Quantifying the Effects of Local and Global Glutamate Expression on Electrical and Calcium Signaling Dynamics

Grant Proposal

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Author Note

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Abstract (RQ) or Executive Summary (Eng)

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease that specifically targets the motor neurons (the neurons that control movement) of the human body and cause cell death. These motor neurons control the functions of breathing, swallowing, walking, and many more. Every year, over 5,000 people die of ALS, as there is no known cure for the disease (Centers for Disease Control and Prevention [CDC], 2024). In ALS patients, glutamate, the main excitatory neurotransmitter which sends signals to communicate with other neurons, is not cleared properly around the motor neurons. This happens because the protein that removes glutamate from around the motor neuron, EAAT2, is located on astrocytes which are found in the brain and spinal cord. Since ALS leads to these brain and spinal cord neuron degeneration due to excitotoxicity, the glutamate around other motor neurons no longer clears up, leading to build up. This build up can cause excessive firing as well as lead to increased calcium in the motor neurons, as glutamate binding to glutamate receptors causes calcium to enter these cells or be released (Le Gall et al., 2020). These factors lead to extreme cell stress and eventually cause motor neurons death. This process of the increase in calcium, excessive firing of neurons due to glutamate receptors, and eventual cell death is called excitotoxicity. Calcium dysregulation is a major part of ALS, and there is evidence of motor nerve cells in ALS patients showing abnormally high calcium levels, impaired calcium regulation, and larger amounts of calcium influx during neuron activity (Grosskreutz, van Den Bosch, & Keller, 2010). Scientists have previously identified how glutamate can increase the influx of calcium through NMDA receptors (a glutamate receptor), and how excess amounts of glutamate contributes to excitotoxicity in ALS, however, the specific relationship between glutamate and calcium dynamics under electrical stimulation is yet to be understood (Spreux-Varoquaux et al., 2002). Using Periplaneta *americana* cockroaches as a neurological model, this project aims to quantify the relationship, locally and globally, between calcium and electrical stimulation, under neuromodulation.

Keywords: ALS, motor neuron, glutamate, calcium, NMDA, EAAT2

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Glutamate Increasing Neuronal Activity

Glutamate receptors have been known to function as channels to allow the entry of certain ions into nerve cells. This occurs because when glutamate binds to these ionotropic receptors, positively charged ions such as sodium and sometimes calcium ions, flow into the cell. When this instance occurs multiple times, a coordinated flow of ions across nerve cell membranes is created, which leads to the generating of nerve signals (National Cancer Institute, 2014). This process, which was originally initiated by glutamate and controlled by glutamate leads to an increase in neuronal activity in the neuromuscular junction (NMJ). The neuromuscular junction is the place in the human body where a motor neuron connects with a muscle, allowing the neuron to signal the muscle to contract. However, an excess amount of activation of ionotropic glutamate receptors such as NMDA by glutamate can lead to a dangerous condition called excitotoxicity. Excitotoxicity is the process in which neurons are damaged or killed by excessive activation of glutamate receptors. This may increase the localized vulnerability of neurons which is a consequence of a changed regional distribution of NMDA receptors (Hynd, 2004).

Glutamate in the Cockroach NMJ

Cockroaches have been established as an excellent model for neuroscience experiments as they have neurons that are very similar to humans. Though their nervous systems are simpler, they still exhibit basic functions like sensing, moving, and learning, which can be easily manipulated using technology. Scientists have identified glutamate to be a primary neurotransmitter in certain neuromuscular junctions of cockroaches, specifically Periplaneta *americana*. They found that L-glutamate, one of the most abundant neurotransmitters in the brain, was able to cause a depolarization of the muscle fiber in cockroaches, showing how glutamate plays a significant role in the cockroach NMJ.

Additionally, the effects that glutamate and GABA (an inhibitory receptor) had on the cockroach were opposite to each other, as the threshold concentration that allowed L-glutamate to increase the amplitude and frequency of the cockroach muscle contractures and Mepps was the same threshold concentration that allowed GABA to decrease the Mepps and contractures of the cockroach muscle (Kerkut & Walker, 1966). These findings show how glutamate has a significant role as a neurotransmitter in the neuromuscular junctions of cockroaches.

Glutamate as a Modulator and Mediator in the NMJ

It is still unknown where glutamate receptors have precisely localized at the presynaptic terminals of neuromuscular junctions, as well as in motoneurons. However, glutamate at the neuromuscular junction has been shown to act on the modulation of cholinergic synaptic transmission (neurotransmission by acetylcholine at synapses) by interacting with presynaptic receptors. In the tail muscles of frogs, glutamate has been found to activate presynaptic ionotropic (ion channels) receptors, such as AMPA and NMDA. This activation leads to an increase in the spontaneous release of acetylcholine. This effect of glutamate on the increased spontaneous release of acetylcholine is controlled by influx of calcium ions that occurs through the NMDA receptors at the opening of voltage activated calcium ion channels. This same effect regarding glutamate and an increase in spontaneous acetylcholine release also occurred in zebrafish larvae (Colombo

& Francolini, 2019). Glutamate can increase neuronal activity through the modulation of the cholinergic synapse, as increased amounts of glutamate leads to larger releases of the neurotransmitter acetylcholine, leading to increased neuron signals and a larger neural output. This is evidence of how glutamate can affect neuronal activity in humans.

Glutamate and Calcium Dynamics

Muscle contraction can be demonstrated by the sliding filament model, which consists of actin and myosin heads that bind to each other to generate a force, and this process is mediated by calcium ions. A calcium transient is the trigger for actin-myosin crossbridge binding, which is where the actin (thin filament) is grabbed by myosin (filament "heads"), and the crossbridge is where the myosin grabs the actin (Figure 1). These cross bridges pull, causing the muscle to contract, which generates a force. More spikes means that there is more calcium, which means there is more actin available for the myosin to grab, and therefore more cross bridges are formed, leading to more/stronger contractions and forces. Recently, studies conducted on ALS have demonstrated that changing glutamate receptor signaling influences calcium dynamics. When too much glutamate was activated, this led to calcium being overloaded and caused neurons to die off. ALS mutations caused more neurons to die off and increased the severity of calcium overloading. They found that calcium resting levels were elevated, there were larger infrequent spikes in muscle force, and the cells could not restore calcium fast enough (Bursch et al., 2019). This study indicates that local and global changes in glutamate influence the baseline, amplitude, and frequency of calcium dynamics. Scientists have previously identified the effect of electrical stimulation on the baseline, amplitude, and frequency of calcium dynamics; however, the effects of neuromodulation on calcium dynamics under electrical stimulation have yet to be studied.

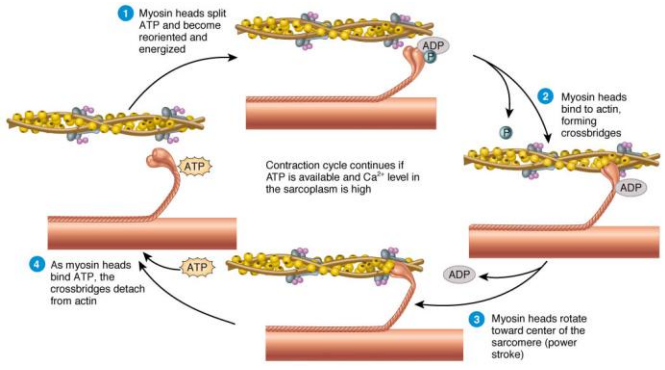

Figure 1. The sliding filament model and the process of cross bridges forming through the binding of actin and myosin fueled by calcium transients (Karki, 2020b).

ALS and Calcium Transients

In ALS, or Amyotrophic Lateral Sclerosis, the motor neurons die off, leading patients to lose functions such as breathing, walking, talking, etc. This occurs due to ALS causing a deficiency in nerve cells' ability to clear up excess glutamate, leading to glutamate build

up. This buildup of glutamate causes the intercellular levels of calcium to change inside the nerve cells, which eventually kills them off. The proteins that clear up glutamate are located on the spinal cord, and since the neurons on the spinal cord die off, the proteins also die off and are unable to complete their function. Scientists have tried multiple medications to reduce the onset of ALS, such as reducing the speed of glutamate being excreted from the cell. However, these medications have lots of toxic side effects and have not been extremely effective in treating ALS (Aggarwal & Cudkowicz, 2008). Scientists have also tried to treat ALS using electrical stimulation, however they have yet to identify how different frequencies of electrical stimulation can affect the calcium dynamics and the specific changes to the calcium amplitude, baseline, and frequency in the motor nerve cells. Though scientists know that lower frequencies of electrical stimulation do decrease calcium transients in a non-neuromodulated environment, they have not yet studied what frequencies can reduce calcium transients in a neuromodulated environment, such as with glutamate added.

Section II: Specific Aims

This proposal's objective is to quantitatively define the characteristics of calcium dynamics under neuromodulation by glutamate in cockroaches.

Our long-term goal is to be able to translate the characteristics of calcium dynamics under neuromodulation by glutamate in P.*americana* to humans and for scientists to be able to apply this relationship in ALS patients through nerve or spinal cord stimulation. The central hypothesis of this proposal is to show that neuromodulation increases the baseline, amplitude, and frequency of the calcium dynamics relationship. The rationale is that since glutamate is the primary neurotransmitter in the cockroach neuromuscular junction, increasing the amount of glutamate would lead to larger electrical spikes under electrical stimulation. The work we propose here will further the understanding of the relationship between glutamate and calcium dynamics, and lead to better ways to treat diseases

affected by unbalanced glutamate and calcium dynamics, such as ALS. The work will also lead to a deeper understanding of how glutamate can affect neuronal activity and allow scientists to refine neuro-prosthetics to have them heal and function at the same time by injecting glutamate into their patients.

Specific Aim 1: Determine the relationship between glutamate and increased electrical output and resemblance of neuron activity

Specific Aim 2: Determine the relationship between glutamate and calcium dynamics

Specific Aim 3: Determine the relationship between calcium dynamics, ALS, and excitotoxicity

The expected outcome of this work is that lower frequencies of electrical stimulation will lead to fewer calcium transients entering the motor nerve cells, which will be shown through the calcium imaging. The highest electrical stimulation will lead to the most amounts of calcium transients, as the cells will be the most active and therefore excrete more glutamate, which adding to the induced increase in glutamate will lead to motor nerve cell death.

Section III: Project Goals and Methodology

Relevance/Significance

Biomedical Significance:

Irregular glutamate signaling and calcium overload contribute to the development of ALS. Patients with ALS experience excitotoxicity which comes from the increased amounts of excess glutamate around their motor nerve cells. Excitotoxicity leads to intercellular levels of calcium being unbalanced in the motor nerve cells, leading to eventual motor neuron cell death. This project will model an ALS environment in cockroaches and help scientists determine how different levels of electrical current can affect intercellular calcium levels, either by reducing them or increasing them. This project will be creating equations that precisely relate specific electrical currents to calcium dynamics, which can be

used by scientists and translated to humans to determine how a similar level of electrical stimulation on the spinal cord or nerves can lead to the progression of ALS slowing down. Scientists are unaware of what electrical frequencies can lead to less calcium transients entering the motor nerve cells. This project will help determine the specific relationship between electrical stimulation and calcium dynamics in a neuromodulated environment using a neurological model such as cockroaches.

Innovation

It has not been previously identified how calcium dynamics are affected during electrical stimulation in an ALS setting – which is mimicked by neuromodulation by glutamate. Scientists can use this information to know which frequencies of electricity can reduce calcium influx into motor nerve cells and reduce the onset of the disease of ALS. Additionally, this project integrates both calcium dynamics under electrical stimulation and calcium dynamics under glutamate which have not been studied together before.

Methodology

Cockroach Preparation Procedure (Local and Global):

1. Chill the cockroach on ice to anesthetize/numb it
2. Secure cockroach with pins and fix the ventral side up
3. Surround the cockroach with cockroach Ringer's solution to keep tissue moist
4. Remove cuticle around the coxa using scalpel to access the nerve/ganglion (local) and brain tissue (global)
5. Keep tracheae and fat as intact as possible

Calcium Indicator Procedure:

1. Prepare Fluo-3 AM
2. Dilute to ~5–10 µM in Ringer's solution
3. Apply it to the exposed tissue and incubate for 30-60 min in the dark
4. Wash gently with Ringer's for 15-30 minutes to remove excess dye

Electrical Stimulation Procedure Local and Global:

1. Use the Roboroach software and spiker box to electrically stimulate the leg (local) and brain (global) at three different levels of electrical stimulation (low, medium, high)
2. Stimulate the leg or brain for around 1 minute and record the electrical output using the Spiker box or RoboRoach
3. Capture video or time-lapse images during electrical stimulation to track motor output

Calcium Dynamics Visualization Procedure:

1. Set up a fluorescence stereomicroscope
2. Record calcium imaging using the fluorescence stereomicroscope

Glutamate Application Procedure Local and Global:

1. Fill a glass micropipette with glutamate solution (around 1-10 mM)
2. Apply short pressure pulses of glutamate near the tissue in the leg (local) and brain (global) with a syringe setup
3. Start with low concentrations to avoid excitotoxicity

Procedure: Local and Global Application:

Part 1: Cockroach Preparation (see local procedure above)

Part 2: Applying Calcium Indicator (see above procedure)

Part 3: Applying Glutamate (see above procedure)

Part 5: Electrical Stimulation, test at the three levels (low, medium, high) (see above local procedure)

Part 6: Record Calcium Dynamics (see above procedure)

Procedure: Local and Global Control Groups

Part 1: Preparing the Cockroaches for testing (see above local procedure)

Part 2: Applying Calcium Indicator (see above procedure)

Part 3: Electrical Stimulation, test at the three levels (low, medium, high) (see above local procedure)

Part 4: Record Calcium Dynamics (see above procedure)

Specific Aim #1: **Determine the relationship between glutamate and increased motor/electrical output + resemblance of neuron activity**

The objective is to understand how glutamate can affect increased motor or electrical output and the inner workings of this process. Our methodology to understand this is to electrocute the cockroaches with glutamate vs without, to observe if electrical output is increased with glutamate vs. without. Our rationale for this approach is that electrical output resembles the activity of the neurons, so electrically stimulating the cockroach will show how active their neurons are with and without extra glutamate added.

Justification and Feasibility. To address this specific aim, the methodology of shocking the cockroaches and observing the electrical output will show evidence for electrical stimulation

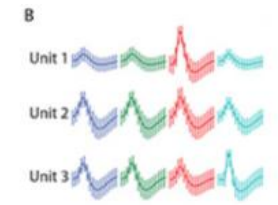

Figure 1.1. Raw voltage traces from single electrodes within one tetrode bundle. There is a difference in the voltage traces among the different electrodes. The units were sorted with MClust.
Guo, P., Pollack, A. J., Varga, A. G., Martin, J. P., & Ritzmann, R. E. (2014). Extracellular wire tetrode recording in brain of freely walking insects. *Journal of Visualized Experiments*, *86*. https://doi.org/10.3791/51337

representing neuron activity. In a related study, scientists used wire tetrodes to record the neurons of the central complex of the cockroach. They connected the wires from the tetrodes into a simulator in order to stimulate activity in the cockroaches and observe the neuron responses that resulted (Guo et al., 2014). The raw voltage traces with electrical stimulation showed higher amounts of neuron activity, as the electrodes in that region picked up higher voltage traces as seen in red in Figure 1.1. This study supports how electrical stimulation can reflect neuronal activity and model movement in cockroaches, as electrical stimulation (movement) led to higher neuron activities as the motor neurons were functioning.

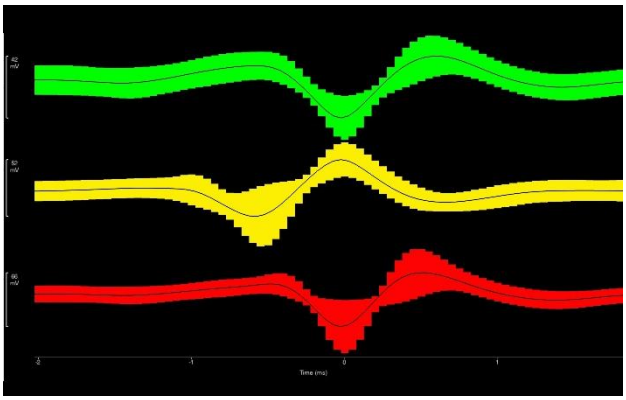

Figure 1.2 Raw Action Potentials of the Cockroach Leg

Summary of Preliminary Data. Preliminary data for this specific aim consists of the raw action potential waves of a cockroach leg using the Spiker Box to stimulate the leg electrically. This preliminary data helps uncover the resting action potentials of the cockroach leg, which would be the local control for this experiment. The red wave indicates the action potential of the cockroach leg without any smoothing or editing of background noise. The yellow wave indicates the action potential of the cockroach leg with light smoothing and editing of background noise. The green wave indicates the edited, most accurate action potential of the cockroach leg. This data provides evidence of how the action potential of the cockroach leg can be recorded and measured, as shown in Figure 1.2. In the future, data will consist of the action potentials of the

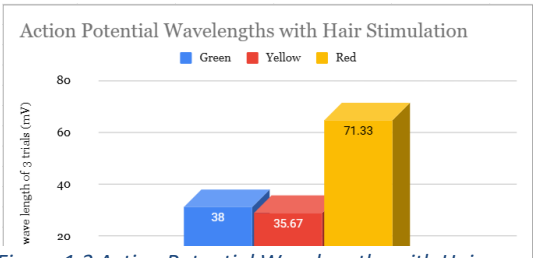

Figure 1.3 Action Potential Wavelengths with Hair Stimulation

cockroach leg, under neuromodulation of glutamate. This would be the local application of glutamate, and it would show how glutamate can increase electrical output (action potentials) locally. Additionally, Figure 1.3 compares the resting action potential wavelengths with stimulation of the hair neurons of the cockroach leg from different channels (red, yellow, green). This preliminary data helps support how the resting action potentials of the cockroach leg can be taken with stimulation of specific neurons as well. In the future, the cockroach leg could be under neuromodulation by glutamate, and the same hair neurons could be electrically stimulated to observe their action potential with glutamate added. This would show how glutamate increases the electrical output locally in all neurons in this area of the leg.

Expected Outcomes. The overall outcome of this aim is to understand that stimulating the cockroach electrically can model motor neuron activity in the cockroach by movement and how glutamate expression can increase this electrical output. This knowledge will be used to model neuronal activity in the cockroach and how that effects glutamate builds up around motor neurons and in turn the intercellular calcium dynamics of the motor neurons.

Potential Pitfalls and Alternative Strategies. We expect the electrical output to be high if there is a high electrical current entering the cockroach. However, this may not be the case under neuromodulation by glutamate, as there is no quantitative data that can reasonably be expected. If this is not the case under neuromodulation by glutamate, then the electrical current frequency (low, medium, or high) that reduces the calcium dynamics and glutamate the largest will be most effective in reducing the factors that contribute to ALS.

Specific Aim #2: **Determine the relationship between glutamate and calcium dynamics**

The objective is to determine how neuromodulation by glutamate can affect the intercellular calcium dynamics of motor neurons. Our approach (methodology) is to increase the amount of glutamate

receptors in a cockroach, both locally and globally for more quantifiable evidence, electrocute the cockroach with different levels of electricity, and then conduct calcium imaging using fluorescence. This would help to identify whether the intercellular levels of calcium increased or decreased with different levels of electricity. These results would then be compared to the resting levels of intercellular calcium without neuromodulation after electrical stimulation. Our rationale for this approach is that it would allow us to observe the levels of calcium visually, and record whether the addition of glutamate increased the amounts of calcium observed by fluorescence or decreased it. This is the only way to quantify the intercellular calcium levels of the nerve cells with evidence.

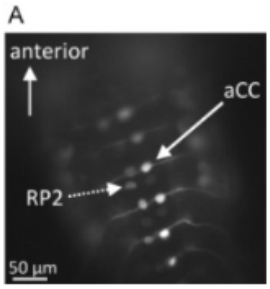

Figure 1.4. The expression pattern of the GCamP5 imaging.
Streit, A. K., Fan, Y. N., Masullo, L., & Baines, R. A. (2016). Calcium imaging of neuronal activity in drosophila can identify anticonvulsive compounds. *PLoS ONE*, *11*(2), e0148461. https://doi.org/10.1371/journal.pone.0148461

Justification and Feasibility. To address this specific aim, the methodology of conducting calcium imaging on the cockroaches after neuromodulation and electrical stimulation will show whether glutamate increased the action potential or decreased them, and if different levels of electrical stimulation generated more/less calcium intercellularly. In a related study, scientists determined how neuronal activity is affected in the CNS of defined seizure mutants using calcium imaging in *Drosophila*. They identified how the calcium imaging technique they used, GCaMP imaging which uses the GCaMP calcium indicator, is mostly efficient for observing neuronal activity, not individual action potentials (Streit et al., 2016). They were able to visualize the calcium in the motor neurons of seizure mutants as shown in Figure 1.4. This study supports how calcium imaging can be used in insects to display neuronal activity, and in turn, show how neuronal activity can change based on factors such as seizure mutants or neuromodulation.

Summary of Preliminary Data. To support this specific aim, future preliminary data would consist of the resting calcium levels of the motor neurons in the cockroach after electrical stimulation

without neuromodulation. This data would help support the specific aim as it would be the control data that the calcium levels of the neuromodulated cockroach motor neurons will be compared to. This data is necessary to observe if the addition of glutamate caused an increase or decrease in the intercellular calcium levels of the motor neurons in the cockroach.

Expected Outcomes. The overall outcome of this aim is to understand the relationship between neuromodulation by glutamate and calcium dynamics intercellularly. This knowledge will be used for evidence on how increasing the glutamate levels of the motor neurons of the cockroach and in turn increasing the intercellular calcium levels is a model for ALS and the processes that occur in ALS. Excitotoxicity is a major factor in ALS, so this evidence will model the motor neurons of an ALS patient.

Potential Pitfalls and Alternative Strategies. We expect calcium dynamics intercellularly to increase because of increased neuromodulation by glutamate. We expect this because glutamate causes the intercellular levels of calcium to increase, so we believe that the same conditions will hold true while the neurons are electrically stimulated by the Spiker Box. However, since there is currently no evidence on how the calcium dynamics measure under electrical stimulation while being neuromodulated by glutamate, this may not occur. If this is not the case, then the calcium dynamics with and without glutamate will be compared, to show if glutamate even influences the calcium dynamics of a cockroach leg under electrical stimulation. Additionally, calcium dynamics could be modeled without electrical stimulation, as there is previous data on how the intracellular levels of calcium increase under neuromodulation of glutamate.

Specific Aim #3: **Determine the relationship between calcium dynamics, ALS, and excitotoxicity**

The objective is to model how the calcium dynamics in an ALS patient are affected by different frequencies of electrical stimulation. Our approach is to use the three electrical stimulation outputs

(high, medium, low) from electrically stimulating the cockroach after neuromodulation by glutamate and input them as well as other variables regarding the intercellular levels of calcium in the cockroaches, into a model to understand which electrical frequency reduces calcium levels the most in motor neurons. Our rationale for this approach is that the modeling of calcium dynamics can allow scientists to apply this model to their studies and patients in the future in order to understand how to treat them.

Justification and Feasibility. To address this specific aim, MATLAB will be used to create equations for the relationships between each level of electrical current –low, medium, high—and the calcium dynamics with that current in the ALS setting. In MATLAB, values for action potentials, calcium levels, and electrical input can be coded to result in precise equations. These equations can help scientists identify what level of electrical stimulation will be the most beneficial for humans, and they can see the exact measurements of calcium dynamics the cockroaches outputted with each electrical input level.

Summary of Preliminary Data.

Expected Outcomes. The expected outcomes of the modeling of the calcium dynamics would be that the equation with the least amount of calcium dynamics would have the least values for calcium dynamics as in non-ALS settings, less electrical stimulation leads to less activity, less neurotransmitters, and therefore less calcium entering the motor neurons.

Potential Pitfalls and Alternative Strategies. Potential pitfalls for this specific aim would be that the modeling may have errors related to what actually occurred, as the values can differ based on external factors. Additionally, the model may not be easily translatable to human systems. Some alternate strategies would be to conduct nerve stimulation on vertebrate muscle with the most effective electrical current, to understand if the equations can be translated to vertebrates.

Section III: Resources/Equipment

Materials List: Cockroaches (P. *americana*), L-glutamate receptors, Roboroach software from Backyard Brains, stereomicroscope/dissection scope, micropipettes/microinjection tools, fine manipulators/micromanipulators, electrodes, high-speed camera, force sensor/micro load cell, stable mounting platform for cockroach, physiological saline, glutamate reagent, video tracking software, Fluo 4AM, fluorescence microscope, super glue, BYB App to control Roboroach, Spike Recorder App to measure outputs from Spiker Box, needles, hot glue.

Section V: Ethical Considerations

Ethical considerations for using the Roboroach include the welfare and safety of the animal, as using the Roboroach might cause the cockroach pain and stress. To use the RoboRoach, the user has to poke a whole into the trachea of the cockroach in order to insert a wire and connect to the neurons there. Therefore, if this is not done properly, the cockroach could be killed. The cockroaches should be given breaks between usage of the Roboroach in order to reduce damage to them. Additionally, when using the Spiker Box, the user will have to cut off one of the cockroach's leg, which is an ethical consideration as the cockroach may die without it.

Section VI: Timeline

October 20-24: Finalize materials and start to purchase or find out where to purchase from

October 27-31: Start ordering all materials

November 3-7: Start to prepare and finalize procedure for pretesting

November 10-14: Pretest in the MAMS Lab

November 17-21: Start to analyze data and make graphs

November 24-28: Analyze data using computational model to model the calcium dynamics

December 1-5: Finish up the computational model analysis and how it correlates with predicted movement outcomes and start poster for December fair

December 8-14: Finish poster for December fair

Post December Fair (January): Order materials for actual testing trials, refine procedure and make necessary changes, begin testing, analyze data with graphs, conduct computational model calcium dynamics analysis if needed, show how the calcium dynamics correlate with predicted movement, make science fair poster for Feb Fair

February: Present project at Feb Fair

Section VII: Appendix

Section VIII: References

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