Mechanically Activated Currents in Mechanosensitive Cells

A Thesis
Presented to
The Academic Faculty

In Partial Fulfillment
of the Requirements for the Degree
B.S. in Biomedical Engineering with Research Option in the
School of Biomedical Engineering

Georgia Institute of Technology

Mechanically Activated Currents in Mechanosensitive Cells

Approved by:
Dr. Costas Arvanitis, Principal Investigator
School of Mechanical Engineering
Georgia Institute of Technology

Dr. Bo Yang
School of Biomedical Engineering
Georgia Institute of Technology

Dr. Anastasia Velalopoulou
School of Biomedical Engineering
Georgia Institute of Technology

TABLE OF CONTENTS

Abstract

The purpose of this study is to analyze the effect of ultrasonic pressure on the mechanosensitive channel activity in the N2A cell line. Prior research has indicated that certain ion channels in the cellular membrane of the N2A cell line can be activated by the application of static pressure. Here, in addition to static pressure, we applied ultrasound pressure to the N2A cells, and assessed the effectiveness of these two stimuli using the patch-clamp method. Results indicate a positive correlation between the application of static pressure and the current response, confirming the piezo-electrical characteristics of these channels. Moreover, the resemblance between the response to static pressure and to that of ultrasound implicates the role of Piezo channels in high frequency mechanosensation and opens up many possibilities in the use of ultrasound for neuromodulation. Identifying the cell populations in the brain that express mechanosensitive channels could provide novel ultrasound-based therapies for neurodegenerative diseases.

Introduction

Parkinson's disease is one of the most common neurodegenerative disorders, affecting approximately 1 million Americans [2]. The progression of this disorder as well as other neurodegenerative diseases can be traumatic and devastating to patient and family as the patient's ability to function and humanity is slowly stripped away. Neurodegenerative diseases advance through the slow loss of neuron function, usually due to an inability to generate or transmit action potentials [2]. There is currently no cure for these diseases, and the therapies that have been developed lack a specific mechanistic understanding as to the complex pathways the therapy affects [9]. This project seeks to characterize the response of brain cells, and notably cells with mechanically activated ion channels, to ultrasound, with the overarching goal to establish new noninvasive methods to generate action potentials and, ultimately, develop new therapies for this disease.

In order for different cells in the body to communicate, cells open and close ion channels, allowing these charged particles to flow in and out of cells to generate a rapid change in the electrical potential of the cell, referred to as action potentials [3]. Along a neural pathway, similar to a telephone wire, the impulse is fired and the signal travels cell by cell to its destination. Most neurons have a resting membrane potential at a negative concentration gradient, meaning there are more positively charged ions outside than inside the cell. In order to fire an action potential, a triggering event must occur to open the ion channels, then the positively charged ions flow into the cell, depolarizing the cell and firing the impulse. These action potentials allow for the transmission of signals, specifically in neurons in the brain, and provide the network to experience sensory input as well as govern neurological processes. Some of these ion channels, called Piezo channels, are sensitive to pressure. When force is applied to

the extracellular membrane, the Piezo channels open, acting as a triggering event and resulting in a flux of ions that mimics or strengthens an action potential. This flux of ions can be measured quantitatively at a cellular level by the current and resistance across the membrane, as is done in a type of experimental setup called the patch clamp [3]. The patch clamp can be attached to cells, yielding a real-time measurement of the electrical potential ion channels.

In landmark study by Coste et al., researchers confirmed the existence of Piezo proteins through DNA knockdown and Piezo overexpression, and concluded that Piezo proteins are essential in the response of mammalian and human cells to mechanical pressure exerted on the cell membrane [1]. As Piezo1 channels respond to fast positive pressure stimuli, they are an ideal candidate for a study involving ultrasound stimulation. Our study replicates the data from Coste et al. as a jumping off point, showing that static pressure can generate a response in N2A cells, and then characterizes the response to ultrasonic pressure. Coste et al. lays out the methods which we will be applying, especially the patch clamp method for generating data.

The application of pressure to generate a response from Piezo proteins has been widely investigated in human platelets [4], T lymphocytes [5], A nociceptors [6], and dental stem cells[7]. A study in the Journal of Endodontics uses in Low-Intensity Pulsed Ultrasound (LIPUS) to trigger the MAPK downstream pathway, which is initially stimulated by Piezo protein activity[7]. This confirms the linkage between ultrasound, Piezo protein activation, and cell proliferation. However, the response of neurons at a cellular level to ultrasound has not yet been flushed out [12]. Ultrasound applications have been trending toward therapy, and recently a study by Yoo et al. confirmed the region specific response to focused ultrasound in the brain, and the ability of sonication to induce functional neuromodulation [8, 9]. Nonetheless, the exact reasoning as to why this is possible has been left undefined, and their association with the

presence of piezo-channels remains elusive. Trials have investigated ultrasound as a neurostimulator through various lenses, attributing its success to thermal effects, acoustic radiation force, and acoustic cavitation [10,11]. This study investigates a promising angle for why ultrasound is a successful neurostimulator: mechanical pressure effects in cells with mechanically activated ion channels. Studying this phenomenon at a cellular level will deepen understanding of the pathway through which ultrasound stimulation in the brain causes neural excitation, and the role that Piezo channels play in this excitation. By establishing the controlled conditions for this excitation to take place, the current study may provide insight as to possible therapies for neurodegenerative diseases by noninvasively modulating brain excitation. Our research seeks to fill these two voids in the field and weave them together to supplement our understanding the cellular work at play, as well more broadly how it may benefit to those suffering from neurodegenerative disorders, such as Parkinson's.

Methods and Materials

The purpose of this study is to analyze the effect of static and ultrasonic pressure on the mechanosensitive channel activity in the N2A cell line.

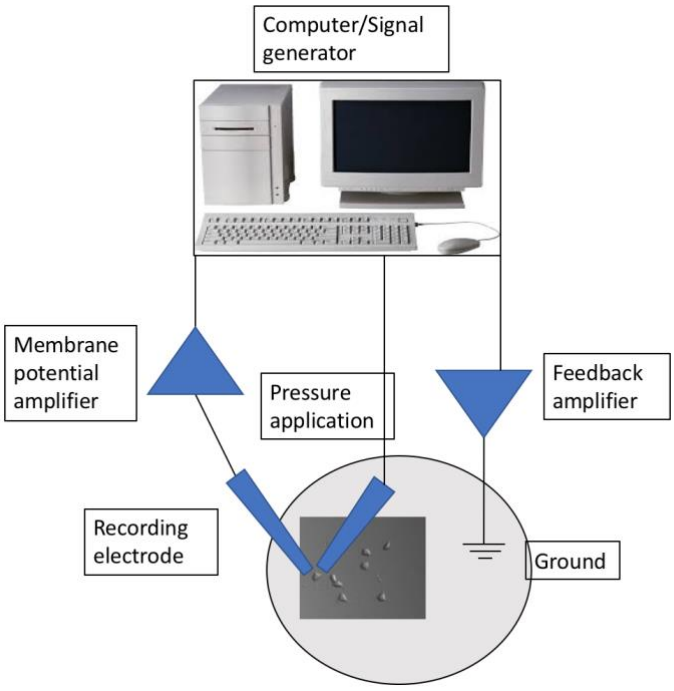
N2A Cell Culture

Neuro2A cells were grown in Eagle's Minimum Essential Medium containing 4.5 $mg.ml^{-1}$ glucose, 10% fetal bovine serum, 50 $units.ml^{-1}$ penicillin and 50 $\mu g.ml^{-1}$ streptomycin. Cells were passaged at 70% confluency. For experiments, cells were plated onto 12-mm round glass coverslips placed in 24-well plates.

Patch Clamp

This research utilizes the patch-clamp method to measure the current response across the extracellular membrane. In this method, a micropipette containing an electrolyte solution is manipulated until it makes contact with the extracellular membrane of the cell. The electrolyte solution inside of the recording electrode is comprised of (in mM) 133 CsCl, 10 HEPES, 5 EGTA, 1 $CaCl_2$, 1 $MgCl_2$, 4 MgATP, and 0.4 Na_2GTP (pH adjusted to 7.3 with CsOH). This electrode forms a high resistance seal, also known as a gigaseal, and then the cell membrane is broken to obtain access to the whole cell, referred to as a whole cell patch. Another electrode is placed in the bath surrounding the cell, and is used as a ground electrode. The bath solution surrounding the cell plate is comprised of (in mM) 127 NaCl, 3 KCl, 1 $MgCl_2$, 10 HEPES, 2.5 $CaCl_2$, 10 glucose (pH adjusted to 7.3 with NaOH). This forms an electrical circuit and allows the system to record the resistance and current across the extracellular membrane. For the application of static pressure, another micropipette placed in close proximity to the cell and is

attached to a system which allows the researcher to apply various quantities of negative pressure



to the extracellular membrane.

Figure 1: Diagram indicating the electrical setup for recording of electrophysiological response and pressure application.

Ultrasound Microsystem

In the application of ultrasound, a built-in house transducer (Cylindrical PZT with Central Frequency 520 KHz) was attached to the cell culture plate using an epoxy and used to deliver low frequency ultrasound to the cells.

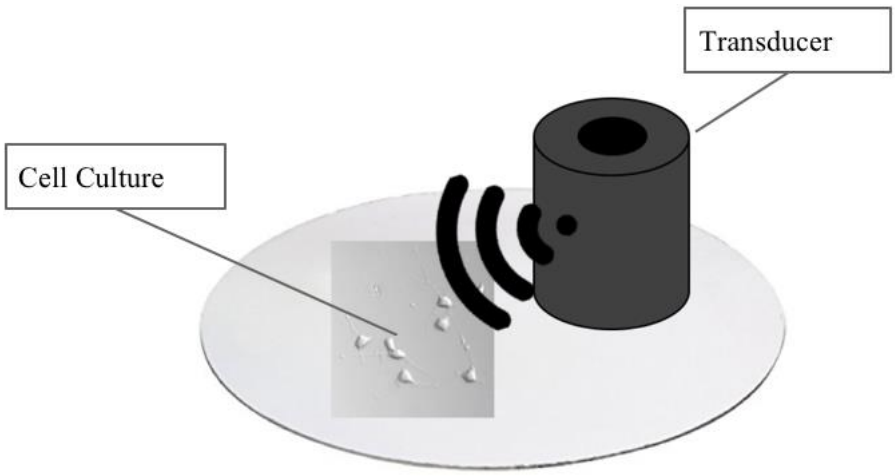


Figure 2: Diagram indicating the setup for ultrasonic activation.

For experiments, the transducer was sterilized using the autoclave and then placed under the hood. Then, fast curing two component epoxy was applied to the bottom of the transducer, which was then adhered to the 12-mm plate and allowed to dry for 12 hours. A waveform generator (Keysight, Santa Rosa, CA, USA) was connected to the transducer to deliver ultrasonic pulses. The current response was recorded again using the patch clamp setup. The parameters adjusted for ultrasound stimulation were the burst (cycles per second) and voltage (Volts peak to peak).

Data Analysis

For analysis of patch clamp data, custom Matlab code was written to parse through the large sets of data and select out data of interest corresponding to the perturbation. This code used a loop to identify when the pressure application passed a threshold and pulled the data set of both the pressure and current response within a certain range. The pressure indicated on the graph was originally in volts required to generate the pressure applied in mmHg, thus a calibration curve was generated to translate this to mmHg.

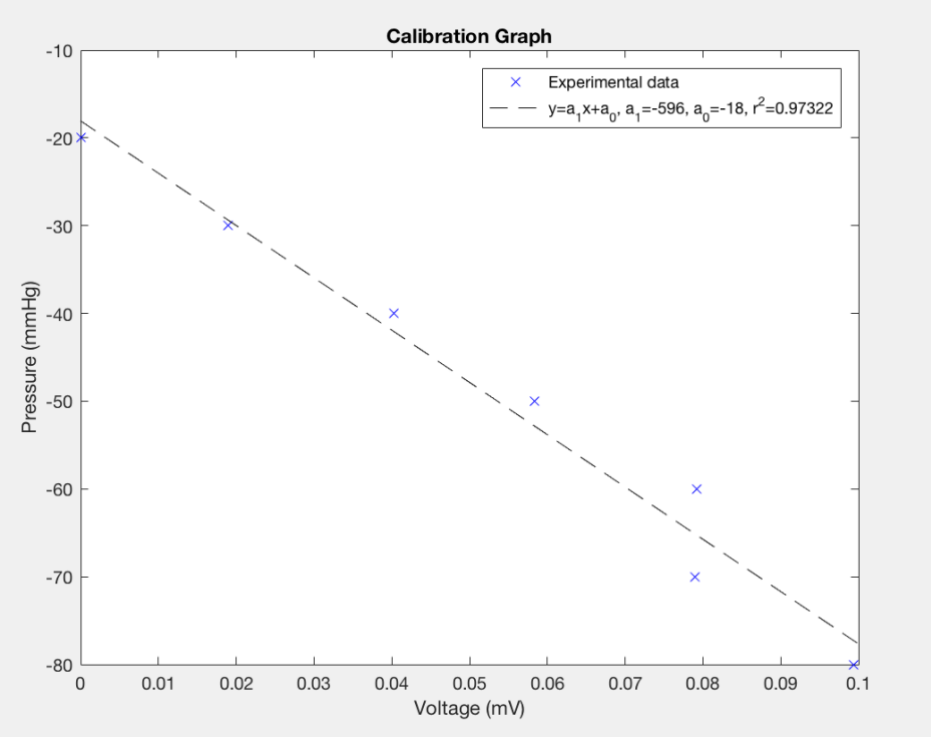


Figure 3: Calibration curve used to adjust data from Voltage (mV) to Pressure (mmHg), generated using a linear best fit.

To accommodate for a shifting baseline and noise, the data was averaged and subtracted from that average.

Results

Static Pressure

From the Coste et al. study, we know the threshold of pressure the cell membrane must pass in order to generate a current response. Too much pressure may break the seal and damage the cell, resulting in unusable data, and too low pressure is not enough to stimulate the cell. The Piezo channels respond best to fast, positive pressure; thus, these pressures were applied over a short time course. Results shown in **Figure 4** from the application of static pressure to wild type cells indicate a response is present and increases with the amount of pressure applied. This range of pressures was chosen to give controlled parameters for excitation that would illustrate the positive linear nature of the response, as shown in **Figure 5**. After stimulation, each graph recovers to the baseline in line with the pressure, however the maximum occurs concurrently with the start of the excitation pressure, indicative of the presence of Piezo channels.

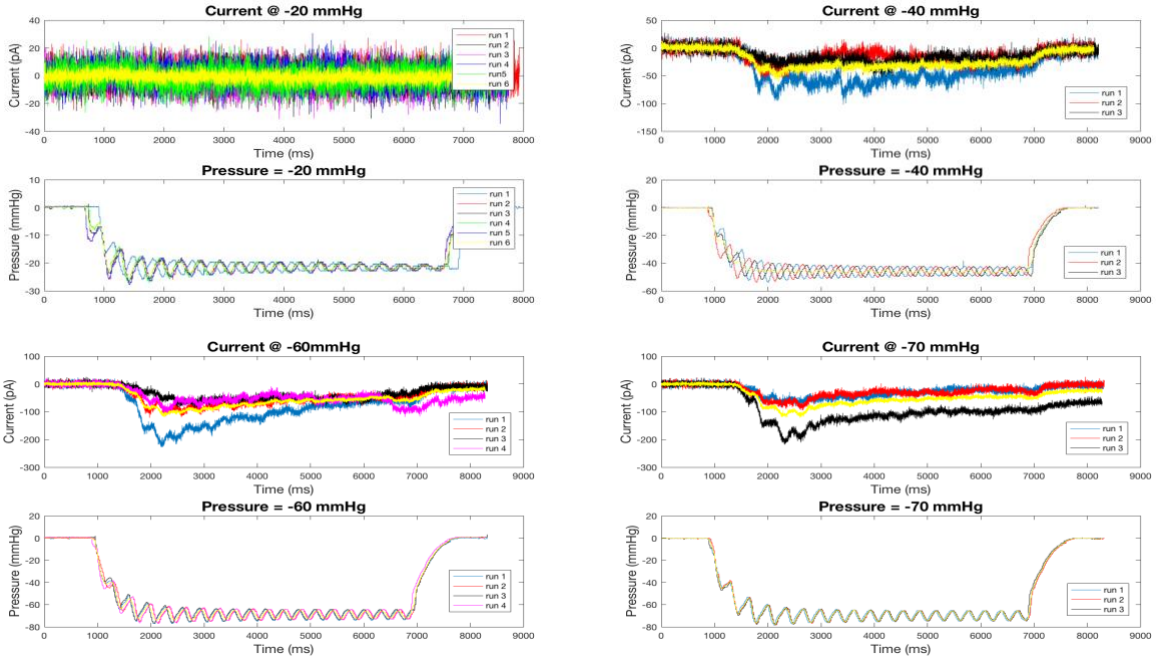


Figure 4: Raw data of current response to various negative pressures. Each top graph indicates the current (pA) with respect to time, while each bottom graph shows the pressure applied (mmHg). From left to right, top down: a) The

current response to a pressure of -20 mmHg is relatively flat and shows no deviation when perturbed. b) The application of -40 mmHg begins to show a response, with a maximum being in the range of -100 pA. c & d) At high pressures such as -60 and -70 mmHg, an observable response is seen, with the maximum ranging over -200 pA. The duration of the stimulation was 600 msec.

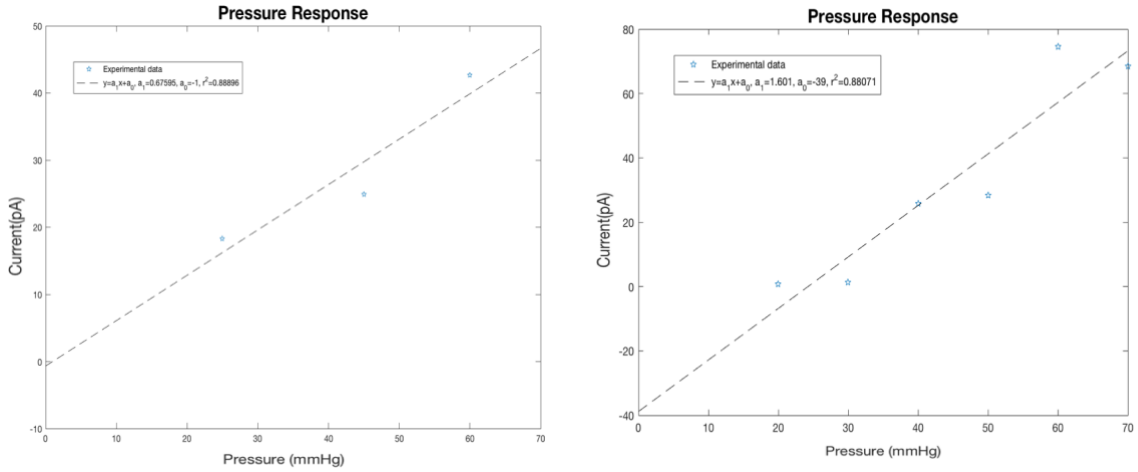


Figure 5: Processed data from 2 different cells indicates a positive correlation between current (pA) and pressure applied (mmHg)

3D Modeling, Ultrasound

A Matlab program was used to generate a modelled pressure field for the plate with the transducer, shown in **Figure 6**. The essential parameters include transducer placement, diameter of the plate, and transducer frequency. The 3D ultrasound model shows the nodes in yellow and the antinodes in blue for the 520 kHz transducer, with the highest and lowest points indicating the peaks in pressure. The antinodes are indicative of the points on the plate that undergo the maximum displacement during each vibrational cycle, thus cells in these areas will be experiencing the most force from the ultrasound. Understanding the location of these absolute maximums in pressure amplitude (nodes and antinodes) helps to describe a spatial variation in the pressure experienced by a cell under the same voltage applied to the transducer.

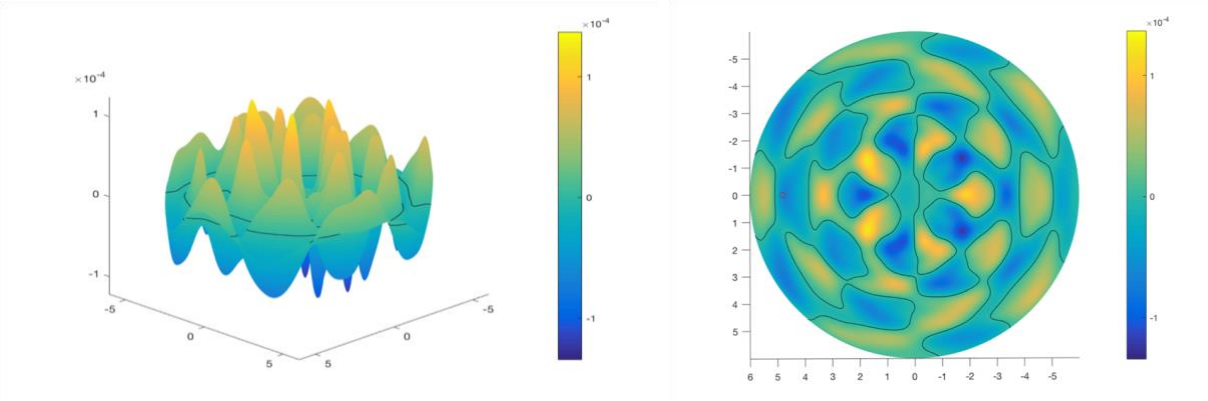


Figure 6: 3D ultrasonic field indicating nodes (yellow) and antinodes (blue) for cell culture plate

Ultrasonic Pressure

Results in **Figure 7** from the application of ultrasonic pressure show a current response remarkably similar to that of static pressure. The graph on the left illustrates a typical experiment time scale, with controlled excitation from the ultrasound shown in the evenly distributed spikes from the thicker bar. Each spike is equidistant from the next, correlating to the timed ultrasonic pulses, and corresponding to a flux in current. The selection magnified from this graph shows a snippet of this larger graph, with that same drop off then return to baseline seen in the static pressure graph. Again, the current shows a linear relationship between magnitude of pressure applied, and magnitude of current expressed, as shown in **Figure 8**.

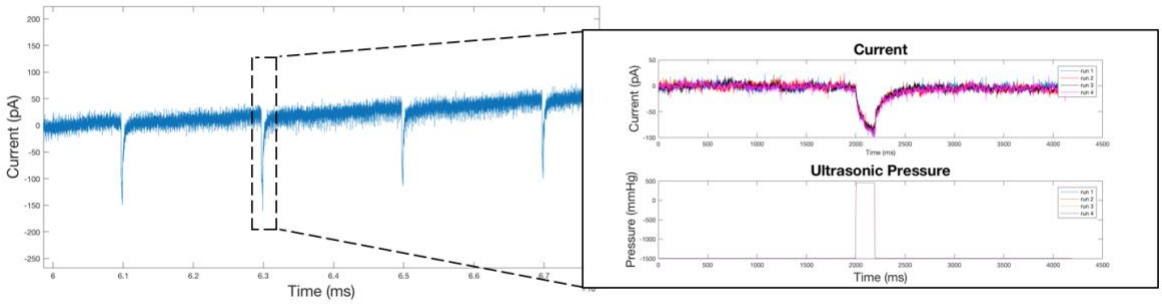


Figure 7: On the left, a larger time span of ultrasound application shows consistent spikes in current with the timed ultrasonic pulses. On the right is magnified image of one of the current spikes shown (pA) with the ultrasound pressure applied on the bottom. The pulse duration was 5,000 cycles.

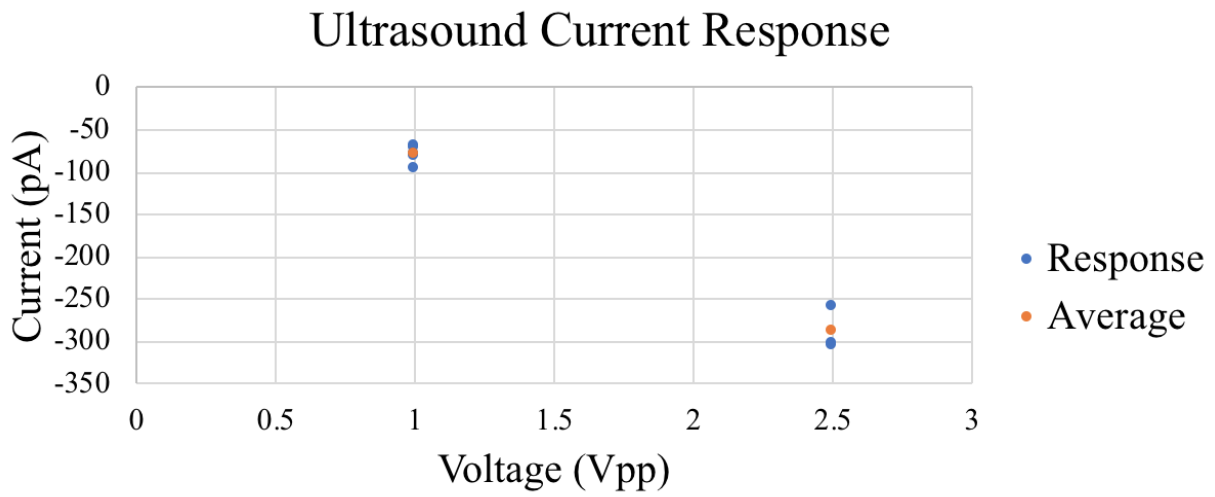


Figure 8: Analyzed data of the minimums of the current graphs for amplitudes of 1 and 2.5 Volts peak to peak. The voltage here is not the same as the voltage mentioned earlier, this voltage corresponds to the setting on the amplitude wave generator. Data is generated from experiments with eight different cells. The average current at 1 Vpp is-79.6 pA and the average current at 2.5 Vpp is -288.3 pA. A paired, two-tailed t-test with an alpha value of 0.05 yielded a p-value of 0.0018, indicating that these two data sets are statistically significantly different, and that higher currents result from higher pressures.

Discussion

In the application of static pressure to wild type N2A cells, an observable current response is generated. This response is proportional to the magnitude of pressure response, with the slope of the correlation graph being positive. This is indicative of the opening of Piezo1 mechanosensitive cells in response to the application of pressure, allowing the flow of ions and changing the membrane potential of the cell. It holds significance by its confirmation of the presence of Piezo channels, and gives a foundation to this research through confirming the method of measurement of this channel response is accurate and appropriate. By comparing this response to that of the ultrasound, we can determine the success of ultrasound in stimulation of mechanosensitive channels.

The application of ultrasonic pressure to wild type N2A cells generates a response comparable of that to static pressure. The resemblances between the current graph are striking and observable, indicating that in both applications of pressure, the N2A cells are opening ion channels, presumably associated with Piezo1 proteins and allowing the flux of ions. The ultrasound also has a positive correlation between the voltage applied and the magnitude of the current response, implying that as the amplitude of the wave is increased and the cell experiences more force, there is a greater amount of channels opening. The amplitude of the maximum current responses in picoamperes are comparable as well, with the ultrasound generating an even stronger response at 2.5 Vpp. This suggests that ultrasound is an even more effective strategy than static pressure for stimulation of these mechanosensitive channels.

The significance of this lies in the possible applications of this knowledge. This data confirms the use of ultrasound for neuromodulation or the control of neural excitation. We can infer that previous uses of ultrasound to generate a response in the brain may have been the result of these mechanosensitive Piezo channels. In diseases such as Parkinson’s or Alzheimer’s the

degeneration or loss of neurons makes neural communication nearly impossible, however stimulation of these ion channels from ultrasound to generate that same current response could help boost weak signals and improve neural performance. For neurodegenerative diseases, this could provide the tools needed to noninvasively stimulate those deteriorating neurons and halt the process of decay. Overall, this data links together the gap in the field by providing the mechanism for ultrasound neuromodulation and opens up many possibilities for ultrasound to be used in this rapidly growing field.

This work is limited in a few facets. This project faced difficulties in many areas and spent a huge portion of time trouble shooting. This work, while reproducible, requires many trials as viable data may only result from 30-40 percent of experiments. Difficulties in cell health and patchability, especially when cultured with the transducer, limited the data and resulted in fewer successful patches than we hoped for. In the static pressure experiments, the parameters of excitation are very limited, as the cell must be stimulated quickly and strongly, and our setup was modified multiple times to accommodate for this. This data is representative of the successful trials, however in future experiments to make these conclusions more robust, it would be beneficial to generate more data, optimize the setup, and have a higher success rate.

The expansion of work done in this study could materialize in many forms. During the course of these experiments, we also expanded our trials to include the transfection of N2A cells to overexpress Piezo channels, allowing for secure confirmation that the response was attributed to the Piezo protein. However, due to the nature of transfection and its disruption of the extracellular membrane, we were rarely able to patch a transfected cell and generate data, so in further work it would be beneficial to successfully transfect the N2A cells and analyze that data. Another protocol that could be explored would be calcium imaging using a microscope that

could capture millisecond scale response, or calcium imaging on sensory neurons (not N2A). The issue with the calcium imaging attempted in these experiments was the time scale, as it could only measure responses on the second scale, which is far too long to see a millisecond scale response. Calcium imaging tags ions with a fluorescent marker and tracks their movement in an observable way either in or out of the cell, and this would be a great tool for viewing the flux of ions through Piezo channels.

Conclusion

In conclusion, this work addresses gaps in the field of ultrasonic neuromodulation by investigating the effect of ultrasound on a cellular level, and how that may play into neural function. The mechanical effects of ultrasound on the brain are staggering, and this research paves the way for a noninvasive neuromodulation technique which understands its cellular mechanism of action and utilizes it to optimize this technique. This opens many doors for the use of ultrasound in the brain and is not limited to therapies for neurodegenerative diseases, as the capacity to control excitation in the brain noninvasively could lead to nearly boundless technological applications. More work in this direction is warranted.

References

1. Coste, B., et al., *Piezo1 and Piezo2 Are Essential Components of Distinct Mechanically Activated Cation Channels.* Science, 2010. **330**(6000): p. 55-60.
2. DeMaagd, G. and A. Philip, *Parkinson's Disease and Its Management: Part 1: Disease Entity, Risk Factors, Pathophysiology, Clinical Presentation, and Diagnosis.* Pharmacy and Therapeutics, 2015. **40**(8): p. 504-532.
3. Rubaiy, H.N., *A Short Guide to Electrophysiology and Ion Channels.* Journal of Pharmacy and Pharmaceutical Sciences, 2017. **20**: p. 48-67.
4. Ilkan, Z., et al., *Evidence for shear-mediated Ca2+ entry through mechanosensitive cation channels in human platelets and a megakaryocytic cell line.* Journal of Biological Chemistry, 2017. **292**(22): p. 9204-9217.
5. Pottosin, I., et al., *Mechanosensitive Ca2+-permeable channels in human leukemic cells: Pharmacological and molecular evidence for TRPV2.* Biochimica Et Biophysica Acta-Biomembranes, 2015. **1848**(1): p. 51-59.
6. Nencini, S. and J. Ivanusic, *Mechanically sensitive A nociceptors that innervate bone marrow respond to changes in intra-osseous pressure.* Journal of Physiology-London, 2017. **595**(13): p. 4399-4415.
7. Gao, Q.H., et al., *Role of Piezo Channels in Ultrasound-stimulated Dental Stem Cells.* Journal of Endodontics, 2017. **43**(7): p. 1130-1136.
8. Yoo, S.-S., et al., *Focused ultrasound modulates region-specific brain activity.* NeuroImage, 2011. **56**(3): p. 1267-1275.
9. Phenix, C.P., et al., *High intensity focused ultrasound technology, its scope and applications in therapy and drug delivery.* J Pharm Pharm Sci, 2014. **17**(1): p. 136-53.
10. Elias, W. J., et al., *A Randomized Trial of Focused Ultrasound Thalamotomy for Essential Tremor.* New England Journal of Medicine, 2016. **375**(8): 730-739.
11. Bertrand, N., et al., *Cancer nanotechnology: the impact of passive and active targeting in the era of modern cancer biology*. Adv Drug Deliv Rev, 2014. **66**: 2-25.
12. Sassaroli, E., & Vykhodtseva, N., *Acoustic neuromodulation from a basic science prospective*. Journal of Therapeutic Ultrasound, 2016. **4**(1).