Introduction

Resting membrane potential results from an electrochemical difference that exists across the neuronal membrane when a neuron is at rest or when an action potential is not being fired (Wright, 2004). This difference or gradient arises due to a few reasons but two big reasons are the role of the sodium-potassium pump and the relative permeability of the membrane to these ions at rest or how easily these ions cross the membrane at rest (Wright, 2004). The sodium-potassium pump works to pump three sodium ions out of the cell and two potassium ions go into the cell, thus creating a difference in charge across the membrane and the inside of the cell being more negative at rest (Wright, 2004). In terms of relative permeability, potassium is more permeable at rest because potassium ion-selective membrane channels are open at rest while sodium ion-selective membrane channels are closed at rest, thus resulting in the efflux of potassium ions down their concentration gradient and less positively charged ions being in the cell (Wright, 2004). This leads to a difference in charge across the membrane, too, and the inside of the cell being more negative at rest (Wright, 2004). Thus, considering these two factors, it makes sense that the resting membrane potential is around -80 mV (Wright, 2004). Lastly, resting membrane potential is crucial in the proper functioning of our nervous system since it helps reset ion concentrations so an action potential can then properly fire (Wright, 2004).

The neurotransmitter glutamate is known to be the main excitatory neurotransmitter in the brain and spinal cord and is involved in many metabolic pathways (Zhou and Danbolt, 2014). Too much glutamate can actually excite these neuronal cells to death and this is referred to as excitotoxicity (Zhou and Danbolt, 2014). This is due to the fact that these cells are not able to make it back to resting membrane potential to reset since glutamate is constantly activating specific types of ionotropic receptors (fast-acting ligand-activated ion channels) called NMDA and AMPA receptors (Mattson, 2019). When glutamate binds to these NMDA receptors and AMPA receptors, this causes sodium and calcium ions to rush into the cell, thus bringing the

membrane potential to a less negative number or closer to 0 mV - it depolarizes the cell (Zhou and Danbolt, 2014). Many studies have actually shown excitotoxicity due to high glutamate levels to be involved in various neurological diseases like Huntington's disease (Lau and Tymianski, 2018). The excitotoxicity hypothesis of Huntington's disease suggests that the neuronal cell death in the striatum is caused by excess glutamate or the overactivation of glutamate receptors (Andre et al., 2010). This is based on studies that would directly inject glutamate receptor agonists, or chemicals that act like glutamate to directly activate receptors, into the striatum - doing this induced neuronal loss and reproduced symptoms similar to those in Huntington's disease (Andre et al., 2010).

The important underlying role of glutamate in resting membrane potential and its effects on neurological conditions like Huntington's disease provided reason to explore further. This is mainly because there seems to be a gap in knowledge in how to target certain regions of the brain, like the striatum, affected by high levels of glutamate and thus, don't allow these cells to return back to resting membrane potential in these neurological diseases like Huntington's disease (Andre et al., 2010). For example, an antiglutamatergic drug called remacemide is a nonselective NMDA antagonist - so it blocks the effects glutamate can have on NMDA receptors by not activating these receptors and not allowing an influx of sodium and calcium (Andre et al., 2010). However, it just blocks all possible receptors without targeting certain ones (Andre et al., 2010). So, the goal of this experiment was to observe the effects of glutamate on resting membrane potential in hopes to build a foundation to create better drugs that target specific regions of the brain in neurological disease affected by higher glutamate concentrations in the brain.

To do this, this experiment tried to replicate the experiment done by Taylor and Frazier in their paper titled *Effect of nicotine and acetylcholine on crustacean muscle membrane potential* (1986). In the 1986 paper, these scientists wanted to see how nicotine and acetylcholine affect resting membrane potential of specific muscle cells in the crayfish that make up a muscle called

the deep extensor abdominal muscle (Taylor and Frazier). Their protocol involved using a crayfish tail and pinning it down to Sylgard in a glass dish (Taylor and Frazier, 1986). With the ventral side up, a single muscle fiber was impaled with glass microelectrodes that record intracellular potentials and after waiting 15 minutes to let things stabilize, the resting membrane potential is recorded (Taylor and Frazier, 1986). Glutamate with a concentration of 0.585 mM was used as a control to test the responsiveness of the muscle cell and once a response was measured, then the muscle was rinsed with saline to allow it to return to a stable resting membrane potential (Taylor and Frazier, 1986). Then the muscle was soaked in a nicotine solution bath, a response was measured, and then rinsed with saline to return to a stable resting membrane potential so then it could be soaked in a glutamate solution bath (Taylor and Frazier, 1986). Again, the muscle was rinsed with saline and returned to a stable resting membrane potential before either a nicotine or acetylcholine solution was added (Taylor and Frazier, 1986). Lastly, a third glutamate treatment was performed (Taylor and Frazier, 1986). Recordings showed that glutamate had a statistically significant effect on resting membrane potential due to it showing a decrease in membrane potential, while nicotine and acetylcholine did not have a statistically significant effect on resting membrane potential (Taylor and Frazier, 1986).

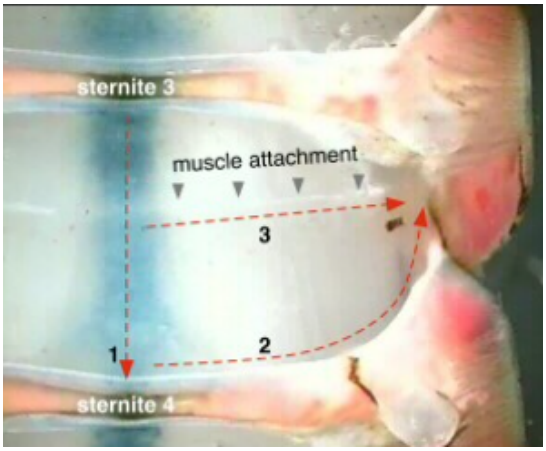
For this experiment, glutamate was the focus of our study and how it affects resting membrane potential. So, resting membrane potentials was measured from an electrode impaling the deep extensor abdominal muscle found on the ventral side of a crayfish tail. Extracellular recordings consisted of the tail being soaked in a 0 mM concentration of glutamate, then a 0.2925 mM concentration of glutamate, and a 0.585 mM concentration of glutamate. It was hypothesized that by increasing extracellular glutamate concentration, this led to resting membrane potential moving from a more negative value to a less negative value that is closer to zero. In other words, it would depolarize the cell due to knowing that glutamate causes sodium and calcium ions to move into the cell.

Methods

Maintenance of Specimens and Dissection:

One crayfish was used to perform this experiment and was provided by MSU NEU 311L instructors. The sex of the crayfish was not an important factor to consider in this experiment since anatomically both sexes have the deep flexor muscle. Before starting the dissection process, the crayfish was anesthetized fully after sitting on ice for 10 minutes. Then the crayfish tail was cut from the rest of its body and with the ventral side up, the tail was pinned to the Sylgard dish. Two pins were put into the fins and two pins were put in the most anterior portion of the tail. In addition, the swimmerets were removed to have an easier time dissecting.

Once completed, the dish was placed under the dissecting microscope and one cuticle segment was chosen to make three cuts in. The first segment was not used since it was damaged from the cut separating the tail from the body. In addition, the last segment was not used since it has an atypical organization of muscle fibers. The muscle attachment was then located to ensure it was not cut or else recordings could not be taken from this segment: the muscle attachment is the white line that intersects the major blood vessel that runs medially down the tail or the blue stripe seen in the diagram above. The first cut was made along the midline, starting at the anterior sternite and ending at the posterior sternite. The next cut was made just above the posterior sternite - the cut went medially to laterally. Lastly, a cut was made just below the white line that is the muscle attachment (if confused about the cuts, the group referenced the red dashed lines in the diagram to the right). Once the cuticle was removed, a tool with a dull end was strummed just below the muscle attachment to make sure the muscle fibers were fully exposed.



Solutions:

All test solutions were made from commercially available (Sigma Aldrich) L-Glutamic acid (reagent plus ≥ 99%) mixed in saline solution (5.4 mM concentration of potassium for the saline). A 0 mM concentration solution of glutamate was made, a 0.2925 mM concentration solution of glutamate was made, and a 0.585 mM concentration solution of glutamate was made. The 0 mM concentration was used as a control or baseline in this experiment to compare to the other higher concentrations. The 0.585 mM concentration was used because we know that this concentration will elicit a depolarized response as mentioned in the Taylor and Frazier, 1986 manuscript that is being replicated. Thus, the 0.2925 mM concentration was chosen because we wanted to see an intermediate response between a baseline concentration and a definite response concentration. To get the highest concentration (0.585 mM), 0.3875 g of glutamate was weighed out and dissolved in 450 mL of saline. For the lowest concentration (0.2925 mM), 150 mL of the 450 mL high glutamate concentration (0.585 mM) was taken and added to 150 mL of water to dilute it. So, in the end, a final volume of 300 mL was obtained for the low concentration (0.2925 mM) and the high concentration (0.585 mM) of glutamate. Solutions were prepared at room temperature (20-25℃) and stored in a refrigerator (4℃) to preserve the integrity of the solution. Lastly, each test solution was added directly to the dish via a syringe and the group tried to use solutions that were still cold and not at room temperature since this can affect a resting membrane potential as mentioned in the Wright, 2004 paper.

Resting Membrane potential measurements:

Three recordings were gathered for each concentration (0 mM, 0.2925 mM, and 0.585 mM). To start gathering recordings, an extracellular recording electrode was inserted into the electrode holder of the headstage of the micromanipulator: both the holder and the microelectrode were filled with 3 M KCl (that was dissolved in water). The group made sure to use extreme caution when handling the electrode tip - it had to remain very sharp in order to penetrate the muscle in order to get a good recording. The wire-wrapped end of the silver pellet ground was then placed in the saline and did not touch the crayfish tail whatsoever.

Once the headstage and manipulator were well-secured, the bath was then zeroed out using the DC offset knob so that the red digital read-out on the amplifier read 0 mV. After this, the Elect Test button was pushed to display the read-out for the resistance (MΩ) of the electrode - this was done when the electrode was in the solution but not in the crayfish tail in order to get an accurate reading. It was decided that in order to keep using the same electrode to get recordings, it must have a resistance value of 2 MΩ or more. If the reading was lower than 2 MΩ, the electrode was replaced with a new one. This process of zeroing the bath and testing the resistance of the electrode was done in between every recording gathered to ensure that precise and accurate data was collected.

To start, the crayfish tail soaked in 0 mM solution of glutamate (or just crayfish saline) for five minutes to allow the solution to penetrate the muscle fibers. The bath was then zeroed out using the DC offset knob and the resistance of the electrode was tested using the Elect Test button. Then the appropriate knobs on the headstage were used to navigate the electrode toward an exposed muscle fiber of the deep flexor muscle. The electrode was advanced slowly into the muscle fiber until a read-out of -50 to -90 mV was noticed signifying resting membrane potential. When resting membrane potential values were collected, the highest negative resting membrane potential value from the read-out was noted for each trial of the specific concentration being tested.

Once recording 1 was gathered for the crayfish tail soaked in 0 mM solution of glutamate, the electrode was re-adjusted to penetrate a different muscle fiber. Again, the DC offset knob was set to 0 mV and the Elect Test button was pushed to check the electrode resistance. Then recording 2 for the 0 mM concentration was gathered. This same process was repeated for recording 3 of the 0 mM solution of glutamate. When all three recordings for 0 mM solution of glutamate were gathered, this solution was then removed using a syringe and the 0.2925 mM solution of glutamate was added using a different syringe.

The crayfish tail would then soak in the 0.2925 mM glutamate solution for five minutes before taking any recordings. Again, the DC offset knob was set to 0 mV and the Elect Test button was pushed to check the electrode resistance before gathering a read-out for recording 1 of the 0.2925 mM glutamate solution. The same steps described above to obtain the three recordings for the 0 mM glutamate solution recordings were performed for the last two recordings for the 0.2925 mM solution concentration. When all three recordings for the 0.2925 mM concentration were gathered, again, the 0.2925 mM glutamate solution bath was removed and replaced with the 0.585 mM glutamate solution. The crayfish tail sat in the solution for five minutes before the same protocol for gathering recordings, as discussed for the 0 mM and 0.2925 mM glutamate solutions, were gathered.

Calculations:

Resting membrane potential (mV) values were analyzed among each of the three different concentrations of glutamate (0 mM, 0.2925 mM, and 0.585 mM). This helped to see if there was a relationship between resting membrane potential (mV) and concentrations of glutamate (0 mM, 0.2925 mM, and 0.585 mM). Both a Pearson Correlation and a Regression was run in Microsoft Excel to analyze the data set. Lastly, the alpha-level used was that of 0.05 to determine statistical significance. So if the p-value was less than 0.05, the data was deemed statistically significant.

Results

Three extracellular recordings of resting membrane potential were collected from the deep flexor muscle of a crayfish for each concentration of glutamate tested (0 mM, 0.2925 mM, and 0.585 mM). These data points collected were then analyzed against each other and were represented graphically with a line of best fit used. A Pearson Correlation and a Regression revealed that there was not a significant correlation or association between extracellular glutamate concentration (0 mM, 0.2925 mM, and 0.585 mM) and resting membrane potential ($r(9) = 0.27513$, $p>0.05$). The data can be seen in Figure 1 below.

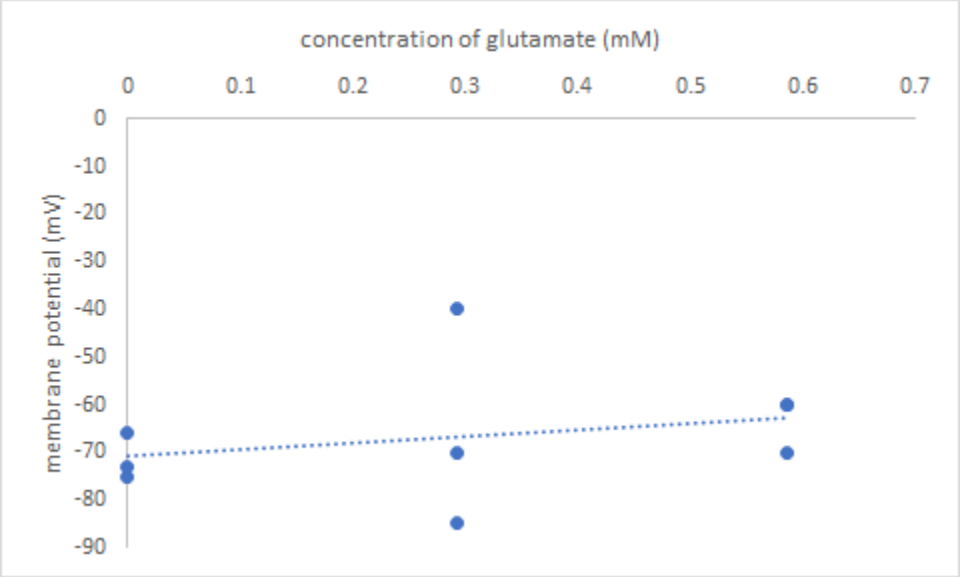


Figure 1. The effect of varying extracellular glutamate concentration on resting membrane potential of the crayfish superficial flexor muscle. A Pearson correlation test and a Regression test revealed that there was not a significant correlation or association between extracellular glutamate concentration (0 mM, 0.2925 mM, and 0.585 mM) and resting membrane potential ($r(9) = 0.27513$, $p>0.05$).

Discussion

The purpose of this experiment was to replicate the 1986 manuscript by Taylor and Frazier in hopes to help build a foundation for drug development focusing on the excitotoxicity hypothesis of Hungtinton's disease. The experiment looked to see if there was an association between various glutamate concentrations (0 mM, 0.2925 mM, and 0.585 mM) and resting membrane potential. Resting membrane potential was measured using an extracellular recording electrode that would then penetrate the cells of the deep flexor muscle found on the ventral side of a crayfish tail. Extracellular recordings consisted of the tail being soaked in a 0 mM concentration of glutamate, a 0.2925 mM concentration of glutamate, and a 0.585 mM concentration of glutamate. It was hypothesized that by increasing extracellular glutamate concentration, this would lead to the cell depolarizing or the resting membrane potential moving

from a more negative value to a less negative value that is closer to zero. This was based on the biological mechanism of glutamate causing sodium and calcium ions to rush into the cell (Boistel and Fatt,1958).

After conducting our experiment, a Pearson Correlation and a Regression revealed that there was not a significant correlation or association between extracellular glutamate concentration (0 mM, 0.2925 mM, and 0.585 mM) and resting membrane potential ($r(9) = 0.27513$, $p>0.05$). This made sense since all of our resting membrane potential recording values were very close together. Ultimately, this meant that our original hypothesis was not supported by the data collected since no correlation was found between increasing glutamate concentrations and resting membrane potential. However, our group did question the validity of these results based on a couple of factors regarding the amplifier used in the experiment. One of these factors is that crazy resistance values were gathered when the Elect Test button was pushed. For example, one electrode that was used for all the recordings gathered for the 0.2925 mM concentration originally had a resistance value of 5 MΩ, then it had a 27 MΩ value, and then a 1 MΩ value when tested in between. Besides this, another factor that made us question the validity of our results was that some of the membrane potential outputs showed a +200 mV value for a couple seconds during some of the recordings. The group knew this didn't make sense, and decided to disregard these values while collecting data, but this still made us question whether this amplifier was reliable to use. Next time, we would make sure to recalibrate the amplifier before using it.

The results found were also puzzling in terms of not matching the results gathered by other researchers. According to the Taylor and Frazier paper that we tried replicating, the use of glutamate was associated with the depolarization of the muscle membrane in their crayfish experiment (1986). Plus, the same concentration was used as Taylor and Frazier for our high concentration of glutamate; it stated in the paper that this concentration would elicit a muscle contraction since it is far above threshold and guarantees a measurable response (Taylor and

Frazier, 1986). This means we were expecting the resting membrane potential to definitely be around 0 mV. Again, this kind of result was not obtained. The group believes that the main methodological difference that contributed to this difference in results is that the crayfish soaked in the designated glutamate concentration for only five minutes compared to the crayfish soaked in glutamate for fifteen minutes in the Taylor and Frazier, 1986 paper. Five minutes appears to not have been a long enough stabilization period based on the data collected.

Other papers also had similar results as the Taylor and Frazier, 1986 manuscript. A paper way back from 1958 found that higher glutamate concentrations caused a greater influx of sodium ions into the cell, meaning the resting membrane potential was closer to 0 mV, too (Boistel and Fatt). Again, our data showed the opposite response -this was also the case with a 1988 paper (Finger, Martian, and Pareto). This paper found that even small crayfish that were 1-3 months old showed cells depolarizing when greater concentrations of glutamate were used going from 1 µmol to 5 µmol (Finger, Martian, and Pareto, 1988).

Even though the results gathered here were different compared to other papers in that no correlation was found between increasing glutamate concentrations and resting membrane potential, this still helps further knowledge in the scientific community. When the excitotoxicity hypothesis was discussed earlier, it was mentioned how this happens from excess glutamate or overly excited glutamate receptors that then would lead to more positive resting membrane potentials (Andre et al., 2010). While looking through the literature, it never gave a specific level of when glutamate excitotoxicity was reached but rather just said when there was excess amounts of glutamate in the synapse (Andre et al., 2010). Based on the data collected in this experiment, it could suggest that the excitotoxicity hypothesis of Huntington's disease is affected possibly by glutamate levels higher than 0.585 mM - further research would have to be done to figure this out. In addition, this data gathered still helps to build a foundation to create better drugs that target specific regions of the brain in neurological diseases affected by this

excitotoxicity hypothesis; it could possibly help to target areas high in glutamate to make drugs for these areas.

As a possible follow-up experiment, this experiment could be redone with one change made to the experimental design. This would be that the saturation period would be increased from five to fifteen minutes like what was done in the Taylor and Frazier manuscript. This would help to ensure that the particular glutamate concentration being tested would fully be soaked into the muscle fibers of the deep flexor muscle of the crayfish. The data collected suggest that five minutes was not enough time since all the data points were close together and not much variation was seen. Another possible follow-up experiment that could be interesting is to use 1H-MRS or 1H magnetic resonance spectroscopy to measure glutamate levels in humans with Huntington's disease and compare this to controls unaffected by Huntington's disease (Marsman et al., 2017). H1-MRS is a non-invasive technique that enables the detection, identification, and quantification of biochemical compounds or metabolites in the brain tissue like that of glutamate (Sidek et al., 2016). Coupling this with the use of an extracellular recording electrode, this could be used to test certain areas of the brain high in glutamate concentration to see if the resting membrane potential gets close to zero or not.

References

Andre VM, Cepeda C, and Levine MS (2010). Dopamine and glutamate in Huntington's Disease: A balancing act. CNS Neurosci Ther. 16:163-178.

Boistel J and Fatt P (1958) Membrane permeability change during inhibitory transmitter action in crustacean muscle. J. Physiol. 144:176-191.

Finger W, Martin C, and Pareto A (1988) Prolonged time course of glutamate-operated single channel currents in neuromuscular preparations of small crayfish and a membrane current triggered by glutamate channel gating. Neurosci Lett. 91:183-188.

Lau A and Tymianski M (2018) 6.06 - Excitotoxicity. In: Biomedical Sciences: comprehensive toxicology (Beal MF, ed), pp515-535. Toronto:Elsevier Ltd.

Marsman A, Boer VO, Luijten PR, Pol HEH, Klomp DWJ, and Mandl RCW (2017) Detection of Glutamate Alterations in the Human Brain Using 1H-MRS: Comparison of STEAM and sLASER at 7 T. Front Psychiatry 8:60.

Mattson MP (2019) Chapter 11 - Excitotoxicity. In: Handbook of Stress Series (Fink G, ed), pp125-134. Baltimore: Academic Press.

Sidek S, Ramli N, Rahmat K, Ramli NM, Abdulrahman F, and Kuo TL (2016) In vivo proton magnetic resonance spectroscopy (1H-MRS) evaluation of the metabolite concentration of optic radiation in primary open angle glaucoma. Eur Radiol. 26:4404-4412.

Taylor F and Frazier DT (1986) Effect of nicotine and acetylcholine on crustacean muscle membrane potential. J Appl Physiol 61:807-809.

Wright SH (2004) Generation of resting membrane potential. Adv Physiol Educ 28:139-142.

Zhou Y and Danbolt NC (2014) Glutamate as a neurotransmitter in the healthy brain. J Neural Transm 121:799-817.