PARKINSON-LIKE SYNDROME IN COCKROACHES STUNG BY A PARASITOID WASP

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ABSTRACT

The parasitoid emerald jewel wasp (*Ampulex compressa*) subjugates its host, the American cockroach (*Periplaneta americana*), through direct envenomation of its brain. Following venom injection, the stung animal falls into a sleep-like state referred to as hypokinesia, whereupon it is led like a dog on a leash by the wasp into its burrow. After depositing a single egg on the metathoracic leg of the host, the wasp departs and seals the burrow entrance. Past collaborative work of Adams lab at UCR and the Libersat lab at Ben Gurion University, Israel, has demonstrated that; 1. Stung cockroaches exhibit reduced descending neuronal activity from the brain to circuitry in the thoracic ganglia, 2. Stung animals allowed to recover are immune to subsequent envenomation, 3. The venom is a cocktail of over 200 proteins and small molecules that program the post-envenomation behavioral sequence of the host. Questions we are concerned with are: does hypokinesia coincide with a reduction in neuronal activity in the central complex (CX) of the brain, an area known to be a control center for locomotory behavior. In particular, does hypokinesia involve altered efficacy of dopamine signaling of the CX? Is behavioral immunity correlated with the insensitivity of CX signaling to envenomation? To address these questions, we will attempt to perform calcium imaging on slices of the cockroach brain containing the CX and determine whether venom exposure in vitro alters spontaneous and evoked neuronal activity.

ACKNOWLEDGEMENTS

I would like to acknowledge and thank my faculty mentor, Dr. Michael Adams, for his role in my growth as a researcher. Under his guidance, I discovered the little joys that came steadily from research and were given a chance to explore what it means to do great research. Hopefully, I can reach greater heights in my graduate studies with the deep insight from working in his lab and towards our project.

My mentors from many different backgrounds were indispensable in my journey to find my passion in neuroscience, and in particular computational neuroscience. For a brief time, I was able to speak to many amazing individuals and professors, and as a consequence, I was able to finally discover and understand where my love was in all of science. Thank you all for the plethora of awe-inspiring advice and insights that emboldened my worldview.

I would also like to thank the modern world for providing such a garden of knowledge and allowing me to appreciate the hidden beauty of the natural world. Community college allowed me to peer into this world with more amazement than the fear usually associated with academics. In contrast, each class I took made me feel more valuable and gave me more confidence that I was finally pursuing something worthwhile. Academics offered me a well-trodden path paved by many impressive individuals giving me the opportunity to flourish as a human being, let alone as a scientist.

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INTRODUCTION

The Emerald Jewel wasp is considered a neuro parasitoid, which propagates by its silent attacks on the common American cockroach (*Periplaneta americana*). Their attack is surprisingly insidious as most predatory wasps would simply eat their prey. However, parasitoid wasps, under the umbrella of parasites, are primarily concerned with taking advantage of their host for the benefit of their descendants. Even more cunning is the method by which these special animals undertake this parasitoid behavior. The Emerald Jewel wasp stings the head of the cockroach into the brain (**Fig. 1**), resulting in a drastic deficit in locomotory motion, specifically causing an inability to initiate movement and hypokinesia. The wasp pulls the cockroach by their antennae, walking the insect into a burrow like a dog on a leash. The wasp then lays an egg on the metathoracic leg and covers the burrow with rocks or debris. Finally, the progeny burrows into the cockroach, eating everything but the nervous system and its gut, and emerges following metamorphosis inside the host as an adult wasp. In the context of this project, this venom is the most salient topic of experimentation.

Figure 1: An image of an adult Emerald Jewel Wasp stinging a cockroach into the head to manipulate its behavior (Taken from Grant Proposal, Parkinson-like syndrome in cockroaches stung by a parasitoid wasp)

The Emerald Jewel wasp, *Ampulex compressa*, attack is focused, precise, and purposeful. The wasp stings towards the head of the american cockroach, *Periplaneta americana*, local to the subesophageal ganglion (SEG). In the first 2-5 minutes following the initial envenomation, the cockroach cannot move. Following for the next 30-40 minutes the cockroach grooms itself

excessively. Afterward, for several weeks the cockroach is in a hypokinetic state where the cockroach faces an inability to initiate movement and reduced escape responses. This hypokinetic state is the central focus of my project. Previous work suggests that the subesophageal ganglion functions to control the excitability of motor networks in the thorax (Altman and Kien 1987; Johnston et al. 1999) Despite this, the wasp's venom does not attack or affect the motoneurons, neuromuscular junction, or sensory interneurons. These regions of the nervous system are affected by neuromodulation from the SEG and, in turn, thoracic interneurons. Due to the site of injection, and how the animal exhibits drastic alterations in behavior, previous research from our collaborators used reserpine, a drug known for depleting monoamines for several weeks (Sloley and Owen 1982), to gain insight into the mechanisms that contribute to these long-term behavioral effects of the stung cockroach. Furthermore, researchers used specific antagonists to determine which of the monoamines were involved in producing these effects, such as dopamine, serotonin, and octopamine. (Weisel-Eichler et al. 2002) In particular, researchers showed that reserpine mimics the behavioral effects of the Emerald Jewel wasp's venom, a monoamine depletor. Furthermore, researchers who treated animals with monoamine antagonists found that functioning of dopamine receptors is required in normal escape-response of the cockroach and lack of dopamine incites grooming in the cockroach, further suggesting that dopamine signaling may underlie the long-term hypokinesia as seen in stung animals. Additionally, symptoms of Parkinson's disease, ailment involving loss of dopamine neurons in the Substantia Nigra in humans, such as freezing or paucity of movement can be seen in stung animals or animals treated with reserpine or dopamine receptor antagonists. (Weisel-Eichler et al. 2002)

Our primary hypothesis is that an upset in dopamine signaling caused by the venom leads

to hypokinesia. Venom-induced suppression of spontaneous locomotory activity in the stung cockroach appears similar to symptoms exhibited by Parkinson's patients. When a cockroach is stung by the parasitoid, the Emerald Jewel wasp, it experiences a variety of symptoms that include deficits to locomotion and an innate insensitivity to adverse stimuli. Collectively, these symptoms are referred to as hypokinesia. Interestingly, there appeared to be parallels between the post-envenomation behavior of the cockroach and Parkinson's disease. Parkinson's disease is primarily characterized by deficits in locomotion, such as rigidity, tremors, and slow movement. Essentially, Parkinson's patients find it difficult to move without the help of another individual. There is an important distinction between inability to move and difficulty in initiating movement, because the former may be a result of physiological deficits whereas the latter is more likely to be symptomatic of a neurological impairment or alteration. Parkinson's patients are, amazingly, able to walk for themselves as long as they are given that initial support, most likely by a third party. More importantly for the purpose of my questions, these patients exhibit a neurological deficit where neurons in the central nervous system begin to degenerate, ultimately causing a deficit in dopamine signaling in the brain, specifically a deficit in levels of dopamine. This is an important issue, because dopamine is not just a molecule that is highly mobilized in the reward circuit, but it is an important signaling molecule for locomotion in animals, including cockroaches. Undercovering the mechanisms induced by the venom that bring about these drastic changes in behavior could prove to be helpful in providing further insight into Parkinson's disease, a rampant human ailment.

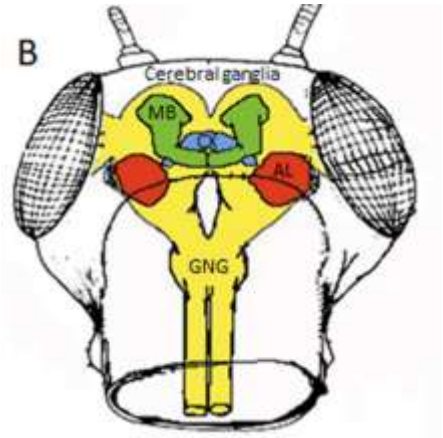

Figure 2: Drawing of a frontal view of the cockroach head ganglia.(CX: central complex, AL: antennal lobes, MB: mushroom bodies, GNG: Gnathal ganglia) (Emanuel S, Kaiser M, Pflueger HJ, Libersat F., 2020)

The central complex (CX) in the cockroach brain (cerebral ganglia) (**Fig. 2**) is considered to be the control unit for locomotion. (Emanuel et al. 2020, Ritzmann 2012) (**Fig. 3**) The relationship between dopamine, locomotion and the central complex sparked a plethora of questions that motivated the research I have been doing at UCR for the past 2-3 years. Do symptoms of hypokinesia in the cockroach coincide with reduced activity in the central complex, an area thought to be the control center for locomotory behavior? Is this reduced activity a consequence of an altered efficacy of dopamine signaling in the central complex? And finally, is the immunity of stung cockroaches to subsequent envenomation due to insensitivity of central complex signaling to the wasp venom?

Calcium is highly mobilized in activated neurons, typically functioning in facilitating the release of synaptic vesicles containing neurotransmitters, and this activation has a direct correlation with activity in the brain. Before we can begin to understand or explore the consequence of venom on dopamine signaling, it is important to see how activity in the brain appears in the first place. From our current understanding, it seems that this study into the interaction between cockroaches and wasps could potentially provide further fundamental insight into Parkinson's disease, a crippling disease that negatively impacts millions of individuals, and possibly provide a new dispensable model to explore this disease across species.

The mechanics and specific interaction between the Emerald Jewel wasp and the American cockroach occur in a deliberate manner to improve the fitness of the parasitoid by essentially attacking the brain using molecular warfare. The wasp stings its host, the cockroach, resulting in symptoms that include a diminishment of spontaneous locomotion and the roach's responsiveness to provocation (Williams 1942; Piek and Spanjer 1986). There are three unique regions where the stinging is localized: the prothoracic ganglion, the subesophageal ganglion,

and the central complex of the brain. (Kaiser & Libersat 2015) Previous work done by our research partners in Israel demonstrated that the highest concentration of venom is localized in and around the central complex, which is a sensory-motor area located in the center of the brain. (Haspel et al. 2003) To further cement the central complex's role in locomotion, researchers also showed that procaine injection into the central complex was effective enough to reduce spontaneous walking. (Kaiser & Libersat 2015) Electrophysiology work also further supported that the central complex was involved in walking since researchers were able to record from the central complex of the cockroaches while performing stationary walking, demonstrating that activity in the central complex is correlated with the stepping rates and that stimulating the same region could either initiate or regulate walking. (Bender et al. 2010)

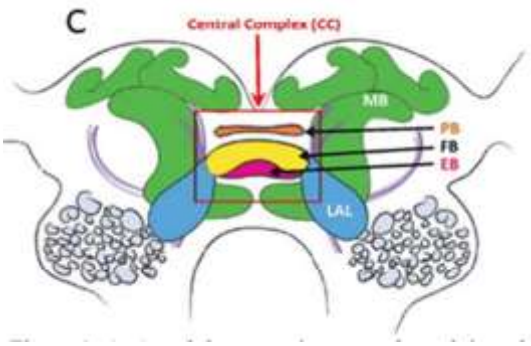

Figure 3: Diagram of the cockroach brain, central complex is inside the red rectangle. (PB: protocerebral bridge, FB: fan-shaped body, EB, ellipsoid body, LAL: lateral accessory lobes, MB: mushroom body.) Emanuel S, Kaiser M, Pflueger HJ, Libersat F., 2020

After considering previous work done by colleagues, our lab, in particular my work, has been focused on revealing the patterns of activity surrounding the central complex using calcium imaging in a live slice of the cockroach brain. Slicing the brain of the cockroach has always been a trivial challenge, as slices could easily be made with a vibratome and some histological techniques, a technique my predecessor, Harvey Sy, frequently used. His work involved injecting venom into the central complex of a live female cockroach in order to observe the physiological and behavioral effects by simulating a wasp sting. Harvey observed paraformaldehyde-fixed slices of these brains post-envenomation. The issue with this method is that fixing the brain involves killing it and, along with it, any opportunity to perfuse with solutions that could help us determine the role of

dopamine in this system or, at the very least, observe the pattern of activity in the slice. For the past 2 years, I have been working with Dr. Michael Adams to develop techniques and methods to take a live section of the brain and capture images of the central complex under different conditions, such as exposure to venom, dopamine agonists, and changes in physiological ion concentrations. As for now, we plan on subjecting slices that show the central complex and examine calcium dynamics by invoking the slice with activity. Eventually, we plan on perfusing the brain with dopamine agonists or with collected venom from the Emerald Jewel wasp to uncover additional insight into the role of dopamine in the central complex.

METHODS

Periplaneta americana collecting:

Cockroach colonies are kept and bred in garbage bins, 55-gallons, kept contained by an 18-volt electrical barrier powered by two 9-volt batteries in series. Additionally, these cockroaches are kept in a controlled environment at an ambient temperature of approximately 25°C and with a typical humidity between 50-60%. Cockroaches were fed *ad libitum* with dry dog kibble and given tap water. Female adult roaches were used and picked instead of males to keep some consistency from the female roaches we use to breed wasps.

Regarding general maintenance, especially due to a recent outbreak of phorid flies, two bins are kept with only one bin keeping an active colony. This allows more frequent cleaning without the significant upheaval of the residing colonies. Egg cartons are used to provide shelter and a surface for the cockroaches to breed and live on. Once an egg carton becomes wet, most likely due to the tap water present in each bin, they are replaced with a brand new one to avoid providing a surface for phorid flies to lay eggs on. Overall, keeping each bin dry will keep the number of unwanted pests away.

Surgical Dissection of the live brain:

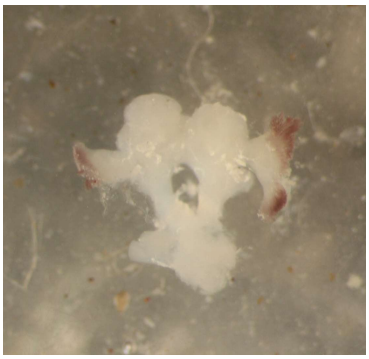

Figure 4: Isolated cockroach brain submerged in low-melting agarose. Photo taken by me.

The ultimate goal for the surgical dissection of the cockroach brain is to embed it in a block of low-melting agarose (**Fig. 4**) to provide structure for vibratome sectioning. The cockroach head is removed and placed in an agar plate for dissection and removal of the brain with the central complex intact. The head is placed on its dorsal surface, and the maxillae and mandibles are removed. Afterward, the esophagus is cut horizontally to vertically cut a small portion of the exoskeleton just underneath the ventral portion of its head. The antennae are also cut short, and two incisions across the eyes from the posterior end are made to allow the dorsal region of the exoskeleton to be removed in two pieces.

The cockroach is flipped ventral side down, and the dish is filled with physiological saline afterward to prepare for brain extraction. First, the esophagus is pulled out. Then, a horizontal cut of the posterior region connecting the two incisions made previously. One more incision is made vertically of the midsagittal dorsal across the exoskeleton (**Fig. 5**), avoiding cutting too deeply into the tissue to avoid brain damage. At this point, microscissors are used to cut into the excess tissue surrounding the brain, which is then slowly extirpated by pulling the antennal lobes once the connective tissue is removed.

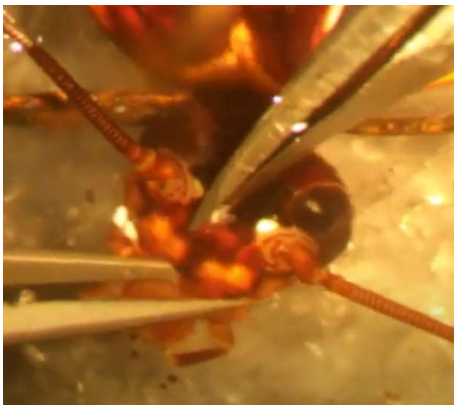

Figure 5: Dissection in progress. Cockroach head is pinned down by its neck and is lying ventral-side down. An incision is currently being made in the midsagittal dorsal region of the exoskeleton. Photo taken by me.

Sectioning of a live brain:

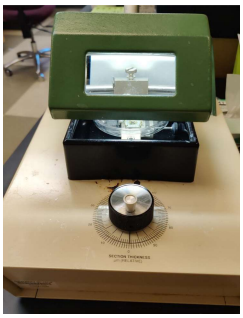

Figure 6: The Vibratome 1000 from Leica as seen in lab and used for all sectioning experiments. Photo taken by me.

After the brain is surgically removed, it is placed parallel to the surface in a mold filled with low-melting agarose (AGR-LM-50, Axygen Bioscience) at an approximate temperature of 35°C and no greater than 38°C due to risking severely damaging the brain or making the brain non-viable for the experiments. The agarose is prepared by microwaving a vial of the solution, removing the vial at the boiling point, and placing the vial in a water bath kept at a temperature of 40°C to prevent the solution from cooling too quickly. After the brain is fully embedded and arranged to be parallel to the bottom of the mold, the brain is placed in a bed of ice to cool for 15-20 minutes. After this allotted time passes, the agarose block with the brain is then removed from the mold and cut with a sharp razor blade into a cube with a length of approximately 1 cm. This 'shaving down' step is important in the realignment of the brain such that we are more likely to produce bilaterally symmetric sections of the cockroach brain. Afterward, we prepare the Leica *Vibratome 1000* by placing a Petri lid with a bolt attached to the center and using a bubble level to ensure bilateral symmetric cuts (Fig. 6). Any typical brand-new double-sided razor is used and attached to the blade holder of the vibratome. Once the apparatus is prepared, a small dot of liquid superglue is applied near the center of the blade and the shaved-down agarose block with the brain is placed such that a side of the agarose block is parallel to the blade. After waiting for

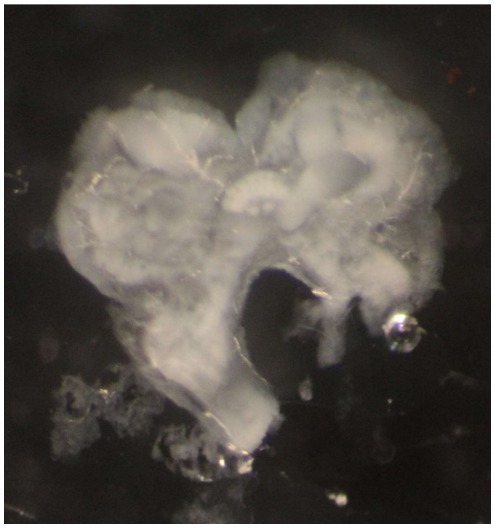

Figure 7: A coronal live section of the cockroach brain. The fan-shaped body can be clearly spotted in the center. Photo taken by me.

around 3-5 minutes to give the superglue time to dry and fully adhere to the block of agarose, the Petri lid is filled with physiological saline that barely covers the top of the agarose block. This makes it easier to move the blade in preparation for slices. Vibratome settings are adjusted to a speed of 2 and a frequency of 8. The blade is lowered until it touches the surface of the water, then the block is incrementally advanced to obtain slices of 150-micrometer thickness. Slices are collected using a small paint brush and placed in a 24-well. This process is repeated until the entire brain is sliced. The slices are observed under a microscope to validate if the central complex has been exposed, particularly the fan-shaped body portion, which is the easiest to identify. (**Figure 7.**)

Calbryte Incubation

Calbryte incubation is vital as it allows the slice to be viewed under a fluorescent microscope, a vital component in calcium imaging. Calbryte is a fluorescent and cell-permeable indicator for the measurement of intracellular calcium. (4) One of the most helpful characteristics of this dye is that it is normally non-fluorescent and non-activatable until it enters the cell, whereupon it is hydrolyzed by an intracellular esterase. Thus it becomes activate and responsive to calcium, more readily available to bind. The hydrolyzed Calbryte is polarized at this stage and unable traverse the cell membraneback into the extracellular space. Once Calbryte binds to the calcium ion, it produces a bright fluorescent signal after being excited by a specific wavelength of light at approximately 488 nm.

A stock solution of Calbryte is prepared by taking a vial containing 50 micrograms and dissolving it in 100 microliters of pluronic DMSO 20%. (Pluronic/DSMO is used to increase the aqueous solubility of Calbryte, helping it dissolve into the more aqueous regions surrounding the cells in the central nervous system.) After vortexing to completely dissolve the Calbryte, it is

stored in a freezer at <- 15 °C and to minimize light exposure. For the experiments, a vial of 300 microliters of physiological saline is prepared, to which 12 microliters of Calbryte stock is added. This solution will be used to incubate the slice.

At this point, slices should have been made, and one slice is chosen for calcium imaging, in particular, the slice that most clearly presents the central complex. The slice is removed and the chamber is filled with physiological saline used for calcium imaging to incubate the slice. The harp is used to keep the slice submerged on the surface of the glass coverslip. This is important because we will perfuse this slice, which would otherwise cause the slice to float away from the objective. The saline is replaced with the physiological saline and Calbryte solution made previously. The chamber is covered and stored at room temperature in a dark environment, minimizing light exposure as much as possible. After a 1 hour incubation in Calbryte, the chamber is rinsed a few times with physiological saline to remove calbryte. The incubation process is finished by filling the chamber with physiological saline.

Calcium Imaging

It is important to note that this step is still in its preliminary stages and is subject to change in the future. There are essential components that are most likely to be present in the later stages of the project as I learn more about the apparatus provided and the factors that contribute to optimal results. The software used to process images from the camera and onto the monitor is called Live Acquisition, which is primarily used to automate and record experiments that involve some fluorescence. The microscope used is called the Olympus BX51WI; this is where all of the calcium imaging will be done. The chamber is placed in the center of the stage, and the entire apparatus is turned on, including the Polychrome V monochrometer (Till Imaging), camera, cube control, XYZ control (for the stage), and the PC with the software.

The fluorescent bulb, placed inside the *Polychrome V monochrometer,* facilitates the emission of specific wavelengths of light that excite the Calbryte indicator, bound to calcium ions, from within the cells in the brain. This excitation will cause Calbryte to emit light at a wavelength of approximately 520 nm, which is green. The microscope contains a cube that filters out the specific wavelength, 520 nm, in this case, preventing any other light and colors from adding noise to the images. At the current stage of the experiments, we are able to excite and record emissions from the microscope, but we have not yet viewed the pattern of emission under different constraints, such as exposing the brain to the peptide tachykinin or high potassium saline. While we may be able to capture the current landscape of calcium ions in neurons, it is even more essential that we view the activity of these calcium ions as they correlate with activity as we perfuse. Especially considering that we aim to view the activity of the brain under the consequence of the venom produced by the parasitoid wasp.

One of the largest drawbacks of not using the perfusion system is the lack of consistency between each image. Previously, a pipette was used to perfuse the brain with varying solutions that aim to excite the neurons in the brain by utilizing the chemical gradient or properties of receptors. This method consistently disturbs the slice from its original resting spot and, as a result, provides inconsistent images, which is essential when comparing fluorescence on a pixel-by-pixel basis. To counteract this, a perfusion system (VC3-4xP Series; ALA Scientific), contains four syringes/reservoirs where the flow is precisely controlled using air pressure. Using this method allows for a constant flow, rather than abrupt, of different solutions delivered directly to the chamber containing the brain. This allows us to switch from normal saline to high potassium saline and then back to normal saline, a level of flexibility that was impossible until now.

RESULTS

Ultimately, there were significant strides that came in the more explorative stage of scientific experimentation. Much of my work has been towards developing a novel protocol that would allow us to peer into the live cockroach brain and visualize activity using calcium imaging techniques, a process that has not been recorded by anyone else to date. The first attempts at dissection and sectioning took a lot of trial and error. The agarose being too hot for the slice, the settings of the vibratome, and even how the slice is oriented in the agarose block are all examples of what could negatively impact the results of the experiments. Altogether, learning how to section took at most a month to perfect. I found that a speed of 2 and an amplitude of 8 was perfect for the vibratome, that a good temperature for the agarose was around 35-38 celsius, and that I could re-orientate the slice by shaving the block down for sectioning.

Once slices were consistent, there were issues that I had to confront before I began the incubation protocol. For instance, how to incubate, what to incubate, what chamber to use, how to hold the slice down, and how to lift slices to move elsewhere. I used an inoculation loop and bent it to transport slices, but this was not consistent enough and would often destroy the slices. In the end, we relied on using a glass micropipette and placing the bulb on the opposite end of where it was usually placed. Initially, for incubation, I tried using the fluorescent indicator Calgreen, which worked, but I found that Calbryte offered an even higher contrast, so this was used instead. For incubation, there were several places used to incubate, ranging from the 24-well to a microscope slide and finally straight into the chamber. To hold the slice down as I perfuse with solution, I used a few techniques. I glued pantyhose fiber to a plastic loop but found this was inconsistent and that the loop kept lifting off the slide. Ultimately, I settled for using a

metal harp (Warner Instruments) (**Fig. 8**) designed for the chamber the slice would be incubated in and would be used for calcium imaging.

After solving the issue of holding the slice to produce more stable calcium images of the cockroach slice with the central complex, there was another recent problem involving the perfusion system. Preliminary trials were identical up until after the slice had been incubated. At that stage, there were attempts made to perfuse the slice with a higher concentration of potassium in physiological saline. Typically in a neuron, potassium has a significantly higher concentration from within the cell relative to the outside of the neuron, approximately 30 times higher in concentration. Despite differing concentrations and the natural tendency for ions to diffuse into regions of lower concentrations, this asymmetrical state is kept stable by an additional, equal but opposite, force from the electrical gradient. Adding an abundance of potassium will disrupt this balance and cause the force from the concentration gradient to overcome the stalemate and result in an inward current of potassium to flow inside of the cell. This increase in voltage allows the neuron to reach a threshold, typically around -55 mV, to initiate an action potential, additionally this causes a mobilization of calcium which we can visualize using the dye and microscope. Early trials involve using a pipette to perfuse this high potassium saline solution and invoke increased levels of activity in the brain. Using a pipette proved to be difficult because of two primary reasons. First, the abrupt and inconsistent flow from manual handling disrupts the position enough to obscure any change we may see from frame to frame when recording with a camera. Secondly, because of this disruption, the brain shifts from the given region of interest, an area we can designate to measure levels of light from pixels of an image, another variable that could be significantly affected by minimal movement. To solve this, a perfusion rig from ALA Science was utilized. This apparatus involves 4 large syringes/reservoirs that can hold different

solutions to perfuse interchangeably, an ALA VC3-4 controller that controls which reservoir will be used by pinching the tubes from a remote valve, and a pressure regulator which allows for an easily manipulated constant flow. As of now, there have yet to be any trials attempted using this new perfusion apparatus.

Discussion

For this project, our goal was to examine the effect of venom on dopamine signaling in the cockroach brain, a simple question that takes a lot of effort to answer. Taking slices of the brain and keeping the cells and systems intact will allow us to closely follow the flow and pattern of activity in the central nervous system, particularly the central complex, as we impose different environments and constraints on the brain using different solutions. Previous work done by our collaborators in the Libersat lab at Ben Gurion University, Israel, has given us additional insight into the functions of the central complex and where we can find the highest concentration of venom. This information is combined with the extensive transcriptomic/proteomic study produced by the Adams Lab (Arvidson et al. 2018) revealing the composition of the venom. The process of fleshing out our methodology was not without trials and tribulations. While a majority of the lengthy process of surgery, slicing, and incubation has been pretty much solved, the problem is now being able to examine the brain under the scrutiny of calcium imaging techniques and establishing consistent perfusion of saline and other agonists that may be included in the perfusion apparatus. Previously, a pipette or syringe was used to perfuse to an exact concentration, but there were issues in keeping the slice still enough to examine changes between the first and the second frames.

Figure 8: Two harps in the shape designed for RC26G chamber. One is 1mm between each thread, and the other is 0.5mm (Photo taken from https://www.warneronline.com/slice-anchors-kits-harps)

In addition to the perfusion complication, I found that it was also necessary to consider the solution in which the slices were produced and incubated. Initially, trials involved using normal physiological saline, but this seemed to produce too much fluorescence and may contribute to the lack of increased levels of fluorescent activity when perfusing with high potassium saline, which, theoretically, should mobilize calcium that would show itself in the corresponding images. One possibility may be due to the process of vibratome sectioning resulting in injured cells which may have disrupted membranes of the neurons allowing a disproportionate amount of calcium from the solution to flow into the damaged cells, resulting in higher calcium-binding ester dyes to bind to calcium and, as a consequence, a higher level of fluorescent emissions when an activating light is emitted onto the slice. To amend this possibility, an identical physiological saline was produced without any calcium to mitigate any potential unwanted calcium binding from injured cells. Another interesting route is incubating the slice in no potassium and no calcium because of the lack of response of introducing high potassium saline during the perfusion steps. It is entirely possible that the potassium already present in the normal physiological saline may be preemptively causing neurons to activate before adding the high-potassium saline.

Afterward, an important step is not just to have the several rigs and apparatuses prepared, but to work with them all in tandem without any issues arising. Additionally, being able to quantitatively and accurately measure the changes in the levels of light in the region of interest is vital. This problem can eventually be resolved after practice and repeated trials using the Live Acquisition software and confirming the functions of each feature of this calcium imaging program. Another piece to the puzzle that we have yet to prepare is the vacuum that helps collect the saline or solutions during perfusion to exit, preventing significant spill or overflow.

Ultimately, there are many problems to face, such as beginning to utilize the calcium imaging rig. Afterwards we can visualize a symphony of activity in the slice, let alone the central complex, where exciting questions brought about at the beginning of this project can be thoroughly explored and answered.

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