electrodes in place. A fine tipped paintbrush was used to apply the substance directly onto the skull at the site of the electrodes.
The acrylic served to secure each electrode in place and to prevent any electrical conductance between the electrodes. The craniotomy site was not exposed to resin during this procedure, as a clear craniotomy was necessary for application of stimulation. Three to five minutes curing time was required after completing application of the resin.

When the resin hardened, the end of each electrode wire was soldered to the pre-built headpiece (refer to the diagram of the preamplifier pin out). For our experiments, channel 1 (pin 3) is the motor cortex EEG, channel 2 (pin 4) is the frontal EEG, and channel 3 (pin 5) is the EMG. The reference EEG is pin 2. All other pin positions were pre-ground together with pins 1 and 11, and soldered to the ground EEG placed in the animal’s skull – not the body ground. The body ground is a separate electrode solely used to ground the animal’s body to the table.

2.4.7 Attaching the preamplifier headpiece

High temperature solder was used to connect the EEG wires with the headpiece montage. Before soldering, small pre-cut pieces of shrink tubing were placed onto the electrode wires. This tubing served to cover the exposed solder joints that result from soldering the electrodes to the headpiece. The soldering iron was used to shrink the tube sufficiently to hold it in place over the joint to reduce electrical noise.

With the wires securely soldered to the headpiece, the Multichannel Systems pre-amplifier was plugged into the correct port on the Multichannel Systems SC8 x 8 console. A large grounding wire was attached from the stereotaxic arm to the surgical table. When the surgical prep was completed, the optogenetic LED stimulation or ultrasound stimulation was applied. The isoflurane/oxygen dosage was reduced to 1.5% during the recording session and the mouse’s breathing was monitored to ascertain that it had returned to a more rapid pace before stimulation occurred.
Grounding was performed immediately prior to the commencement of recording. A wire was attached to the stereotaxic device and connected directly to the surgical table. The entire stereotaxic plate was covered with the small cube shaped Faraday cage – approximately 18 inches long, 18 inches wide, and 18 inches deep. The Faraday cage was built to help eliminate unwanted noise in the signal. The cage was also connected to the surgical table via a grounding wire and alligator clip. The surgical table must be grounded to the ‘earth’ ground present in the electrical outlet in the wall with more grounding wire.

2.5 Optogenetic stimulation apparatus

Our optogenetic stimulation system consisted of a TTL stimulus generator (STG4002), a light source, a power supply, and a fiber optic cable. The TTL generator controlled the frequency and duration of timed light pulses. An LED driver (Doric Lenses, product # D480-1003; description is LED_DRV_1CH, version 2012 with an independent power cord) was the power source for the LED light source. A 1.5-meter length of cable with the corresponding M8-4 pin male and female connectors provided the electrical connection to the LED source. This cable attached to the connectorized high brightness single LED light source (LEDC1-B_FC_M8). The light source was a blue LED with a wavelength of 465 nm, and a bandwidth (full width at half-maximum; FWHM) of 25 nm. The fiber output power at 1000 mA (meaning the power actually delivered by LED driver) for this blue light is 5.0 mW, with a fiber optic core diameter of 200 micrometers and a numerical aperture of 0.48. The numerical aperture is a characteristic number representing a range of angles over which the LED can emit light.

A fiber optic cable was attached to the LED source via a ferrule connector/physical contact (FC/PC) connectorized optical cable from ThorLabs (BFL37 – 200) which was partially jacketed with furcation tubing (part no. FT030, ThorLabs). This furcation tubing formed the
outer jacket of our completed cables and served to protect the fiber optic cable. The tubing itself was comprised of the PVC outer jacket and Kevlar protective threading. The end was fitted with an FC connector, an FC Connector Boot and an FC/PC multimode connector set. An 18-inch section of the fiber optic cable was left bare, the end was flat cleaved with a diamond-scribing tool and polished using ThorLabs polishing paper. The measured power from the fiber optic cable (via a ThorLab optical power meter) was approximately 9.8 microwatts. The power output maximum for this LED, 5.0 mW, can be achieved by lengthening pulse duration, stimulating with continuous waves (CW) versus pulsed waves (PW), and adjusting the frequency and duty cycle. For optogenetic stimulation to be appropriately timed, the LED driver (power supply) is used in TTL triggering mode. A BNC cable conveys the TTL signal from the STG4002 stimulus generator to the LED driver.

2.6 Application of optogenetic stimulation

The stereotaxic arm was used to secure the fiber optic cable into the probe holder and to position the cable over the animal’s skull. The bare fiber end protruded from the stereotaxic device with its exposed end directly over the craniotomy site (without directly contacting the dura mater) for application of the optogenetic stimulation.

2.7 Ultrasound stimulation apparatus

Procedures were modified from Tufail, et al for the assembly and programming of the frequency generators and the radio frequency (RF) amplifier used to power the ultrasound transducer. This process was rigorous, as the transducer is quite sensitive and can be damaged if high-amplitude driving voltages are delivered to the RF amplifier. We used a GMP-based contact/immersion transducer from the Ultran Group in State College, PA (Model # GS350-D19). The transducer’s nominal frequency was 350 kHz, with an observed bandwidth center
frequency of 320 kHz – as reported from laboratory analysis of the transducer from the Ultran Group.

We utilized two Agilent Waveform Generators – 33220A, 20 Hz (Techni-Tool, Worcester, PA; product #431TE3322). The first frequency generator served as a pulse trigger and was used to establish the ultrasound pulse repetition frequency and the number of ultrasound pulses for the desired stimulation. The second frequency generator produced the acoustic frequency, in addition to a set number of acoustic cycles per pulse for each of the ultrasound pulses that created the stimuli.

![Schematic diagram of ultrasound apparatus](image)

Figure 2.9: Schematic diagram of ultrasound apparatus. Acoustic frequency ($A_f$) is the operating frequency of the ultrasonic transducer.

The first frequency generator was set to drive a 5 V<sub>pp</sub> square pulsed wave with the
appropriate number of pulses (each of 0.5 ms duration) to be delivered at a pulse repetition frequency of 2.0 kHz. The generation of a 5 V<sub>pp</sub> signal serves as a TTL input to the second frequency generator. The second frequency generator was adjusted to create the desired ultrasound pulses as sine waves when triggered by the square waves of the first frequency generator. These individual ultrasound pulses consisted of 75 acoustic cycles per pulse at an acoustic frequency of 350 kHz (yielding a pulse duration of approximately 0.214 ms). The output of this acoustic wave frequency generator varied across experiments from 0.25 to 1.0 V<sub>pp</sub>. An oscilloscope connected to the frequency generators via BNC cable allowed for visualization of the ultrasound waveforms. An RF amplifier (240L Power Amplifier from Electronics and Innovation, Ltd. in Rochester, NY; Item description – E&I 240L) was controlled by the second frequency generator via a BNC cable. The amplifier was placed inside a Faraday cage to eliminate the excessive noise introduced into the recording room from this piece of equipment. A cable exiting the side of the Faraday cage conveyed electrical signal to the transducer head.

2.8 Application of ultrasound stimulation

The transducer head was covered with gel before use to provide the necessary medium for the transduction of the generated waves. In an attempt to narrow the focus of the transducer, a coupling column was used. This column was made by clipping the tip from a 1.0 mL plastic syringe. A transducer cap was fitted with the small syringe tip. The transducer cap was filled with ultrasound gel to one-half of its volume and placed firmly over the end of the transducer. Pressure was applied to the transducer cap and syringe tip until the gel began to exude from the syringe tip. The ultrasound transducer was placed into a circular holder accessory and attached to the stereotaxic device. The stereotaxic arm was used to position the ultrasound transducer over the animal’s skull at the site of the craniotomy.
The stereotaxic arm was used to lower the opening of the gel filled syringe tip onto the craniotomy site until a depression of the skull was seen, creating a contact between the syringe tip and the exposed dura. Once the transducer was positioned, the electrical connection from the programmed STG4002 to frequency generator 1, to frequency generator 2, to RF amplifier and to the transducer was established, and MC_Stim stimulation protocols were used to apply the desired ultrasound stimulation parameters.

2.9 Recording optogenetic and ultrasound electrophysiological responses

After placement of the stimulation apparatus (fiber optic cable or transducer), the signal was tested. A five-minute 10 Hz stimulation protocol was applied. Visual inspection of a fast Fourier transformation of the motor-frontal derived signal, generated in the MATLAB programming environment (see below), was used to determine whether the stimulus triggered an electrophysiological response. In the absence of such a response, the stimulation apparatus was
repositioned and the process repeated. When responses to stimulation were detected by visual inspection, experimental recordings were performed.

2.10 Using MATLAB for real time and post recording analysis

Electrophysiological signals collected at 5000 Hz were processed in the MATLAB programming environment. To account for drift in the signal, data were subjected to normalization, first by subtracting the mean of all sampled values across the recording from each individual sampled value in the recording. Next, the data were subjected to a smoothing algorithm, in which the mean of the potential across a 3 ms window centered on each data point was subtracted from the value at that data point. This function only nominally changes the physiological response (i.e. the amplitude is slightly adjusted). Because of the size of the window chosen for smoothing, the operation itself tended to further center the signal around zero. A single vector consisting of 15000 data points (3-seconds of data) was generated from these transformed EEG potential values by averaging values across 3-second segments of data centered on every TTL-triggered stimulus onset in the file. For the purpose of data visualization (though not data analysis) this averaged curve for the recording was subjected to a 60-Hz bandstop elliptic filter. The 60 Hz electrical noise that was present from various sources in the surgical suite was removed through this process. However, the averaged triggered biological response was more robust than the 60 Hz noise and this filtering was not used in the final data analysis. In addition to the TTL-triggered data segments, an equivalent number of randomly timed 3-second segments of data from the same file were subjected to the same smoothing, normalization and averaging procedures in order to yield a randomized data vector as an experimental control.
The resulting 3-second segments of triggered and random data were subjected to fast Fourier transform (FFT) with the MATLAB FFT function. The 3-second data segments and the resulting FFTs for both triggered and randomly-timed data were plotted within the MATLAB environment immediately after data collection. This procedure allowed us to visualize the response to stimulation and adjust stimulation sites accordingly.

![Graphs showing FFTs of triggered and randomly-timed data](image)

**Figure 2.11:** Example of smoothed and filtered plot of electrophysiological response to ultrasound stimulation in one representative animal.

Finally, an output routine for exporting the average response of all stimuli in one recording into an Excel spreadsheet allowed for statistical analysis across the entire data set of animals. The program Statistica, a statistics and analysis software package produced by StatSoft (Tulsa, OK) then enabled calculation of descriptive and inferential statistics across n=8 animals per experiment. SigmaPlot, a scientific graphing and statistical analysis platform from Systat Software, Inc. (San Jose, CA) was used to generate graphs.
CHAPTER 3: VERIFICATION OF ELECTROPHYSIOLOGICAL RESPONSE TO ULTRASOUND STIMULATION

In order to address the hypothesis that ultrasonic neuromodulation can be used to manipulate slow wave sleep, it was necessary to establish reliable collection of an electroencephalogram in response to cerebral cortical stimulation by ultrasound. The following questions were asked:

- Is there a quantifiable EEG response to the TTL trigger?
- How do responses to varying stimulation frequencies and stimulation types compare – is one frequency more reliable/robust than another?
- Are the electrical responses to ultrasound stimulation physiologically-mediated?

We have published experimental evidence of optogenetically-triggered EEG events. Optogenetic stimulation of the cerebral cortex with a 473 nm LED-generated blue light delivered via a fiber-optic probe was used as a positive control. The response to ultrasound stimulation was then measured using the same electrophysiological recording system, stimulus timing mechanisms and algorithmic processing to verify the physiological basis for evoked physiological responses to ultrasound stimulation.

This technical challenge proved to be tedious in its execution. While there are examples of collection of electromyogram (EMG) response to ultrasound stimulation, current literature did not provide specific examples of electrophysiological responses to ultrasound stimulation within the cerebral cortex. The evidence of evoked responses to ultrasound available in the literature to date consist of either visual or electromyographic detection of skeletal muscle contractions.\textsuperscript{11, 16} Previous reports described difficulty with excess electrical noise present in the biological signal when using ultrasound transducers that require high-powered radio frequency (RF) amplifiers.
and frequency generators. The presence of this electrical noise required a specifically constructed experimental system that enabled careful grounding of the animal and the recording equipment.

In our previous optogenetic experiments, we worked with freely moving mice fitted with a surgically implanted cannula designed to house a fiber optic cable for optogenetic manipulation of the cerebral cortex. In order to show that ultrasound can be used as a non-invasive alternative to the optogenetic technique, it was necessary to perform head-to-head comparisons of each stimulation type in the same animal. The use of an ultrasound transducer prohibited the application of stimulation in freely moving mice. Instead, the stimulations were applied to lightly anesthetized mice in a surgical setting. Animals received an initial dosage of 5% isoflurane, reduced to 3.5% during surgery, and maintained at 1.5% isoflurane during the experimental application of stimuli.

Prior optogenetic experiments in our lab required application of light via a fiber optic cannula applied intra-cortically. To provide a parallel noninvasive optogenetic stimulus for comparison to noninvasive ultrasound, it was necessary to demonstrate electrophysiological responses to light applied via a fiber optic cable placed on or above the dura mater overlaying the cerebral cortex. This was achieved by performing a 3 mm by 3 mm craniotomy over the site of the whisker barrel cortex. Placement of the electrodes relied upon stereotaxic coordinates as identified in Franklin and Paxinos The Mouse Brain in Stereotaxic Coordinates.²⁰

It was necessary to use Thy1-ChR2 transgenic mice expressing the blue light sensitive cation channel, Channelrhodopsin2, in cerebral cortical neurons for optogenetic stimulation of electrophysiological responses. Because ultrasound neuromodulation did not require the use of a specific genetic makeup, but rather utilizes physical mechanisms independent of any genetic modification, we do not believe that our use of this transgenic line impacted the
electrophysiological response to ultrasound stimulation. Using methods described in chapter two, we applied both optogenetic stimulation at 1 Hz and 7 Hz, and ultrasound stimulation at 1 Hz and 7 Hz to each of eight mice (see table 3.1 for the sequence of stimulation sessions for each animal). All stimulus pulses were 10 ms in duration in this experiment. A transistor-transistor logic (TTL) trace collected from the stimulus generator in parallel with the motor-frontal derived electrical potential was used to collect event-triggered potential events. The 1 Hz stimulus condition thus generated 600 TTL-event triggered potentials; The 7 Hz stimulus condition thus generated 4200 TTL-event triggered potentials. An averaged curve was generated across all TTL-event triggered potentials within each 10-minute recording session. Electrical potential extracted from an equivalent number of randomly timed events was used to generate an averaged random event curve for statistical comparison, the fluctuations of which are unrelated to stimulation. Statistical comparison of the stimulus-triggered averaged curves vs. random averaged curves were used to discriminate physiological responses from noise.

The 1 Hz variable proved to be difficult to detect in the initial optogenetic and ultrasound records. The FFT showed aliasing at 1 Hz intervals and made verification of a 1 Hz signal unclear (Figure 3.1). In order to avoid aliasing from the 60 Hz noise in the room, we choose a 7 Hz stimulation frequency. The 7 Hz stimulation frequency lies within the range of typical neuronal activity in the mammalian cerebral cortex. In order to confirm that the response to the stimulus was biological, each animal was subjected to stimuli after post-surgical euthanasia. This is denoted by post mortem in table 3.1.

Offline analysis of the data allowed us to generate averaged curves within each recording session representing the changes in electrical potential at time points relative to stimulus onset. Individual examples of the offline analysis from each of the eight experimental conditions (1 vs.
7 Hz stimulation; optogenetic vs. ultrasound stimulation; live animals vs. post mortem tissue) are shown in figures 3.1 through 3.16. As expected, the FFT of the event-triggered modulation of electrophysiological responses of the motor cortex to 7 Hz stimulation by either optogenetic (Figure 3.5) or ultrasound (Figure 3.13) stimuli yielded a peak in the FFT at 7 Hz, with aliasing at multiples of 7 Hz. However, these were obvious and allowed for unambiguous verification of the response to 7 Hz stimulation. This experiment served to establish the use of 7 Hz stimulation frequency for the remainder of this dissertation project.

In order to quantify the magnitude of the response to optogenetic and ultrasound stimulation, the positive and negative deflections in event-triggered potentials were detected algorithmically from each curve representing the averaged event triggered potential within 72 ms of stimulus onset (corresponding to a full cycle of 7 Hz stimulation; the shorter in duration of the two stimulus frequencies). Negative deflections were defined as the interval between each downward zero cross and the subsequent upward zero cross. Positive deflections were defined as the interval between each upward zero cross and the subsequent downward zero cross. For both positive and negative deflections, the area under the curve (AUC; Figures 3.21, 3.22, 3.29, 3.30) was calculated by summation of all electrical potential values across the duration of that deflection. For both positive and negative deflections, the mean voltage (MV; Figures 3.23, 3.24, 3.31, 3.32) was calculated by division of the AUC values by the number of data points in that deflection. For negative deflections, the peak voltage (PV; Figures 3.25, 3.26, 3.33, 3.34) was the minimum value detected during that negative deflection. For positive deflections, the peak voltage (PV; Figures 3.25, 3.26, 3.33, 3.34) was the maximum value detected during that positive deflection. For both positive and negative deflections, the event duration (ED; Figures
3.27, 3.28, 3.35, 3.36) was the time lapsed from the zero cross defining deflection onset and the zero cross defining deflection offset.

To verify that the response to ultrasound stimulation was physiological in nature, and not due to a mechanical signal from the electricity needed to power the ultrasound transducer, we compared the potential versus time graphs of live animals (Figures 3.1, 3.5, 3.9, 3.13) with those of the animals after euthanasia was performed (Figures 3.3, 3.7, 3.11, 3.15). The triggered evoked potentials were plotted against time and were compared to similar graphs generated by choosing random time samples present in the potential record, which were devoid of stimulation, but with equal number of data points. This was done for both optogenetic (Figures 3.1, 3.3, 3.5, 3.7) and ultrasound (Figures 3.9, 3.11, 3.13, 3.15) stimulation. Negative and positive deflections were detected from these averaged curves (Figures 3.17-3.20). Event-triggered parameters detected from the positive and negative deflections from live vs. dead tissue are compared in figures 3.21-3.28. Comparison of these parameters from event-triggered potentials to those of randomly selected segments of electrophysiological potential data from the same recordings are presented in figures 3.29-3.36.

Analysis of variance (ANOVA) was used to determine whether the response to optogenetic (Table 3.2) or ultrasound (Table 3.3) stimulation was dependent on living tissue. ANOVA indicated statistically significant effects of physiological state (live vs. dead tissue) on the AUC, MV, PV and ED for negative deflections (Table 3.2 all F statistics > 10; P < 0.006) and positive deflections (Table 3.2 all F statistics > 8; P < 0.011) in electrical potential in response to optogenetic stimulation. Similarly, ANOVA indicated statistically significant effects of physiological state (live vs. dead tissue) on the AUC, MV, PV and ED for negative deflections
(Table 3.3 all F statistics > 4; P < 0.047) and positive deflections (Table 3.3 all F statistics > 12; P < 0.003) in electrical potential in response to optogenetic stimulation. These comparisons indicate that the modulations in electrical potential that occur in association with optogenetic stimulation are a function of living tissue. Responses to both optogenetic and ultrasound stimulation are physiological in nature. The physiological nature of the response is not a function of specific stimulus frequencies. With the exception of AUC values for positive deflections occurring in response to ultrasound stimulation (F=7.24, P= 0.014), ANOVAs failed to detect a statistically significant interaction of physiological state (live vs. dead tissue) and stimulus frequency in affecting the magnitude of responses to stimulation. Thus, for all other parameters, the dependence of the electrophysiological response on living tissue was intact for either 7 or 1 Hz stimulation.

The application of the optogenetic stimulation (for example Figure 3.1, Figure 3.5) resulted in a much more robust response when compared to the ultrasound stimulation (for example Figure 3.9, Figure 3.13). For instance, the peak positive voltage evoked by optogenetic stimulation at 1 Hz (Figure 3.25) was approximately ten-fold greater than peak positive voltage evoked by ultrasound stimulation at 1 Hz (Figure 3.33), although both stimuli were dependent on live tissue for their peak responses. The magnitude of the optogenetic response reflects the vast number of neurons being affected by optogenetic stimulation in this transgenic line of mice. Given that the light is placed directly on the dura, we hypothesize that millions of neurons are being stimulated with each pulse. Additional work was undertaken (see next chapter) to determine whether modifications in stimulus intensity would result in more equivalent responses to these two distinct stimulation modalities.
Figure 3.0: This graph displays visual representation of the values used in the statistical analyses performed for each experiment.
Figure 3.1: Graphs from one representative sample of the response to LED
Conventions are identical to those in Figure 3.1.

Figure 3.2: Graphs taken from a zoomed in trace from one representative sample of the response to LED optogenetic stimulation at 1 Hz. Format and labeling equal.
Labeling conventions are identical to those in figure 3.1. For example, simulation at 1 Hz applied subsequent to euthanasia. Format and orientation were smoothed in GIMP 2.12.16

**Figure 3.3** Graphs from one representative sample of the response to LED
Format and labeling conventions are identical to those in Figure 3.1.

**Figure 3.4**: Graphs taken from a zoomed-in trace from one representative sample
Figures 3.5: Graphs from one representative sample of the response to LED.

Figures 3.5:

A.

B.

C.

D.

E.
Conventions are identical to those in Figure 3.1. Format and labeling of the response to LED optical stimulation at 7 Hz. Plots taken from a zoomed in trace from one representative sample.
Labeling conventions are identical to those in Figure 3.1.

Figure 3.7: Graphs from one representative sample of the response to LED
Formal and labeling conventions are identical to those in Figure 3.1.

Figure 3.8: Graphs taken from a zoomed in trace from one representative sample.
Identical to those in Figure 3.1.

Figure 3.9: Graphs from one representational sample of the response to ultrasound stimulation at 1 Hz in a living animal. Format and labeling conventions are identical to those in Figure 3.1.
Labeling conventions are identical to those in Figure 3.1.

Figure 3.10: Graphs taken from a zoomed in trace from one representative sample
conventions are identical to those in Figure 3.1.

Figure 3.11: Graphs from one representational sample of the response to ultrasound

D. 

E. With corroboration in L. D. 0. C. 2. 1. 3. C. 5. Content. How.
Figure 3.12: Graphs taken from a zoomed-in trace from one representative sample.

Format and labeling conventions are identical to those in Figure 3.1.
Identical to those in Figure 3.1.

Simulation at 7 Hz in a living animal. Format and labeling conventions are identical to those in Figure 3.1.

Figure 3.13: Graphs from one representative sample of the response to ultrasound.

A.

B.

C.

D.
Labeling conventions are identical to those in Figure 3.1.

**Figure 3.14:** Graphs taken from a zoomed-in frame from one representative sample.

**A.**

**B.**

**C.**

**D.**
Figure 3.15: Graphs from one representative sample of the response to ultrasound stimulation at 7 Hz applied subsequent to euthanasia. Formal and labeling conventions are identical to those in Figure 3.1.
Form and labelling conventions are identical to those in Figure 3.1.

**Figure 3.16:** Graphs taken from a zoomed in face from one representative sample.
Figure 3.17: Voltage versus time graphs of the trajectories of positive and negative deflections upon application of ultrasound stimulation. Data are mean curves of all triggered responses from a single representative animal. Black symbols denote response in the living animal. White symbols denote responses subsequent to euthanasia.
A. Positive deflection in response to 1 Hz ultrasound stimulation.
B. Positive deflection in response to 7 Hz ultrasound stimulation.
C. Negative deflection in response to 1 Hz ultrasound stimulation.
D. Negative deflection in response to 7 Hz ultrasound stimulation.
Figure 3.18: Voltage versus time graphs of the trajectories of positive and negative deflections upon application of optogenetic stimulation. Data are mean curves of all triggered responses from a single representative animal. Black symbols denote response in the living animal. White symbols denote responses subsequent to euthanasia.

A. Positive deflection in response to 1 Hz optogenetic stimulation.
B. Positive deflection in response to 7 Hz optogenetic stimulation.
C. Negative deflection in response to 1 Hz optogenetic stimulation.
D. Negative deflection in response to 7 Hz optogenetic stimulation.
Figure 3.19: Voltage versus time graphs comparing the trajectories of positive and negative deflections upon application of ultrasound stimulation to randomly selected event traces from the same recording. Data are mean curves of all triggered responses from a single representative animal. Black symbols denote stimulus-triggered responses. White symbols denote randomly selected event traces.

A. Positive deflection in response to 1 Hz ultrasound stimulation.
B. Positive deflection in response to 7 Hz ultrasound stimulation.
C. Negative deflection in response to 1 Hz ultrasound stimulation.
D. Negative deflection in response to 7 Hz ultrasound stimulation.
Figure 3.20: Voltage versus time graphs comparing the trajectories of positive and negative deflections upon application of optogenetic stimulation to randomly selected event traces from the same recording. Data are mean curves of all triggered responses from a single representative animal. Black symbols denote stimulus-triggered responses. White symbols denote randomly selected event traces.

A. Positive deflection in response to 1 Hz optogenetic stimulation.
B. Positive deflection in response to 7 Hz optogenetic stimulation.
C. Negative deflection in response to 1 Hz optogenetic stimulation.
D. Negative deflection in response to 7 Hz optogenetic stimulation.
Figure 3.21: Area under the curve (AUC) values for optogenetic stimulus-triggered positive and negative events shown for data collected from n = 8 animals in live (black bars) versus post-euthanasia (gray bars) conditions. A. AUC of positive deflection from 1 Hz stimulation. B. AUC of positive deflection from 7 Hz stimulation. C. AUC of negative deflection from 1 Hz stimulation. D. AUC of negative deflection from 7 Hz stimulation. Asterisks indicate a significant difference from dead condition according to post hoc analysis by Fisher’s Least Significant Difference.
Figure 3.22: Area under the curve (AUC) values for ultrasound stimulus-triggered positive and negative events shown for data collected from n = 8 animals in live (black bars) versus post-euthanasia (gray bars) conditions. A. AUC of positive deflection from 1 Hz stimulation. B. AUC of positive deflection from 7 Hz stimulation. C. AUC of negative deflection from 1 Hz stimulation. D. AUC of negative deflection from 7 Hz stimulation. Asterisks indicate a significant difference from dead condition according to post hoc analysis by Fisher’s Least Significant Difference.
Figure 3.23: Mean voltage (MV) values for optogenetic stimulus-triggered positive and negative events shown for data collected from n = 8 animals in live (black bars) versus post-euthanasia (gray bars) conditions. 

A. MV of positive deflection from 1 Hz stimulation. 
B. MV of positive deflection from 7 Hz stimulation. 
C. MV of negative deflection from 1 Hz stimulation. 
D. MV of negative deflection from 7 Hz stimulation. Asterisks indicate a significant difference from dead condition according to post hoc analysis by Fisher’s Least Significant Difference.
Figure 3.24: Mean voltage (MV) values for ultrasound stimulus-triggered positive and negative events shown for data collected from $n = 8$ animals in live (black bars) versus post-euthanasia (gray bars) conditions. A. MV of positive deflection from 1 Hz stimulation. B. MV of positive deflection from 7 Hz stimulation. C. MV of negative deflection from 1 Hz stimulation. D. MV of negative deflection from 7 Hz stimulation. Asterisks indicate a significant difference from dead condition according to post hoc analysis by Fisher’s Least Significant Difference.
Figure 3.25: Peak voltage (PV) values for optogenetic stimulus-triggered positive and negative events shown for data collected from \( n = 8 \) animals in live (black bars) versus post-euthanasia (gray bars) conditions. **A.** PV of positive deflection from 1 Hz stimulation. **B.** PV of positive deflection from 7 Hz stimulation. **C.** PV of negative deflection from 1 Hz stimulation. **D.** PV of negative deflection from 7 Hz stimulation. Asterisks indicate a significant difference from dead condition according to post hoc analysis by Fisher’s Least Significant Difference.
Figure 3.26: Peak voltage (PV) values for ultrasound stimulus-triggered positive and negative events shown for data collected from \( n = 8 \) animals in live (black bars) versus post-euthanasia (gray bars) conditions. A. PV of positive deflection from 1 Hz stimulation. B. PV of positive deflection from 7 Hz stimulation. C. PV of negative deflection from 1 Hz stimulation. D. PV of negative deflection from 7 Hz stimulation. Asterisks indicate a significant difference from dead condition according to post hoc analysis by Fisher’s Least Significant Difference.
**Figure 3.27**: Event duration (ED) values for optogenetic stimulus-triggered positive and negative events shown for data collected from $n = 8$ animals in live (black bars) versus post-euthanasia (gray bars) conditions. **A.** ED of positive deflection from 1 Hz stimulation. **B.** ED of positive deflection from 7 Hz stimulation. **C.** ED of negative deflection from 1 Hz stimulation. **D.** ED of negative deflection from 7 Hz stimulation. Asterisks indicate a significant difference from dead condition according to *post hoc* analysis by Fisher’s Least Significant Difference.
**Figure 3.28**: Event duration (ED) values for ultrasound stimulus-triggered positive and negative events shown for data collected from n = 8 animals in live (black bars) versus post-euthanasia (gray bars) conditions.  
**A.** ED of positive deflection from 1 Hz stimulation.  
**B.** ED of positive deflection from 7 Hz stimulation.  
**C.** ED of negative deflection from 1 Hz stimulation.  
**D.** ED of negative deflection from 7 Hz stimulation.  
Asterisks indicate a significant difference from dead condition according to *post hoc* analysis by Fisher’s Least Significant Difference.
Figure 3.29: Area under the curve (AUC) values for optogenetic stimulus-triggered (black bars) vs. random (gray bars) positive and negative events shown for data collected from n = 8 animals.  

**A.** AUC of positive deflection from 1 Hz stimulation.  

**B.** AUC of positive deflection from 7 Hz stimulation.  

**C.** AUC of negative deflection from 1 Hz stimulation.  

**D.** AUC of negative deflection from 7 Hz stimulation.  

Asterisks indicate a significant difference from dead condition according to *post hoc* analysis by Fisher’s Least Significant Difference.
Figure 3.30: Area under the curve (AUC) values for ultrasound stimulus-triggered (black bars) vs. random (gray bars) positive and negative events shown for data collected from n = 8 animals.  
A. AUC of positive deflection from 1 Hz stimulation.  
B. AUC of positive deflection from 7 Hz stimulation.  
C. AUC of negative deflection from 1 Hz stimulation.  
D. AUC of negative deflection from 7 Hz stimulation.  
Asterisks indicate a significant difference from dead condition according to post hoc analysis by Fisher’s Least Significant Difference.
Figure 3.31: Mean voltage (MV) values for optogenetic stimulus-triggered (black bars) vs. random (gray bars) positive and negative events shown for data collected from n = 8 animals. 

A. MV of positive deflection from 1 Hz stimulation. 

B. MV of positive deflection from 7 Hz stimulation. 

C. MV of negative deflection from 1 Hz stimulation. 

D. MV of negative deflection from 7 Hz stimulation. 

Asterisks indicate a significant difference from dead condition according to *post hoc* analysis by Fisher’s Least Significant Difference.
Figure 3.32: Mean voltage (MV) values for ultrasound stimulus-triggered (black bars) vs. random (gray bars) positive and negative events shown for data collected from n = 8 animals. A. MV of positive deflection from 1 Hz stimulation. B. MV of positive deflection from 7 Hz stimulation. C. MV of negative deflection from 1 Hz stimulation. D. MV of negative deflection from 7 Hz stimulation.
Figure 3.33: Peak voltage (PV) values for optogenetic stimulus-triggered (black bars) vs. random (gray bars) positive and negative events shown for data collected from n = 8 animals. A. PV of positive deflection from 1 Hz stimulation. B. PV of positive deflection from 7 Hz stimulation. C. PV of negative deflection from 1 Hz stimulation. D. PV of negative deflection from 7 Hz stimulation. Asterisks indicate a significant difference from dead condition according to post hoc analysis by Fisher’s Least Significant Difference.
Figure 3.34: Peak voltage (PV) values for ultrasound stimulus-triggered (black bars) vs. random (gray bars) positive and negative events shown for data collected from n = 8 animals. A. PV of positive deflection from 1 Hz stimulation. B. PV of positive deflection from 7 Hz stimulation. C. PV of negative deflection from 1 Hz stimulation. D. PV of negative deflection from 7 Hz stimulation.
Figure 3.35: Event duration (ED) values for optogenetic stimulus-triggered (black bars) vs. random (gray bars) positive and negative events shown for data collected from n = 8 animals.  A. ED of positive deflection from 1 Hz stimulation.  B. ED of positive deflection from 7 Hz stimulation.  C. ED of negative deflection from 1 Hz stimulation.  D. ED of negative deflection from 7 Hz stimulation. Asterisks indicate a significant difference from dead condition according to post hoc analysis by Fisher’s Least Significant Difference.
Figure 3.36: Event duration (ED) values for ultrasound stimulus-triggered (black bars) vs. random (gray bars) positive and negative events shown for data collected from n = 8 animals. A. ED of positive deflection from 1 Hz stimulation. B. ED of positive deflection from 7 Hz stimulation. C. ED of negative deflection from 1 Hz stimulation. D. ED of negative deflection from 7 Hz stimulation.
and 1 Hz and opposite stimuli all 1 Hz and 1 Hz to n = 8 animals in a randomized order.

### Table 3.1: Stimulation Protocol used in the 1 vs 7 Hz Experiments

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<th>7 Hz</th>
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First Stimulation Module | Second Stimulation Module
---|---
Post-Mortem Stim. Module
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<th>Positive Deflection</th>
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<td>F(df)</td>
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<tr>
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</tr>
<tr>
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<tr>
<td>Interaction</td>
<td>N.S.</td>
<td>N.S.</td>
<td></td>
</tr>
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**Table 3.2:** Analysis of variance for effects of physiological state (live animal vs. dead animal), stimulus frequency (1 Hz Vs. 7 Hz) and their interaction in affecting four parameters extracted from optogenetically-triggered potentials in the motor-frontal derivation. Separate ANOVAs were performed for parameters derived from the negative deflections and positive deflections. ANOVA F values that were statistically significant (P <0.05) are shown in red. N.S. indicates non-significant experimental effects.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Effect</th>
<th>Negative Deflection</th>
<th>Positive Deflection</th>
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<td>F(df)</td>
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<tr>
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<td>N.S.</td>
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<tr>
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<td>0.046</td>
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<td>Stimulus Frequency</td>
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<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>Interaction</td>
<td>N.S.</td>
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<tr>
<td>Physiological State</td>
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</tr>
<tr>
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<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>Interaction</td>
<td>N.S.</td>
<td>N.S.</td>
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</tr>
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</table>

Table 3.3: Analysis of variance (ANOVA) for effects of physiological state (live animal vs. dead animal), stimulus frequency (1 Hz Vs. 7 Hz) and their interaction in affecting four parameters extracted from ultrasound-triggered potentials in the motor-frontal derivation. Separate ANOVAs were performed for parameters derived from the negative deflections and positive deflections. ANOVA F values that were statistically significant (P < 0.05) are shown in red. N.S. indicates non-significant experimental effects.
CHAPTER 4: ESTABLISHING AN INTENSITY-RESPONSE CURVE

The second experiment sought to determine first, whether there existed an intensity proportionate electrophysiological response to transcranial ultrasound, and second, whether the electrophysiological responses to optogenetic and ultrasound stimuli of equivalent magnitude were similar in their temporal profiles. Further optimization of the electrophysiological signal in this experiment resulted in markedly improved signals that were more easily detected (Figures 4.1-4.8). Once we achieved dependable physiological responses to ultrasound stimuli, we used the 7 Hz stimulation frequency to allow for reliable detection of event-triggered fluctuations in electrical potential. It was necessary to design an experiment that allowed us to explore whether both the LED optogenetic and transcranial ultrasound stimuli could produce responses of the equivalent amplitude and temporal profile. This would prove significant in order to establish ultrasound neuromodulation as an effective alternative to the optogenetic technique.

Because the optogenetic and ultrasound stimuli are generated by distinct physical mechanisms (conversion of electricity to light in the first case and conversion of electricity to ultrasonic vibration in the second), it was necessary to use distinct means to vary the intensity of stimulation. Ultrasound stimulation intensity was varied by altering the voltage of the output of the radio frequency (RF) amplifier that drives the vibration of the ultrasound transducer. The limiting factor of a maximum voltage output of the ultrasound transducer at 1.0 V_pp yielded the choice of ultrasound voltages of 250 mV, 500 mV, 750 mV and 1000 mV. The 9.8 microwatt blue light stream emitted by the fiber optic cable was invariable due to constraints of the experimental system that we use. It was necessary to alter the placement of the fiber optic cable relative to the level of the craniotomy in order to vary the number of photons reaching the...
cerebral cortex below the craniotomy. Accordingly, the cable was positioned at distances 2 cm, 4 cm, 6 cm, and 8 cm above the craniotomy for the duration of each experimental animal’s electrophysiological recording. The stereotaxic device was used to accurately measure the desired positions. The power delivered to the fiber optic cable remained constant at 9.8 microwatts. Each animal received 10 minutes of continuous stimulation of both stimulus types at each level of intensity in a randomized order. See table 4.1 for the experimental protocol for n = 8 male mice.

Deflections in electrical potential were apparent in both the averaged event triggered potentials and the associated fast Fourier transforms (FFTs; Figures 4.1-4.4) of optogenetic stimulation data. The magnitude of evoked response parameters was inversely proportionate to the distance between the fiber optic tip and the surface of the cerebral cortex (Figures 4.9-4.13). Statistical analyses of the evoked response parameters by repeated measures ANOVA (Table 4.2) indicated significant effects of stimulation on area under the curve (F > 11; P < 0.003), mean voltage (F > 11; P < 0.003) and peak voltage (F > 10; P < 0.004) for both positive and negative deflections induced by stimulation. These data demonstrate a significant electrophysiological response to optogenetic stimulation. Additionally, repeated measures ANOVA (Table 4.2) yielded a significant interaction of optogenetic stimulus intensity and event type in affecting the magnitude of response parameters (F > 2.9, P < 0.05), with the exception of the duration of the positive event-triggered deflection. In planned comparisons, significant electrophysiological responses were only significant in response to higher intensity optogenetic stimuli (panels A and B of Figures 4.10-4.13). These data are evidence of an intensity-proportionate response to optogenetic stimulation within the range of stimulus intensities delivered.
Triggered potential fluctuations were apparent in both the averaged event triggered potentials and the associated FFTs of ultrasound stimulation data (Figures 4.5-4.8), verifying a response to the 7 Hz stimulus. Statistical analyses of the evoked response parameters by repeated measures ANOVA (Table 4.3) indicated significant effects of stimulation on area under the curve (F > 11; P < 0.003), mean voltage (F > 11; P < 0.003) and peak voltage (F > 10; P < 0.004) for both positive and negative deflections induced by stimulation. These data demonstrate a significant electrophysiological response to ultrasound stimulation. However, repeated measures ANOVA (Table 4.3) did not detect a significant interaction of ultrasound stimulus intensity in affecting the magnitude of these response parameters (panels C and D of Figures 4.10-4.13). Therefore, there is no evidence of an intensity-proportionate response to ultrasound stimulation within the range of stimulus intensities delivered.

A decrease in distance of placement of fiber optic cable above the skull resulted in an increase in the amplitude of the physiological response to optogenetic stimuli. With the optogenetic stimulations, we found that the magnitude of the response was greater when the fiber optic cable was placed closer to the craniotomy site. In fact, the magnitude of the response was approximately four times greater when the cable was placed at 2 cm above the site when compared to the magnitude of the response when the cable was placed 8 cm above the site. By contrast, a four-fold increase in the voltage output of the ultrasound transducer from 250 mV to 1000 mV did not yield a significant increase in amplitude of the physiological response to ultrasound stimuli.

The primary purpose for this portion of the project was to identify a ‘matching’ set of stimulus parameters that would produce relatively equivalent responses in order to proceed with non-invasive ultrasound as a replacement for the more invasive optogenetic technique. Having
established an intensity proportionate curve, we found that we could induce responses of equivalent magnitude for both types of stimuli. This further allowed us to compare the shapes of the waveforms induced by stimuli of equivalent intensities. We examined changes in potential across time relative to stimulus onset, which occurs at time zero in figure 4.14. These data represent results from the averages of values from eight animals subjected to both types of stimulation at equivalent intensity. The red curve indicates the response to a 10 ms ultrasound stimulus at 1000 mV, the black curve represents the response to a 10 ms optogenetic stimulus with a placement of 6 cm above the exposed dura. We can see near total overlap in the fluctuation subsequent to stimulus onset at time zero.

It is known that the ChR2 neurons are pyramidal cells and the responses to optogenetic stimulation are mediated by a focal activation of neuronal ion channels. The striking similarity of the ultrasound-induced response to the optogenetically induced response leads us to suspect that it is also mediated by ion channel dependent processes in neurons. Because we are the first group to measure EEG effects of ultrasound stimulation, we are offering, with these overlaid curves, new insight into the possible mechanisms of ultrasound neuronal modulation.
A. Voltage versus time graph showing patterns of data sequences extracted from the simulation on an electrode located 5 cm above the surface of the brain.

B. Voltage versus time graph illustrating patterns for LED opogeneal simulation occurring at 7 Hz.

C. A representative example of the response to LED opogeneal stimulation at 7 Hz.

D. Magnitude versus time graph illustrating patterns of the mean integrated waveforms.
those in figure 4.1. Superficial (6 cm above the surface of the brain), FOM, and labeling conventions are identical to
7 Hz. Opposite stimuli were delivered via a fiber optic cable positioned at a distance of 6 cm
FIGURE 4.2: Graphs from one representative sample of the response to TCD. Opposite stimuli
EL I with smoothing in 0.5-15 Hz. Opp bends.
In Figure 1, those in Figure 4.1...
Those in Figure 4.1.

Figure 4.4: Graphs from one representative sample of the response to LED ophthalmic stimulation at 7 Hz. Ophthalmic stimuli were delivered via a Fiber optic cable positioned a distance of 2 cm supranumeral (2 cm above the surface of the brain). Formal and labeling conventions are identical to

Figure 4.3 with smoothing in 0.1-15 Hz. ICP or EEG.
Figure 4.5: Graphs from one representative sample of the response to ultrasound stimulation at 7 Hz. Ultrasound stimuli (250 mw) were applied epidurally via a transducer positioned in contact with the cranium, site. Form and labeling conventions are identical to those in Figure 4.1.
with the chromatographic flow. Form and labeling conventions are identical to those in Figure 4.1. 

HZ ultrasound stimuli (500 mW) were applied epidurally via a transducer positioned in contact.

Figure 4.6: Graphs from one representative sample of the response to ultrasound stimulation at 7
Figure 4.7: Graphs from one representative sample of the response to ultrasound stimulation at 7 Hz.
with the ceramic-only site. Figure 4.1 shows the results of HZ ultrasound stimuli (1000 MV) with absorbed energy, HZ ultrasound stimuli. The absorbed energy was applied for 1000 MV of ultrasound stimulation at 4 Hz. Figures 4.8: Graphs from one representative sample of the response to ultrasound stimulation at 4 Hz, with smoothing in 0.1-1.1 MHz.
Figure 4.9: Voltage versus time graphs of the trajectories of positive and negative deflections upon application of optogenetic (left) or ultrasound (right) stimulation. Data are mean curves of all triggered responses from a single representative animal. Each graph contains a representative curve for each of the four stimulus intensity levels represented in figures 1-8 and a single curve representing randomly selected data events collected from the same recording session.

A. Positive deflection in response to 7 Hz optogenetic stimulation.
B. Positive deflection in response to 7 Hz ultrasound stimulation.
C. Negative deflection in response to 7 Hz optogenetic stimulation.
D. Negative deflection in response to 7 Hz ultrasound stimulation.
Figure 4.10: Area under the curve (AUC) values for optogenetic (A, B) and ultrasound (C, D) stimulus-triggered (black bars) vs. random (white bars) positive and negative events shown for data collected from N= 8 animals.

A. AUC of positive deflection from 7 Hz optogenetic stimulation.
B. AUC of negative deflection from 7 Hz optogenetic stimulation.
C. AUC of positive deflection from 7 Hz ultrasound stimulation.
D. AUC of negative deflection from 7 Hz ultrasound stimulation.

Asterisks indicate a significant difference from dead condition according to post hoc analysis by Fisher’s Least Significant Difference.
Figure 4.11 Mean voltage (MV) values for optogenetic (A, B) and ultrasound (C, D) stimulus-triggered (black bars) vs. random (white bars) positive and negative events shown for data collected from N= 8 animals.

A. MV of positive deflection from 7 Hz optogenetic stimulation.
B. MV of negative deflection from 7 Hz optogenetic stimulation.
C. MV of positive deflection from 7 Hz ultrasound stimulation.
D. MV of negative deflection from 7 Hz ultrasound stimulation.

Asterisks indicate a significant difference from dead condition according to post hoc analysis by Fisher’s Least Significant Difference.
Figure 4.12 Peak voltage (PV) values for optogenetic (A, B) and ultrasound (C, D) stimulus-triggered (black bars) vs. random (white bars) positive and negative events shown for data collected from N= 8 animals.

A. PV of positive deflection from 7 Hz optogenetic stimulation.
B. PV of negative deflection from 7 Hz optogenetic stimulation.
C. PV of positive deflection from 7 Hz ultrasound stimulation.
D. PV of negative deflection from 7 Hz ultrasound stimulation.

Asterisks indicate a significant difference from dead condition according to post hoc analysis by Fisher’s Least Significant Difference.
Figure 4.13 Event duration (ED) values for optogenetic (A, B) and ultrasound (C,D) stimulus-triggered (black bars) vs. random (white bars) positive and negative events shown for data collected from N= 8 animals.

A. ED for positive deflection from 7 Hz optogenetic stimulation.
B. ED for negative deflection from 7 Hz optogenetic stimulation.
C. ED for positive deflection from 7 Hz ultrasound stimulation.
D. ED for negative deflection from 7 Hz ultrasound stimulation.

Asterisks indicate a significant difference from dead condition according to post hoc analysis by Fisher’s Least Significant Difference.
**Figure 4.14:** Overlay of electrophysiological response to optogenetic and ultrasound event-triggered responses. Data from ultrasound stimulation delivered at maximal intensity (red curve; 1000 mV) are compared to optogenetic stimulation delivered at medium-low intensity (black curve; 6 cm supradural). These two data sets were chosen for comparison because the response parameters were shown to be equivalent in magnitude in figures 4.10-4.13. The near total overlap in the fluctuation of potential subsequent to stimulus onset at time zero demonstrates that the responses are temporally equivalent as well as proportionate in magnitude.
and ultrasound stimulation at these intensities to \( N = 8 \) animals at 20 minute intervals.

Table 4.1: Experimental Protocol for Intensity-Response Experiment. Following the detailed

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<thead>
<tr>
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<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
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**Table 4.2:** Analysis of variance for effects of stimulus intensity, event type (triggered vs. random) and their interaction in affecting four parameters extracted from optogenetically-triggered potentials in the motor-frontal derivation. Separate ANOVAs were performed for parameters derived from the negative deflections and positive deflections. ANOVA F values that were statistically significant (P <0.05) are shown in red. N.S. indicates non-significant experimental effects.
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Table 4.3: Analysis of variance for effects of stimulus intensity, event type (triggered vs. random) and their interaction in affecting four parameters extracted from ultrasound-triggered potentials in the motor-frontal derivation. Separate ANOVAs were performed for parameters derived from the negative deflections and positive deflections. ANOVA F values that were statistically significant (P < 0.05) are shown in red. N.S. indicates non-significant experimental effects.
CHAPTER 5: MEASURING TEMPORAL CONSTRAINTS ON THE RESPONSE TO ULTRASOUND STIMULATION

Neurons have a refractory period that is a function of their ability to re-establish ion concentration gradients following stimulus onset. The refractory period is an interval time during which the neurons cannot undergo another action potential after stimulation induced action potentials have occurred. The refractory period is manifested during optogenetic stimulation frequencies greater than 40 Hz (i.e., when the interstimulus interval is $\leq 25$ ms). At stimulation frequencies lower than 40 Hz, nearly 100% of optogenetic stimuli result in action potentials in ChR2-expressing pyramidal cells (the cells being stimulated in our experiments). At stimulation frequencies higher than 40 Hz, the probability that a stimulus will elicit an action potential declines. If ultrasound mediated responses are, in fact, ion channel mediated responses, ultrasound induced responses should exhibit temporal constraints similar to those imposed on optogenetically-induced responses.

The biophysical constraints on a given physiological event can additionally be defined by the minimal duration of stimulation necessary to produce a detectable response. If ultrasound mediated responses are, in fact, ion channel mediated responses, ultrasound induced responses should exhibit a dependence on stimulus duration that is similar to optogenetically-induced responses. Here, we sought first to establish the minimum duration of ultrasound and optogenetic stimulation that is sufficient to evoke an electrophysiological response (Experiment 1). Second, we applied stimuli in pairs to determine whether the cerebral cortex was refractory to stimulation or exhibited additive electrophysiological responses to paired-pulses delivered at varying inter pulse intervals (Experiment 2).
For Experiment 1, n = 8 Thy1-ChR2 male mice were subjected to 10-min sessions of optogenetic stimulations at each of five LED pulse durations and each of five ultrasound pulse durations for direct head-to-head comparison. Pulse durations within each stimulus modality were 0.5 ms, 1.0 ms, 2.5 ms, 5.0 ms, and 10 ms. Animals were subjected to 10 continuous minutes of stimulation at each pulse duration. The order of stimulation types (ultrasound vs. LED) was varied in a counterbalanced fashion with 50% of the animals subjected to LED followed by ultrasound stimulation, and 50% of the animals subjected to US stimulation followed by LED stimulation (Table 5.1).

Systematic variation of pulse duration was achieved by distinct means for the two stimulus modalities. For optogenetic pulses, the duration of the TTL-on signal produced by the STG4002 stimulus generator (MultiChannel Systems) was programmed to each of the five stimulus duration values. While the duration for the optogenetic manipulation was established solely with the MultiChannel Systems generator, the duration of ultrasound manipulations was altered by modulating the number of output cycles generated by the radio frequency generator. The cycle repetition frequency of the radio frequency generator is 2.0 kHz; and thus cycle duration is 0.5 ms. The generator was therefore programmed to undergo 1, 2, 5, 10 or 20 cycles in order to achieve the intended range of ultrasound stimulus pulse durations (0.5 ms, 1 ms, 2.5 ms, 5 ms, 10 ms, respectively).

Examples of evoked responses to optogenetic stimuli of varying durations are shown in figures 5.1-5.5; samples of negative and positive deflections extracted from these evoked responses are shown in figure 5.11. Statistical analyses of the optogenetically-evoked response parameters by repeated measures ANOVA (Table 5.2) indicated significant effects of optogenetic stimulation on peak voltage ($F > 4; P < 0.05$) for both positive and negative
deflections. Other response parameters were not consistently affected by stimulation. The attenuated response to stimulation in this experiment relative to prior measurements is reflective of the reduced stimulus duration: planned comparison of evoked response parameters with cognate random values (panels A and B in Figures 5.12-5.15) indicated significant evoked responses for only stimulus durations of 2.5 ms or greater. Thus, the threshold stimulus duration for significant responses to optogenetic stimulation in our experimental system is approximately 2.5 ms.

Evoked responses to ultrasound stimuli of varying durations are shown in figures 5.6-5.10; samples of negative and positive deflections extracted from these evoked responses are shown in figure 5.11. Statistical analyses of the ultrasound-evoked response parameters by repeated measures ANOVA (Table 5.3) indicated significant effects of ultrasound stimulation on peak voltage (F > 8; P < 0.008) for both positive and negative deflections. Other response parameters were not significantly affected by stimulation. The attenuated response to stimulation in this experiment relative to prior measurements is reflective of the reduced stimulus duration: planned comparison of evoked response parameters with cognate random values (panels C and D in Figures 5.12-5.15) indicated significant evoked responses for only stimulus durations of 2.5 ms or greater. The biophysical constraints on ultrasound stimulation, like those for optogenetic stimulation, dictate that the threshold stimulus duration for significant responses to ultrasound stimulation is approximately 2.5 ms.

Experiment 1 established that 1.0 ms stimulus duration was sub-threshold for producing a reliable, robust response to both optogenetic and ultrasound stimuli. For both optogenetic and ultrasound stimuli of durations of 0.5 ms or 1 ms, area under the curve, mean voltage, peak voltage and event duration of evoked responses were not significantly greater than random
values. Having established this temporal constraint on the responses, we continued the investigation to determine in Experiment 2 whether paired-pulses of sub-threshold duration would cumulatively produce significant evoked responses. This experimental protocol design included five paired 1.0 ms stimuli occurring at interstimulus intervals of 1.0 ms, 2.0 ms, 4.0 ms, 16.0 ms, and 64.0 ms and a single 1.0 ms stimulus, which served as a control. The application of the stimulation was randomized with respect to the order in which the paired stimuli were applied, as well as type of stimulation. Table 5.4 presents the order of presentation of stimulus protocols for each of the eight animals. Each animal was subjected to ten consecutive minutes of stimulation of each type at each of the five interstimulus intervals.

Pairs of 1 ms optogenetic stimulus pulses induced statistically significant increases in mean voltage and peak voltage for both positive and negative evoked responses relative to randomly-selected events (repeated measures ANOVA; Table 5.5; F > 5; P < 0.03). Sample evoked responses are shown in figures 5.16-5.20; samples of negative and positive deflections extracted from these evoked responses are shown in figure 5.26. Other response parameters were not consistently affected by stimulation. Planned comparison of evoked response parameters with cognate random values (panels A and B in Figures 5.27-5.30) indicated significant evoked responses for interstimulus intervals of 1 to 16 ms. Neither a single 1 ms pulse, nor a pairing of pulses separated by 64 ms resulted in detectable evoked responses. Thus, optogenetic stimuli of sub-threshold duration separated by 16 ms or less cumulatively produce a statistically significant modulation of cerebral cortical neuronal activity.

Evoked responses to paired ultrasound stimuli of varying interstimulus intervals are shown in figures 5.21-5.25; samples of negative and positive deflections extracted from these evoked responses are shown in figure 5.26. Statistical analyses of the ultrasound-evoked
response parameters by repeated measures ANOVA (Table 5.6) indicated significant effects of ultrasound stimulation on mean voltage and peak voltage of evoked potentials ($F > 9; P < 0.013$) for both positive and negative deflections. Other response parameters were not significantly affected consistently by ultrasound stimulation. Planned comparison of evoked response parameters with cognate random values (panels C and D in Figures 5.27-5.30) indicated significant evoked responses only when the paired-pulses were delivered at interstimulus intervals of 16 ms or less. Neither a single pulse of 1 ms duration nor a pair of pulses separated by 64 ms generated statistically significant response parameters. Thus, the temporal constraints on evoking supra-threshold responses by the cumulative effects of paired-pulses are identical for ultrasound and optogenetic stimuli across the range of interstimulus intervals tested. paired-pulse
D. Magnitude versus voltage fast Fourier transform of the randomly mixed waveforms.
C. Magnitude versus voltage fast Fourier transform of the mean filtered waveforms.
B. Voltage versus time graph chosen from randomly selected snippets of data segments extracted from
the same line during the simulation.
A. Voltage versus time graph following the trigger onset for LED otoacoustic stimulation occurring at
a distance of 6 cm superior and 6 cm above the surface of the brain.

Figure 2.1: Graphs from one representative sample of the response to LED otoacoustic stimulation at
10 Hz.
D. Magnitude versus volume fast Fourier transform of the randomly timed waveforms.
C. Magnitude versus volume fast Fourier transform of the mean filtered waveforms.
B. Volume versus time graph chosen from randomly selected snippet of data segments extracted from the same 4 lines devoid of stimulation.
A. Volume versus time graph following inferred onset for LED opotgenetic stimulation accounts at 7 Hz, 1 ms pulse duration. Opopogenetic stimuli were delivered via a hypocycloidal cable positioned at a distance of 6 mm subradial (6 mm above the surface of the brain).

Figure 5.2: Graphs from one representative sample of the responses to LED opotgenetic
A. Voltage versus time graph showing integer number for LED optical image simulation occurring at 7 Hz. 2.5 ms pulse duration. Opposite stimuli were delivered via a fiber optic cable positioned at a distance of 6 cm supradural (6 cm above the surface of the brain).

B. Voltage versus time graph showing integer number for LED optical image simulation occurring at 7 Hz. 2.5 ms pulse duration. Opposite stimuli were delivered via a fiber optic cable positioned at a distance of 6 cm supradural (6 cm above the surface of the brain).

C. Magnitude versus time graph showing integer number for LED optical image simulation occurring at 7 Hz. 2.5 ms pulse duration. Opposite stimuli were delivered via a fiber optic cable positioned at a distance of 6 cm supradural (6 cm above the surface of the brain).

D. Magnitude versus time graph showing integer number for LED optical image simulation occurring at 7 Hz. 2.5 ms pulse duration. Opposite stimuli were delivered via a fiber optic cable positioned at a distance of 6 cm supradural (6 cm above the surface of the brain).
D. Magnitude versus Voltage Fast Fourier Transform of the randomly timed waveforms.
C. Magnitude versus Voltage Fast Fourier Transform of the mean high-level waveforms.
B. Voltage versus Graph chosen from randomly selected snippets of data segments extracted from the same file at times devoid of stimulation.
A. Voltage versus Graph following integer outer integer for LED optronic stimulation occurring at 7 Hz. A pulse duration. Optic nerve stimulus were delivered via a fiber optic cable positioned at a distance of 5 cm superiorly (6 cm above the surface of the brain).

Figure 5.4: Graphs from one representative sample of the response to LED optronic stimulation at 6 of smoothing in 0.2-4.13 LED 5.0 Hz.
D. Magnitude versus voltage Fast Fourier transform of the randomly timed waveforms.
C. Magnitude versus voltage Fast Fourier transform of the mean filtered waveforms.
B. Voltage versus graph chosen from randomly selected snippet of data segments extracted from the same file at times devoid of simulation.
A. Voltage versus graph showing integer pulse for LED opposite simulation occurring at 7 Hz. 10 ms pulse duration. Opposing stimulus were delivered via a trigger pulse positioned at a distance of 6 cm superiorly (6 cm above the surface of the brain).

Figure 5.2 Graphs from one representative sample of the response to LED opposite stimulation at 7 Hz.
D. Magnitude versus volume Fast Fourier transform of the radially aligned waveforms.
C. Magnitude versus volume Fast Fourier transform of the mean inclined waveforms.
B. Volume versus time Graph chosen from randomly selected snippets of data segments extracted
A. Volume versus time Graph following higher onset for ultrasound stimulation occurring at 7 HZ.

Figure 5.6: Graphs from one representative sample of the response to ultrasound stimulation at 7 HZ.
D. Magnitude versus voltage Fast Fourier transform of the randomly timed waveforms.
C. Magnitude versus voltage Fast Fourier transform of the mean summed waveforms.
B. Voltage versus time graph chosen from randomly selected snippet of data segments extracted
A. Voltage versus time graph following integer onset for ultrasound stimulation occurring at 7 Hz. 

Figure 5.7: Graphs from one representative sample of the response to ultrasound stimulation at 7 Hz 

110
B. Amplitude versus volume fast Fourier transform of the mean filtered waveforms.
C. Amplitude versus volume fast Fourier transform of the mean filtered waveforms.

From the same still image data of simulation.

2.5 ms pulse duration.

A. Voltage versus time graph following trigger onset for ultrasound simulation occurring at 7 Hz.

Figure 5.8: Graphs from one representative sample of the response to ultrasound simulation at 7 Hz.
D. Magnitude versus time for a random waveform.
C. Magnitude versus time for a random waveform.
B. Magnitude versus time for a random waveform.
A. Magnitude versus time for a random waveform.

The same data is shown in Figure 3.9, graphs from one representative sample of the response to ultrasound stimulation at 7 Hz.

**Figure 3.9:** Graphs from one representative sample of the response to ultrasound stimulation at 7 Hz.
D. Magnitude versus voltage Fast Fourier transform of the randomly timed waveforms.
C. Magnitude versus voltage Fast Fourier transform of the mean filtered waveforms.
B. Voltage versus time graph chosen from randomly selected snippets of data segments extracted from pulse duration.
A. Voltage versus time graph following integer offset for ultrasound stimulation occurring at 7 Hz; 0.1 ms pulse duration. Ultrasound stimulus generated by 1000 mW ultrasound with initial duty factor 5% and frequency 7 Hz.
**Figure 5.11**: Voltage versus time graphs of the trajectories of positive and negative deflections upon application of optogenetic (left) or ultrasound (right) stimulation at varying pulse durations. Data are mean curves of all triggered responses from a single representative animal. Each graph contains a representative curve for each of the varying pulse durations represented in figures 5.1-5.10 and a single curve representing randomly selected data events collected from the same recording session.

A. Positive deflection in response to 7 Hz optogenetic stimulation at pulse durations of 0.5 - 10 ms.

B. Positive deflection in response to 7 Hz ultrasound stimulation at pulse durations of 0.5 - 10 ms.

C. Negative deflection in response to 7 Hz optogenetic stimulation at pulse durations of 0.5 - 10 ms.

D. Negative deflection in response to 7 Hz ultrasound stimulation at pulse durations of 0.5 - 10 ms.
Figure 5.12: Area under the curve (AUC) values for optogenetic (A, B) and ultrasound (C, D) stimulus-triggered (black bars) vs. random (white bars) positive and negative events shown for data collected from N= 8 animals.

A. AUC of positive deflection from 7 Hz optogenetic stimulation at pulse durations of 0.5 -10 ms.
B. AUC of negative deflection from 7 Hz optogenetic stimulation at pulse durations of 0.5 -10 ms.
C. AUC of positive deflection from 7 Hz ultrasound stimulation at pulse durations of 0.5 -10 ms.
D. AUC of negative deflection from 7 Hz ultrasound stimulation at pulse durations of 0.5 -10 ms. Asterisks indicate a significant difference from dead condition according to post hoc analysis by Fisher’s Least Significant Difference.
Figure 5.13 Mean voltage (MV) values for optogenetic (A, B) and ultrasound (C, D) stimulus-triggered (black bars) vs. random (white bars) positive and negative events shown for data collected from N = 8 animals.

A. MV of positive deflection from 7 Hz optogenetic stimulation at pulse durations of 0.5 -10 ms.
B. MV of negative deflection from 7 Hz optogenetic stimulation at pulse durations of 0.5 -10 ms.
C. MV of positive deflection from 7 Hz ultrasound stimulation at pulse durations of 0.5 -10 ms.
D. MV of negative deflection from 7 Hz ultrasound stimulation at pulse durations of 0.5 -10 ms.
Asterisks indicate a significant difference from dead condition according to post hoc analysis by Fisher’s Least Significant Difference.
Figure 5.14 Peak voltage (PV) values for optogenetic (A, B) and ultrasound (C,D) stimulus-triggered (black bars) vs. random (white bars) positive and negative events shown for data collected from N= 8 animals.

A. PV of positive deflection from 7 Hz optogenetic stimulation at pulse durations of 0.5 -10 ms.
B. PV of negative deflection from 7 Hz optogenetic stimulation at pulse durations of 0.5 -10 ms.
C. PV of positive deflection from 7 Hz ultrasound stimulation at pulse durations of 0.5 -10 ms.
D. PV of negative deflection from 7 Hz ultrasound stimulation at pulse durations of 0.5 -10 ms.

Asterisks indicate a significant difference from dead condition according to post hoc analysis by Fisher’s Least Significant Difference.
Figure 5.15 Event duration (ED) values for optogenetic (A, B) and ultrasound (C, D) stimulus-triggered (black bars) vs. random (white bars) positive and negative events shown for data collected from N=8 animals.

A. ED for positive deflection from 7 Hz optogenetic stimulation at pulse durations of 0.5 - 10 ms.
B. ED for negative deflection from 7 Hz optogenetic stimulation at pulse durations of 0.5 - 10 ms.
C. ED for positive deflection from 7 Hz ultrasound stimulation at pulse durations of 0.5 - 10 ms.
D. ED for negative deflection from 7 Hz ultrasound stimulation at pulse durations of 0.5 - 10 ms.
Waveforms.

D. Amplitude versus voltage Fast Fourier transform of the random signal.
E. Graph of the same line length evolved on simulation.
F. Voltage versus time graph chosen from randomly selected snippet of data segments extracted from the same line length evolved on simulation above the surface of the brain.

Figure 5.16: Graphs from one representative sample of the response to paired-pulse LED-opponent stimulation with an inter-stimulus interval of 1 ms. Pairs of pulses were delivered at 7 Hz. 

Duration of all pulses was 1 ms. Opponent stimuli were delivered via a fiber optic cable positioned at a distance of 6 cm superficial to the brain.
Waveforms

A. Amplitude versus voltage just before transformation of the mean filtered waveforms.
B. Voltage versus time graph chosen from randomly selected sample of data segments.
C. Amplitude versus voltage just before transformation of the mean filtered waveforms.
D. Amplitude versus voltage just before transformation of the mean filtered waveforms.

Opposinwave stimulation occurring at 7 Hz above the surface of the brain.

Figure 5.17: Graphs from one representative sample of the response to periodic pulse LED.
Figure 2.18: Graphs from a representative sample of the response to paired-pulse LED optical stimulation with an inter-stimulus interval of 4 ms.

A. Voltage versus time graph following the onset for paired-pulse LED stimulation delivered 6 cm above the surface of the brain.

B. Voltage versus time graph following the onset for paired-pulse LED stimulation occurring at 7 Hz.

C. Magnitude versus time graph fast Fourier transform of the signal recorded.

D. Magnitude versus time graph fast Fourier transform of the signal recorded after the stimulation.
waveforms.

D. Magnitude versus Volume Fast Fourier Transform of the Randomly Timed

waveforms.

C. Magnitude versus Volume Fast Fourier Transform of the Mean Filtered
segments extracted from the same file times deemed to be subject of
stimulation.

B. Volume versus Time Graph Follows The Upper onset for paired-pulse LED
opposing stimulation occurring at 7 Hz.

A. Volume versus Time Graph Follows The Upper onset for paired-pulse LED
supradural (6 cm above the surface of the brain).

Supradural(6 cm above the surface of the brain). Volume versus time delivered via a therapeudic cable positioned at a distance of 6 cm.

Supradural(6 cm above the surface of the brain). Duration of all pulses was 1 msec. Opposing stimulation with an inter-stimulus interval of 16 msec. Parts of
LED opposing stimulation with an inter-stimulus interval of 16 msec. Parts of

Figure 5.19: Graphs from one representative sample of the response to paired-pulse
Waveforms:

D. Magnitude versus time Fourier transform of the random input
C. Magnitude versus time Fourier transform of the mean filtered
sequences extracted from the same time series data obtained from stimulation.

B. Voltage versus time graph chosen from randomly selected snippets of data
opposing stimulation occurring at 7 Hz
A. Voltage versus time graph following longer onset for paired-pulse LED

Opsonic stimulation were delivered via a fiber optic cable positioned at a distance
of 6 cm superior to the surface of the brain.

Pairs of pulses were delivered at 7 Hz. Duration of all pulses was 1 ms.

Figure 2: Graphs from one representative sample of the responses to paired-

Waveforms.

A. Amplitude versus time graph showing the variation of the random signal.

B. Amplitude versus time graph showing the mean filtered segments extracted from the same data, with peaks above and below the mean level.

C. Amplitude versus time graph showing the mean filtered segments extracted from the same data, with peaks above and below the mean level.

D. Amplitude versus time graph showing the mean filtered segments extracted from the same data, with peaks above and below the mean level.

E. Amplitude versus time graph showing the mean filtered segments extracted from the same data, with peaks above and below the mean level.

F. Amplitude versus time graph showing the mean filtered segments extracted from the same data, with peaks above and below the mean level.

G. Amplitude versus time graph showing the mean filtered segments extracted from the same data, with peaks above and below the mean level.

H. Amplitude versus time graph showing the mean filtered segments extracted from the same data, with peaks above and below the mean level.

I. Amplitude versus time graph showing the mean filtered segments extracted from the same data, with peaks above and below the mean level.

J. Amplitude versus time graph showing the mean filtered segments extracted from the same data, with peaks above and below the mean level.

K. Amplitude versus time graph showing the mean filtered segments extracted from the same data, with peaks above and below the mean level.

L. Amplitude versus time graph showing the mean filtered segments extracted from the same data, with peaks above and below the mean level.

M. Amplitude versus time graph showing the mean filtered segments extracted from the same data, with peaks above and below the mean level.

N. Amplitude versus time graph showing the mean filtered segments extracted from the same data, with peaks above and below the mean level.

O. Amplitude versus time graph showing the mean filtered segments extracted from the same data, with peaks above and below the mean level.

P. Amplitude versus time graph showing the mean filtered segments extracted from the same data, with peaks above and below the mean level.

Q. Amplitude versus time graph showing the mean filtered segments extracted from the same data, with peaks above and below the mean level.

R. Amplitude versus time graph showing the mean filtered segments extracted from the same data, with peaks above and below the mean level.

S. Amplitude versus time graph showing the mean filtered segments extracted from the same data, with peaks above and below the mean level.

T. Amplitude versus time graph showing the mean filtered segments extracted from the same data, with peaks above and below the mean level.

U. Amplitude versus time graph showing the mean filtered segments extracted from the same data, with peaks above and below the mean level.

V. Amplitude versus time graph showing the mean filtered segments extracted from the same data, with peaks above and below the mean level.

W. Amplitude versus time graph showing the mean filtered segments extracted from the same data, with peaks above and below the mean level.

X. Amplitude versus time graph showing the mean filtered segments extracted from the same data, with peaks above and below the mean level.

Y. Amplitude versus time graph showing the mean filtered segments extracted from the same data, with peaks above and below the mean level.

Z. Amplitude versus time graph showing the mean filtered segments extracted from the same data, with peaks above and below the mean level.
Figure 5.22: Graphs from one representative sample of the response to pulsed ultrasound.
Figure 5.23: Graphs from one prospective sample of the response to paired-pulse ultrasound.
Waveforms.

C. Magnitude versus voltage fast Fourier transform of the raw data.

B. Volatge versus time graph chosen from randomly selected snapshot of data simulation occurring at 7 Hz.

A. Voltage versus time graph following trigger onset for paired-pulse ultrasound delivered via a transducer positioned on a cadaveric with intact dura mater. Pulses were delivered at 7 Hz. Duration of all pulses was 1 ms. Ultrasound pulses were delivered at 7 Hz. Duration of all pulses was 1 ms. Ultrasound pulses were delivered at 7 Hz. Duration of all pulses was 1 ms. Ultrasound pulses were delivered at 7 Hz. Duration of all pulses was 1 ms.

Figure 2.4: Graphs from one representative sample of the response to paired-

---
Waveforms C. Magnitude versus voltage Fast Fourier Transform of the random signal

B. Voltage versus time graph chosen from randomly selected snippet of data simulation occurring at 7 Hz.

A. Voltage versus time graph following integer offset for paired-pulse ultrasonic

Figure 5.25: Graphs from one representative sample of the response to paired-
Figure 5.26: Voltage versus time graphs of the trajectories of positive and negative deflections upon application of optogenetic (left) or ultrasound (right) stimulation. Data are mean curves of all triggered responses from a single representative animal. Each graph contains a representative curve for each of the five inter-stimulus interval levels represented in figures 5.16-5.25 and a single curve representing randomly selected data events collected from the same recording session.

A. Positive deflection in response to 7 Hz paired-pulse optogenetic stimulation.
B. Positive deflection in response to 7 Hz paired-pulse ultrasound stimulation.
C. Negative deflection in response to 7 Hz paired-pulse optogenetic stimulation.
D. Negative deflection in response to 7 Hz paired-pulse ultrasound stimulation.
Figure 5.27: Area under the curve (AUC) values for optogenetic (A, B) and ultrasound (C, D) paired-pulse stimulus-triggered (black bars) vs. random (white bars) positive and negative events shown for data collected from N= 8 animals.

A. AUC of positive deflection from 7 Hz optogenetic paired-pulse stimulation.
B. AUC of negative deflection from 7 Hz optogenetic paired-pulse stimulation.
C. AUC of positive deflection from 7 Hz ultrasound paired-pulse stimulation.
D. AUC of negative deflection from 7 Hz ultrasound paired-pulse stimulation.

Asterisks indicate a significant difference from dead condition according to post hoc analysis by Fisher’s Least Significant Difference.
Figure 5.28 Mean voltage (MV) values for optogenetic (A, B) and ultrasound (C,D) paired-pulse stimulus-triggered (black bars) vs. random (white bars) positive and negative events shown for data collected from N=8 animals.

A. MV of positive deflection from 7 Hz optogenetic paired-pulse stimulation.
B. MV of negative deflection from 7 Hz optogenetic paired-pulse stimulation.
C. MV of positive deflection from 7 Hz ultrasound paired-pulse stimulation.
D. MV of negative deflection from 7 Hz ultrasound paired-pulse stimulation.

Asterisks indicate a significant difference from dead condition according to post hoc analysis by Fisher’s Least Significant Difference.
Figure 5.29 Peak voltage (PV) values for optogenetic (A, B) and ultrasound (C,D) paired-pulse stimulus-triggered (black bars) vs. random (white bars) positive and negative events shown for data collected from N=8 animals.

A. PV of positive deflection from 7 Hz optogenetic paired-pulse stimulation.
B. PV of negative deflection from 7 Hz optogenetic paired-pulse stimulation.
C. PV of positive deflection from 7 Hz ultrasound paired-pulse stimulation.
D. PV of negative deflection from 7 Hz ultrasound paired-pulse stimulation.

Asterisks indicate a significant difference from dead condition according to post hoc analysis by Fisher’s Least Significant Difference.
Figure 5.30 Event duration (ED) values for optogenetic (A, B) and ultrasound (C,D) paired-pulse stimulus-triggered (black bars) vs. random (white bars) positive and negative events shown for data collected from N= 8 animals.

A. ED for positive deflection from 7 Hz optogenetic paired-pulse stimulation.
B. ED for negative deflection from 7 Hz optogenetic paired-pulse stimulation.
C. ED for positive deflection from 7 Hz ultrasound paired-pulse stimulation.
D. ED for negative deflection from 7 Hz ultrasound paired-pulse stimulation.

Asterisks indicate a significant difference from dead condition according to post hoc analysis by Fisher’s Least Significant Difference.
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<th>0.15 mm</th>
<th>0.25 mm</th>
<th>0.25 mm</th>
<th>0.5 mm</th>
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<td>20.0</td>
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<td>15.0</td>
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<td>5 ms</td>
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<td>15.0</td>
<td>10.0</td>
<td>10.0</td>
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</tr>
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<td>2.5</td>
<td>2.5</td>
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Table 5.1: Stimulation protocols applied in the varying pulse duration investigation.
<table>
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<th>Positive Deflection</th>
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<td>F(df)</td>
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<td>F(df)</td>
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<tr>
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<td>N.S.</td>
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<td>N.S.</td>
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<td>N.S.</td>
</tr>
<tr>
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<td>N.S.</td>
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</tr>
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<td></td>
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<td><strong>0.042</strong></td>
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<td>Interaction</td>
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<tr>
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*Table 5.2*: Analysis of variance for effects of pulse duration, event type (triggered vs. random) and their interaction in affecting four parameters extracted from optogenetically-triggered potentials in the motor-frontal derivation. Separate ANOVAs were performed for parameters derived from the negative deflections and positive deflections. ANOVA F values that were statistically significant (P < 0.05) are shown in red. N.S. indicates non-significant experimental effects.
<table>
<thead>
<tr>
<th>Parameter</th>
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<th>Positive Deflection</th>
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<tr>
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<td>Interaction</td>
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<td>Interaction</td>
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Table 5.3: Analysis of variance for effects of pulse duration, event type (triggered vs. random) and their interaction in affecting four parameters extracted from ultrasound-triggered potentials in the motor-frontal derivation. Separate ANOVAs were performed for parameters derived from the negative deflections and positive deflections. ANOVA F values that were statistically significant (P <0.05) are shown in red. N.S. indicates non-significant experimental effects.
Table 5.4: Simulation protocols applied in the paired-pulse investigation.
<table>
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<tr>
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Table 5.5: Analysis of variance for effects of inter-stimulus interval, event type (triggered vs. random) and their interaction in affecting four parameters extracted from optogenetically-triggered potentials in the motor-frontal derivation. Separate ANOVAs were performed for parameters derived from the negative deflections and positive deflections. ANOVA F values that were statistically significant (P < 0.05) are shown in red. N.S. indicates non-significant experimental effects.
<table>
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<th>Parameter</th>
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<th>Positive Deflection</th>
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<tr>
<td></td>
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<td>P</td>
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<td>Event Type</td>
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<tr>
<td></td>
<td>Interaction</td>
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<tr>
<td>Mean Voltage</td>
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</tr>
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</tbody>
</table>

Table 5.6: Analysis of variance for effects of inter-stimulus interval, event type (triggered vs. random) and their interaction in affecting four parameters extracted from ultrasound-triggered potentials in the motor-frontal derivation. Separate ANOVAs were performed for parameters derived from the negative deflections and positive deflections. ANOVA F values that were statistically significant (P <0.05) are shown in red. N.S. indicates non-significant experimental effects.
CHAPTER 6: DISCUSSION

While sleep is important to physical and mental health, the biological basis mediating the restorative effects of sleep requires further investigation. Classification of sleep stages via electroencephalograms, which measure electrical potentials driven by changes in neural activity, allow scientists to monitor predictable changes in the wake/sleep cycles in mammals. Our lab seeks to manipulate sleep slow wave activity in an effort to understand the restorative impacts of sleep. Our previous investigations show significant correlations between reduced rates of cerebral glucose utilization and the amount of slow wave activity in the rodent cerebral cortex.

Optogenetic stimulation is routinely used in our lab to manipulate electrical activity of pyramidal neurons, the majority population of neurons in the cerebral cortex, without disrupting the subject’s global sleep state. This technique requires the use of genetically modified mice and invasive surgical implantation procedures. Directing blue LED light onto targeted cerebral cortex coordinates allows us to manipulate local activity in freely moving animals and study the effects of the stimuli relative to spontaneous sleep. While these methods will allow for study of the biological mechanisms of sleep and may lead to therapies correcting abnormal cortical activity in human conditions, it is necessary to develop non-invasive techniques for manipulation of slow wave activity within the cerebral cortex.

Although several non-invasive methods have been used to study sensory, motor, and cognitive functions, transcranial pulsed ultrasound can offer better spatial and temporal resolution. Using transducers to exploit the piezoelectric effect, ultrasound has been used in medical imaging applications for several decades. It has been successfully used in the treatment of intracranial and somatic conditions. Recently, ultrasound stimulation has been used to elicit action potentials \textit{in vivo} as a way of manipulating intact brain tissue in animal models. Testing
the electrophysiological parameters of electroencephalograms associated with sleep slow wave activity in response to non-invasive low frequency transcranial ultrasound stimuli may provide insight into the restorative effects of sleep slow waves in humans.

Five experiments were conducted for this doctoral project. Having established evidence of motor responses to transcranial stimulation of the sensory cortex with ultrasound, we compared the responses at the electroencephalographic level to optogenetic and ultrasound stimuli. We then established, by manipulating stimulus intensities for optogenetic and ultrasound stimuli, stimulus parameters that yielded equivalent electrophysiological responses. We measured the effects of varying pulse durations for both types of stimuli on electrophysiological response magnitude in order to determine threshold stimulus durations. Finally, we applied paired-pulse stimulation to determine the temporal limits to paired-pulse facilitation. Recognizing that ultrasound and optogenetic stimuli are characterized by distinct energy transfer mechanisms, this project tested whether those divergent energy transfer mechanisms confer distinct biophysical constraints on the electrophysiological response of the cerebral cortex.

At the outset of this project, electrophysiological responses to ultrasound stimulation at the level of the electroencephalogram had not been documented. It was essential to overcome the technical challenge of recording electroencephalograms in response to ultrasound stimulation in order to establish this method as a viable alternative to the optogenetic technique. The use of specially constructed electrodes and careful elimination of electrical noise during application of ultrasound stimulation made it possible to collect evoked responses in the electroencephalogram and discriminate them from noise. While it was evident that neither bone density of the skull, nor brain tissue of the cerebral cortex would hinder the transmission of transcranial ultrasound, the
use of the optogenetic technique required a craniotomy. This limitation necessitated the application of the LED and ultrasound stimuli in an anesthetized animal. Electrophysiological measurements were made using an in vivo recording system. MATLAB was used for real time and post recording analysis. Establishing this method of effective cortical stimulation allowed us to compare the physiological data collected from ultrasound-stimulated responses with data collected from the optogenetic studies in the same anesthetized preparation.

Preliminary work established a protocol in which 10 ms stimulus pulse durations were applied at either 1 Hz or 7 Hz frequencies for a period of 10 minutes. Real time comparisons of TTL-event triggered potentials with an equivalent number of randomly timed events, and associated fast Fourier transforms, enabled us to discriminate physiological responses from electrical noise in these 10 min recordings. Average curves generated specific to individual recording sessions in offline analysis allowed us to examine the electrical potentials at time points relative to our stimulus onsets. Similar comparisons were made with responses recorded from stimulus applications –both ultrasound and optogenetic- in post mortem tissue, to ensure that the responses were dependent on electrical excitability of brain tissue. The 7 Hz stimulation paradigm offered the most robust response and was chosen for the remainder of the investigation. The magnitude of the optogenetic response was much larger than that of the ultrasound response in this experiment. Given that the transgenic mouse line used was specifically designed for application of optogenetic stimulation, this increase in magnitude likely stems from the response of millions of light sensitive neurons in the cerebral cortex. Nonetheless, the unambiguous electrophysiological signals recorded in response to ultrasound stimulation provide verification that the electrical responses are physiologically-mediated.
We next sought to adjust stimulus parameters, such that the magnitude of electrophysiological responses to optogenetic and ultrasound stimuli could be made equivalent for the purpose of direct comparison of response profiles. The methods of varying stimulation intensities required different means given that optogenetic and ultrasound stimuli are generated by distinct physical devices. Intensity of optogenetic stimulation was varied by changing the distance of the fiber optic cable placement relative to the craniotomy. Intensity of ultrasound stimulation was varied by changing the voltage of the radio frequency amplifier driving the ultrasound transducer. While the magnitude of the evoked response parameters to optogenetic stimulation resulted in an inversely proportionate relationship with the distance between the fiber optic tip and the surface of the cerebral cortex, there was no evidence of intensity-proportionate responses to ultrasound stimulation within the range of stimulus intensities chosen for these experiments. However, the variation in the magnitude of the response to LED stimulation allowed us to establish a method to induce responses of equivalent magnitude for both stimulation types. This experiment demonstrated that LED optogenetic and transcranial ultrasound stimuli produce responses in equivalent amplitude and temporal profiles.

Our data show that the response to 10 ms optogenetic stimulation resulting from the placement of the fiber tip 6 cm above the exposed dura, and the response to 10 ms ultrasound stimulation at 1000 mV, yielded the desired equivalence in the magnitude of the response. The data from animals subjected to both stimulation types at equivalent intensities show a nearly total overlap in the shape and magnitude of the waveforms produced subsequent to stimulus onset at time zero. Given that optogenetically-responsive neurons are pyramidal cells with electrophysiological responses mediated by light activated neuronal ion channels, the striking similarity of the ultrasound-induced responses to the optogenetic responses suggests that
ultrasound stimulation may also be mediated by an ion channel dependent process in cerebral cortical pyramidal neurons.

Neuronal excitation is in its simplest, most fundamental form, dependent on electrical currents. The biophysical mechanism for electrical excitation of neurons places temporal constraints on physiological responsiveness. In an effort to determine whether ultrasound stimulation is mediated by ion channel dependent responses, we compared temporal constraints on the electrophysiological responses to ultrasound and optogenetic stimulation of the cerebral cortex. By subjecting animals to optogenetic and ultrasound stimulation periods with pulse durations of 0.5 ms, 1.0 ms, 2.5 ms, 5 ms and 10 ms, we demonstrated that both stimulation modes exhibited equivalent duration thresholds for inducing electrophysiological responses. Confirmations of significant evoked responses to stimulus durations $> 2.5$ ms established this value as the duration threshold.

Once the temporal constraints on the responses were identified, we sought to determine whether paired-pulses of sub threshold duration would facilitate significant evoked responses. A 10-minute application of paired 1.0 ms stimuli occurring at varying inter-stimulus intervals was administered. The data demonstrated that whereas a single 1 ms pulse of ultrasound or LED stimulation did not evoke a significant response, paired 1 ms pulses separated by intervals of 16 ms or less evoked significant increases of mean and peak voltages for positive and negative evoked responses. By contrast, paired-pulses of ultrasound or LED stimulation separated by 64 ms did not result in detectable evoked responses. This experiment established that paired-pulse facilitation exists for optogenetic sub threshold durations separated by 16 ms or less.

In summary, these experiments have collectively established an electrophysiological basis for low frequency transcranial ultrasound manipulations of the cerebral cortex. Although
intracellular electrophysiological recordings of cerebral cortical neuronal activity will ultimately be needed to document the biophysical basis for this phenomenon, several parallels exist between the electrophysiological responses to optogenetic stimulation of pyramidal cells and ultrasound. Ultrasound stimulation requires living, electrically-excit able tissue in order to propagate in nervous tissue. When ultrasound and optogenetic stimuli are applied at electrophysiologically-equivalent intensities, the temporal profile of the evoked response is identical. The temporal constraints for responsiveness to short duration ultrasound and optogenetic stimuli and pairs of stimuli were identical within the parameter set applied in this project. These observations validate the concept that low frequency transcranial ultrasound stimulation directly impacts the electrical excitability of cerebral cortical neurons.
References


