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Neuroscience DMP Thesis

Imaging Neural Circuits in Absence Epilepsy Using Miniaturized Microscopes

Abstract

Epilepsy is the fourth most common neurological disorder that is caused by an abnormal and sudden rush of electrical activity in the brain. It is characterized by unpredictable seizures with different types and intensities, such as generalized seizures, which affect the entire brain, and focal seizures, which affect just one part of the brain. Such seizures arise due to complex interactions between different neural circuits throughout various regions in the brain. This research project aims to better understand the function of the thalamus in amplifying the excitatory stimulation that occurs during a seizure, which can prolong the duration of electrical activity within a region of the brain during a seizure. By using GCaMP, a green fluorescent protein that fluoresces brighter when neurons fire, a miniature fluorescence microscope (MiniScope) will be used to record neuronal activity in awake, freely moving mice. The MiniScope will be inserted above a mouse's thalamus to observe neurons firing at different times and sites during a seizure. Using a living animal that will undergo epileptic seizures will enable the visualization of neurons firing within an intact brain, as opposed to viewing neurons in a brain slice from an animal that has been sacrificed, which does not allow for as much information about the thalamus' role in seizures. By utilizing the MiniScope, this research allows for an in vivo method to record neuronal activity, leading to a better understanding of the thalamus' importance in epileptic seizures.

Introduction

Epilepsy, the fourth most common neurological disorder, is caused by an abnormal and sudden rush of electrical activity throughout the brain. In general, epilepsy is characterized by unpredictable seizures on a broad spectrum ranging from different types and controls. Currently, about forty to fifty types of epilepsy exist, each with their own unique mechanism. Epilepsy is usually comprised of two main types of seizures: general, which results in widespread seizure activity in both hemispheres of the brain, or focal/partial, which are limited to just one part of the brain which subsequently affects the part of the body controlled by that section of the brain[1]. The severity of seizures can range from mild to strong. Mild seizures often go unnoticed and the person may lose conscious awareness for a few seconds, while strong seizures can consist of uncontrollable muscle spasms that may last a few seconds to a few minutes[2].

This project specifically examines the most common pediatric epilepsy, absence epilepsy, which is characterized by generalized nonconvulsive seizures and is mostly found in children. A sudden, brief, loss of consciousness with an abrupt ending is the main feature of absence seizures. These seizures most often cause children to undergo short bouts of unawareness of their surroundings accompanied by staring blankly into space. Such seizures may occur frequently and affect attention, memory, and learning[3]. Absence epilepsy is usually diagnosed around ages four or five with juvenile onset occurring at around ages six or seven. Although absence epilepsy has historically been viewed as benign, its remission rates have recently become exaggerated due to lasting side effects from the blunt usage of pharmacological drugs. Constant exposure to antiepileptic drugs (AEDs) such as Lamotrigine and Ethosuximide can inhibit the normal development of the childhood brain. AEDs possess numerous mechanisms of action, such as the

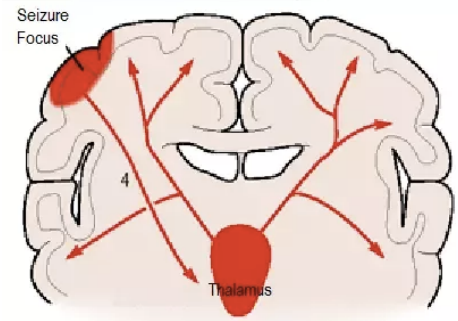
blockage of voltage-gated sodium ions and calcium ions, stimulating gamma-aminobutyric acid (GABA)–ergic neurotransmission, and also inhibiting glutamatergic neurotransmission[4]. Most AEDs block voltage-gated sodium channels, effectively delaying the recovery of such channels from their inactivated state, which consequently inhibits the firing of neurons during epileptic seizures. Such drugs have been found to influence mood and behavior, sometimes resulting in long-term affective disorders[5]. Moreover, AEDs that function through enhancing GABA-ergic neurotransmission have resulted in the development of aggressive behavior[6]. As a result, many labs, including our own, are focusing on the mechanisms through which these drugs function in order to prevent seizures and how they contribute to the development of the brain.

Several fMRI techniques such as event-related analysis and time-course analysis have revealed a common network of structures involved in the propagation of absence seizures. The network's structures include the thalamus, lateral parietal cortex, caudate nuclei, and the reticular structure of the pons[7]. Although absence epileptic seizures propagate throughout the cerebral cortex, it is believed that the thalamus is the primary component of the neuronal circuits in generating and spreading the seizures[8]. Absence seizures consist of bilaterally synchronous spike-and-wave discharges (SWDs) in an electroencephalogram, which showcases abnormal oscillating electric wave patterns within the brain[9]. GABAergic projections from the substantia nigra pars reticulata to thalamocortical neurons of the ventral medial thalamic nucleus serve as the network for the control of absence seizures[10].

Thalamic connections with the cerebral cortex and limbic system relay both sensory and motor signals that may be involved in the generation of generalized seizures. In general, a sensory impulse travels from the body's surface towards the thalamus, which receives the impulse as a sensation. The thalamus then passes this sensation onto the cerebral cortex for interpretation as touch, pain, or temperature. The role of the thalamus in producing seizures must be understood within the context of an epileptic network, because neurons rarely function in isolation, but are instead organized into circuits that process specific kinds of information. Epilepsy itself is a collection of numerous disorders of the brain, and attempts to designate one mechanism as the source of the seizures often results in failed targeted therapies. Overall, seizures arise from complex interactions between circuits throughout various regions in the brain.

The epileptic network consists of the seizure focus, which triggers a seizure, and the initiating circuit, which converts the initial spark into a seizure. The seizure focus lies in the cerebral cortex, providing the initial excitatory projections that physiologically ignite the seizure. The initiating circuit is a set of interconnected regions between the thalamic relay nuclei that support the spark from the seizure focus and convert the drive into an epileptic, spike wave pattern. Essentially, the key circuit for these discharges involves an interaction between the cortex and several nuclei of the thalamus. The thalamus supplies additional, late-arriving excitatory projections that prolong the duration of the excitatory stimulation in the target region of the brain, which ultimately results in a seizure[11] (**Figure 1**).

Figure 1: Seizure Focus and Initiating Thalamic Circuits



Many different methods have been used to observe the network patterns associated with absence seizures. One common approach in studying seizures consists of recording extracellular activity from neuronal populations in targeted brain areas during experiments where subjects perform certain tasks[12]. Such recordings fail to communicate how neuronal activity is regulated outside of a task framework and during various behavioral states. Also, these brief, sporadic recording sessions are not dependable for tracking the same group of neurons over time. Alternatively, the imaging of activity-dependent indicators in neurons from brain slicing also produces an inaccurate understanding of neuronal populations during a seizure, because the brain has been extracted and modified through multiple conditions. Both approaches stray away from the more authentic *in vivo* process that can be achieved using miniature microscope (Miniscope) technology.

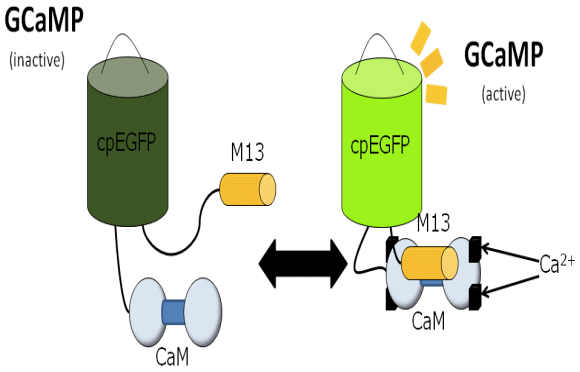
Additionally, the development of calcium indicators has allowed investigators to record *in vivo* neuronal activity using microscopy. Although early organic fluorescent calcium indicators assisted in revealing important information about calcium-dependent neuronal processes, their power was limited due to these molecules needing to be introduced physically. However, the development of genetically encoded calcium indicators allowed for neuronal activity to be monitored more efficiently along with the development of different instruments used to record such calcium imaging.

Traditional brain imaging utilizing multiphoton microscopy requires head fixation to a large tabletop microscope, limiting the behaviors that can be studied and potentially inducing changes in behavior due to restraint stress[1314]. The Miniscope resolves such barriers by integrating a standard light microscope's capabilities for *in vivo* brain imaging by visualizing neuronal populations and recording calcium-dependent activity in deep brain areas of mice freely behaving in their environment. At the crux of this project, **the Miniscope allows a neuronal population in a seizing mouse to be recorded over a long duration, allowing for a more dependable interpretation of neuronal function during seizures, and ultimately leading to a better understanding of the role of the thalamus in absence epilepsy.**

Methodology

The DBA/2J strain of seizure prone mice will be virally transfected to express the genetically encoded calcium indicator GCaMP6, allowing seizure activity to be recorded using a Miniscope system. The two main components of GCaMP are green fluorescent protein (GFP) and calmodulin, a calcium binding protein. The GFP is modified, fusing the N-terminus and C-terminus together to form a barrel-like structure. Calmodulin is linked to the GFP, such that when calcium rushes through neurons during an action potential, the calcium ions cause the calmodulin to change its shape around a myosin linker, which holds calmodulin to GFP (**Figure 2**). Therefore, during an action potential, where everything is brought together through a myosin linker, the GFP fluoresces brighter, permitting visualization for GFP brightness and neuronal activity.

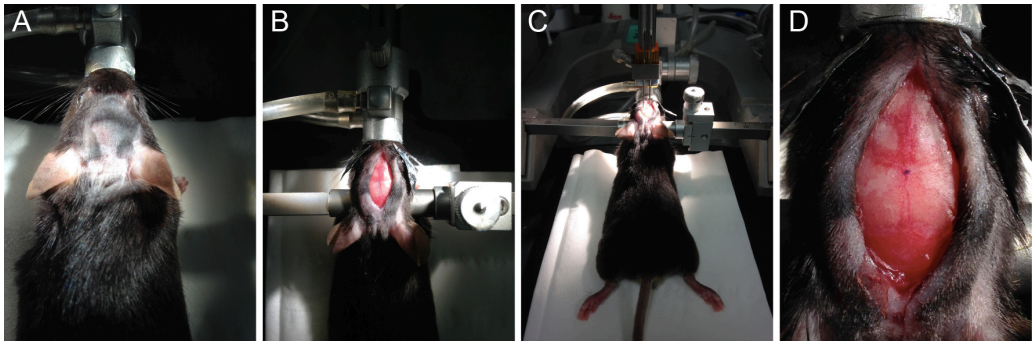
Figure 2: GCaMP Interaction



The Miniscope itself is a miniaturized, wide-field fluorescence imaging system, designed to record neuronal activity in awake, freely-moving mice. The design and procedure for implementing the Miniscope are available open source[15]. In the Miniscope, the tabletop objective lens of the standard microscope is replaced with an implanted imaging objective lens, and the excitation light source is a bright LED rather than a lamp or a laser. This allows us to record brain activity in mice without the negative constraints of other techniques.

Before placing the Miniscope on the head of the mouse, a virus injection will be performed to transfect the gene that expresses GCaMP6 at specific coordinates within the thalamus. In order to transfect the virus, a mouse is first obtained, anaesthetized with isoflurane, and fixed on a stereotax in order to perform a craniotomy (**Figure 3**). Next, the mouse's skull is exposed and drilled into at the following coordinates: X = 2.1, Y = -2.1, Z = -1.7. At these coordinates, which were localized to the hippocampus, the GCaMP6 virus is transfected into the mouse's brain, and the mouse's skull is then stitched shut using sutures.

Figure 3: Mouse preparation for craniotomy

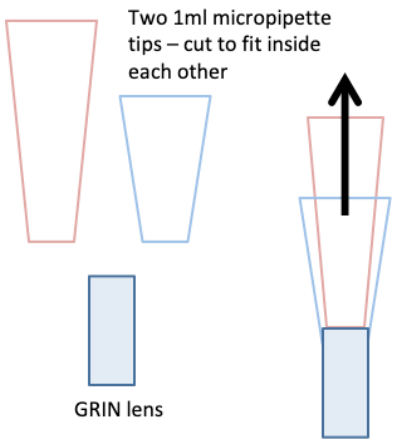


The gradient index of refraction (GRIN) lens system for the Miniscope will then be inserted into the same coordinates a week later. The steps consist of anesthetizing and shaving the mouse's scalp, applying Betadine on the mouse's skull to ensure a sterile environment, removing the skull, cleaning the skull, and scoring the skull. Moreover, a skull screw is inserted on the opposite side of the skull in order to increase the stability of the implant. Next, using a drill, four guide holes are made in a circular fashion to outline the lens placement. Once the skull fragment has been removed around the target region where the GCaMP6 had been transfected, it

is necessary to remove any excess bone or dura and to aspirate to the corpus callosum while constantly flushing the surrounding area with cortex buffer. The cortex should be continuously removed until it is possible to visualize the white striations of the corpus callosum. Afterwards, it is necessary to carefully remove the horizontal white striations of the corpus callosum until the vertical striations are in sight. The aspirator consists of a vacuum line, a liquid trap, a 1 mL syringe with a hole to control suction, blunt needles, tubing, and connectors (**Figure 4**). Next, the corpus callosum is carefully peeled back, and the lens is inserted using a suction holder that holds onto the GRIN lens (**Figure 5**). The lens must be aligned with the top of the skull at the posterior region of the skull fragment that had been removed and the lens is then inserted 1.35 mm below the top of the skull. The lens is then glued to the skull while the suction is removed, and the skull is sealed and supported with dental cement while the lens is covered with Kwik-sil to stabilize the lens in the target region. The mouse is then monitored over the next week and given analgesics consisting of 0.1 mL injections of Dexamethasone and Carprofen for pain relief and blood thinning in order to clear the window to better visualize neuronal firing through the Miniscope.

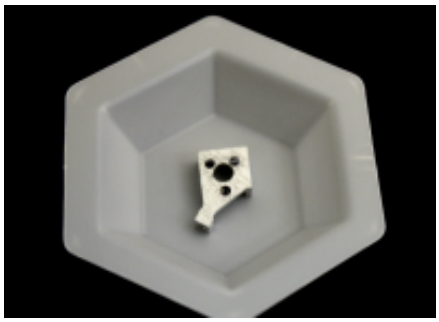
Figure 4: Aspirator Set-up

Figure 5: Lens Holder



An aluminum baseplate for the Miniscope is then placed on the mouse's head, and the Miniscope itself can be connected to this baseplate. The basic equipment needed for baseplating includes a stereotax, an isoflurane vaporizer, and a drill. Prior to fixing the baseplate on the animal, the first step consists of inserting magnets into the correct holes on the baseplate. Next, a layer of cyanoacrylate glue is applied to the bottom side of the baseplate to stabilize the magnets, and the baseplate's bottom and sides are then scored (**Figure 6**).

Figure 6: Scored Baseplate with Magnets



The animal is then anesthetized and fixed along the ear bars of the stereotax and the Kwik-Sil on top of the animal's GRIN lens is removed with blunt forceps while ensuring that the lens is not scratched. Using the stereo surgical microscope, the top of the GRIN lens is wiped down using a sheet of lens paper dampened with distilled water to remove any excess debris. After the lens has been cleaned, it is necessary to explore the Miniscope's field of view by adjusting for LED brightness, focus, gain, and exposure. After detecting the ideal view, one must practice taking the Miniscope on and off the lens and finding the same view. Once the experimenter feels comfortable with repeatedly finding the ideal view with the Miniscope, it is necessary to apply viscous dental cement in a "C" pattern around the GRIN lens while ensuring that the lens itself does not come in contact with the glue. Next, hold the Miniscope on top of the lens at its ideal view as the dental cement dries, ensuring that the ideal view through the Miniscope does not change. Apply more dental cement on the posterior region of the baseplate, ensuring that the baseplate seals to the skull.

From here, the thalamic neurons expressing GCaMP6 will be visualized using the Data Acquisition (DAQ) software that is compatible with the Miniscope. This software detects neurons present in the imaged field and can extract the dynamic changes in the GCaMP6 signal during the course of the recording. The software additionally gives the user full control of camera properties in addition to control of the power of the excitation LED. Overall, the Miniscope is used for imaging the activity of hundreds of neurons in deep regions of the brain via a miniature lens, and this allows us to simultaneously capture the activity of hundreds of neurons in the thalamus and correlate this to the seizure activity of a mouse.

Figure 7: GRIN Lens System

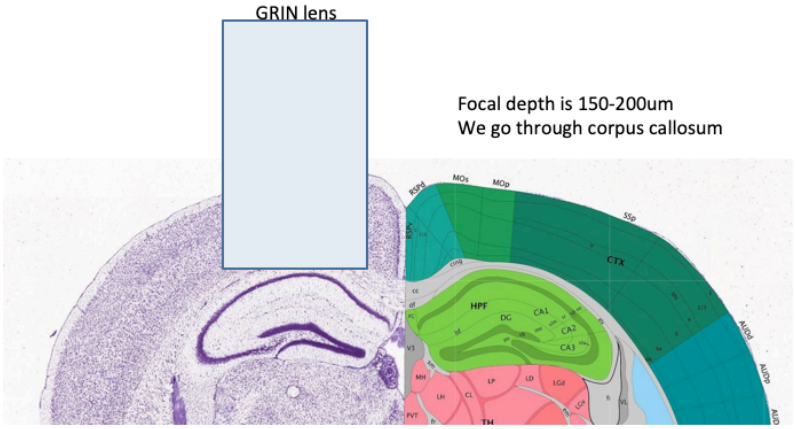
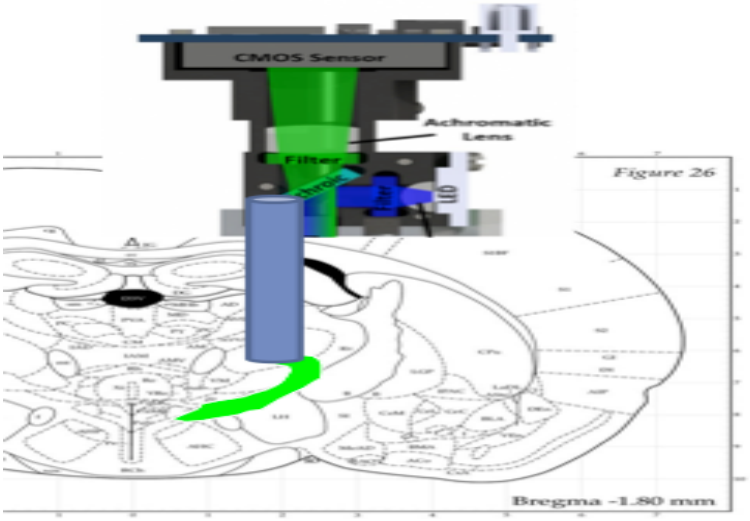


Figure 8: Miniscope Set-up



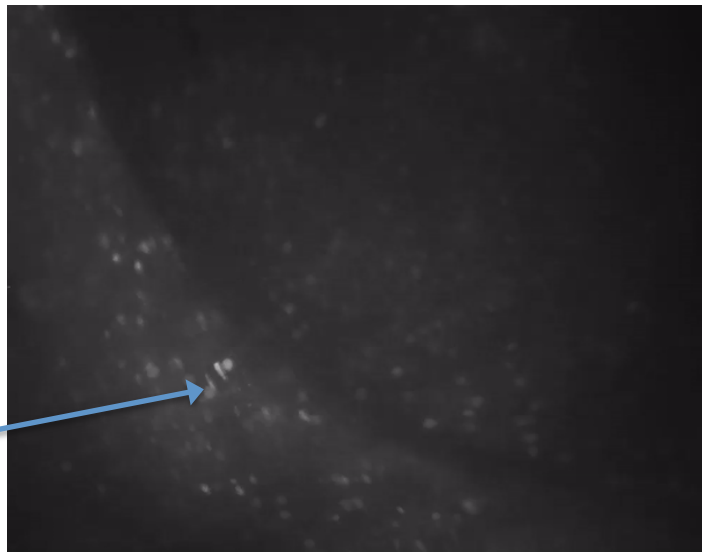
Additionally, several mice were perfused to visualize the tract along which the GRIN lens had been implanted and to visualize GCaMP signaling by staining for GFP through using an anti-GFP antibody. Mice were perfused using a 0.2M Phosphate Buffer solution of pH 7.40 and 4% Paraformaldehyde of a pH of 7.40. The perfused brain's cerebellum was sliced off and the remaining portion of the brain was covered in agar in order to more easily manage the sectioning of the brain. From here, ~100 um sections were initially taken using a vibratome. The thickness of the sectioning decreased as the vibratome's blade came in closer contact with the site of transfection. From here, multiple sections were taken in order to stain for GFP.

After slicing, the brain tissue sections were stained for GFP using a primary antibody (chicken anti GFP), secondary antibody (donkey anti chicken 488), TBS (3% 5M sodium chloride, 10% 1M tris-buffer pH 7.4, Q.S. deionized water) and blocking buffer (10% horse serum, 0.1% triton-X, Q.S. TBS). The brain tissue was washed 3x5 minutes in TBS, then incubated in blocking buffer for one hour, and then incubated in primary antibody prepared in blocking buffer for one hour at room temperature until incubations continued overnight at 4° Celsius. The following day, the brain tissue was washed 3x5 minutes in TBS, incubated in the secondary antibody prepared in TBS for one hour at room temperature, and then washed 3x5 minutes in TBS and placed on slides for viewing on a microscope.

Results

In collaboration with Peter Klein of the Mark Beenhakker lab, we were able to visualize GCaMP signaling in acute brain slices localized to the reticular thalamic nucleus. Initially, we were interested in determining whether GCaMP signaling was successful, so we undertook a reductionist approach by staining the brain with GCaMP, slicing the brain, and then placing the brain sections on a microscope where we could visualize calcium imaging during neuronal firing (**Figure 9**).

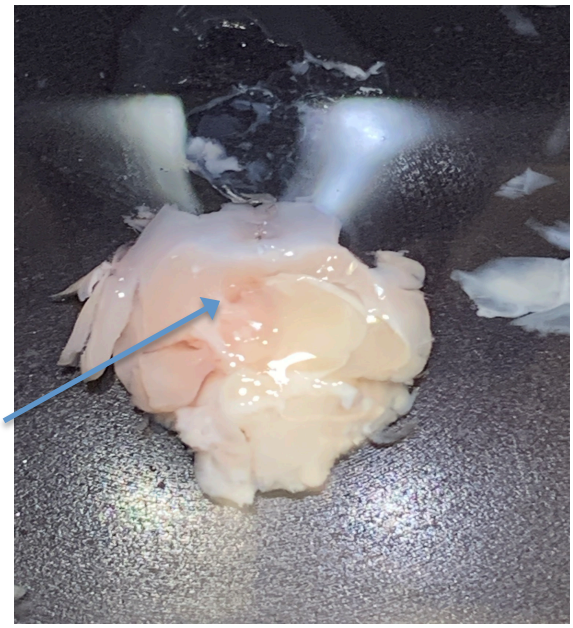
Figure 9: Neuronal Activity Localized to the Reticular Thalamic Nucleus in a Brain Slice



After *in vitro* methods, Peter Klein and I attempted to execute *in vivo* imaging utilizing the Miniscope system. After implanting the GRIN lens, the baseplate for the Miniscope was secured along with the Miniscope connected to the DAQ software. However, neuronal activity was difficult to visualize due to challenges in running the DAQ software and errors stemming from an inaccurate transfection of the GCaMP virus or inaccurate placement of the GRIN lens.

Consequently, several mice containing the GCaMP virus and GRIN lens were perfused in order to visualize the tract along which the GRIN lens had been placed. Additionally, the mice were perfused to stain for GFP by using the anti-GFP antibody. However, GFP staining was also difficult to visualize in the brain tissue sections, most likely due to the brain tissue being damaged along the tract where the GRIN lens had been implanted (**Figure 10**).

Figure 10: GRIN Lens Tract Above the Thalamus within a Coronally Sectioned Brain



Discussion

In light of the results, certain modifications are necessary to properly accomplish *in vivo* imaging using the Miniscope system. Firstly, a more robust strain of mice or rats should be used to better counteract the issue of the GRIN lens damaging the target brain tissue upon implantation. Additionally, staining for GFP should be executed prior to GRIN lens implantation to ensure that GCaMP has been amplified in the target region of the brain so that the GRIN lens is placed in the matching coordinates upon implantation. Furthermore, the neck muscle should be detached from the skull during GRIN lens implantation in order to reduce the pull on the skull and prevent muscle growth that can make the implant less stable. During lens implantation, one must also ensure that bleeding has stopped after removal of the skull, otherwise blood at the bottom of the GRIN lens can increase scar tissue formation, which subsequently interferes with imaging[16]. Most importantly, the GRIN lens implantation is a relatively invasive surgery. Future directions should include devising methods that ensure a safer and less invasive surgery that accounts for the animal's health post-surgery and causes less internal damage to the brain tissue.

Although neuronal firing was not clearly visualized, the goals of this research included transfecting the GCaMP virus into the mouse's brain, implanting a lens above the coordinates where the virus was transfected, and placing a Miniscope on the head of a mouse. I additionally aimed to see the GCaMP protein expressed in thalamic neurons as they undergo neuronal firing. Overall, the main goal of this project was to use the Miniscope to look into the thalamus of a living animal.

This research allows for the visualization of neurons firing at different times and sites in a living, freely moving mouse with an intact brain. This permits the visualization of neurons during a seizure that occurs in real-time, in a much more natural context rather than in brain slice experiments. Moreover, undergoing multiple intermittent recordings of single neuron populations tends to be a burdensome task that consists of perpetually transporting animals to and from recording chambers, which can induce unnecessary stress upon the animal. The Miniscope resolves such a laborious process by permitting repeated recordings of neuronal activity over a time period that can last up to months. As a result, more accurate inferences about neural circuits and neuronal activity patterns according to certain conditions can be made.

Additionally, the brain consists of numerous different neuron subtypes that can be targeted using promoter-driven viral constructs. GCaMP could be expressed in thalamic cells, while the red-shifted calcium indicator RCaMP could be expressed in a different cell type, and the Miniscope can enable the visualization of interactions between cell types during a seizure. Such a task is difficult to achieve with only electrophysiological tools and EEG recordings.

Moreover, Miniscopes allow for large-scale neuronal activity to be recorded from genetically defined populations of neurons. Through establishing the target region of neurons and registering activity at this target location across days or even weeks, Miniscopes permit more certainty in the longitudinal study of neurons than an electrophysiological recording could[17].

Besides Miniscopes, other calcium imaging methods that exist for *in vivo* recording include two-photon microscopy and fiber photometry. Two-photon microscopes have the potential of imaging several hundreds of micrometers into brain tissues, permitting 3D optical imaging of brain tissues through using a GRIN lens. However, two-photon microscopy involves fixing the animal's head, which limits the range of behavioral tests. On the other hand, fiber photometry can allow for freely moving behavior, but only records signals from a certain population of neurons around its fiber tip with little resolution, resulting in less clear behavioral

information[18]. Miniscope technology accounts for the pitfalls of these two *in vivo* recording methods by allowing animals to freely move while also capturing relatively high-resolution recordings of neuronal populations transfected with a genetically encoded calcium indicator.

In general, the field of systems neuroscience is rapidly developing with respect to the imaging of cellular events, which will usher in a deeper understanding of certain diseases and complex behaviors. The Miniscope represents a critical step forward in the way that neuroscientists will image neuronal structures in freely moving animals. In the context of this experiment, using a Miniscope allows for an *in vivo* imaging process that will help to better understand the role of the thalamus in seizures, which can possibly lead to better avenues for therapy and information regarding the thalamus' importance in epilepsy. Overall, the findings collected from this project would advance the understanding of the neural mechanisms that underlie epilepsy, and how utilizing a Miniscope permits for a more reliable characterization of the behavior of neurons over a long period of time.

Acknowledgements

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References

[1] Epilepsy Ontario. (2018). Types of Seizures. Retrieved from http://epilepsyontario.org/about-epilepsy/types-of-seizures/

[2] Mayo Foundation for Medical Education and Research (MFMER). (2018, June 13). Seizures. Retrieved from https://www.mayoclinic.org/diseases-conditions/seizure/symptoms-causes/syc-20365711

[3] Carney, P. W., & Jackson, G. D. (2014). Insights into the Mechanisms of Absence Seizure Generation Provided by EEG with Functional MRI. *Frontiers in Neurology,5*. doi:10.3389/fneur.2014.00162

[4] Thigpen, J., Miller, S. E., & Pond, B. B. (2013). Behavioral Side Effects of Antiepileptic Drugs. *US Pharmacist,38*(11), 15-20.

[5] Thigpen, *US Pharmacist,* 15-20.

[6] Thigpen, *US Pharmacist,* 15-20.

[7] Carney, *Frontiers in Neurology*

[8] Mayo Foundation for Medical Education and Research (MFMER). (2018, May 08). Absence seizure. Retrieved from https://www.mayoclinic.org/diseases-conditions/petit-mal-seizure/symptoms-causes/syc-20359683

[9] Paz, J. T., Chavez, M., Saillet, S., Deniau, J., & Charpier, S. (2007). Activity of Ventral Medial Thalamic Neurons during Absence Seizures and Modulation of Cortical Paroxysms by the Nigrothalamic Pathway. *Journal of Neuroscience,27*(4), 929-941. doi:10.1523/jneurosci.4677-06.2007

[10] Paz, *Journal of Neuroscience,* 929-941

[11] Bertram, E. H. (2013). Neuronal circuits in epilepsy: Do they matter? *Experimental Neurology,244*, 67-74. doi:10.1016/j.expneurol.2012.01.028

[12] Dhawale, A. K., Poddar, R., Kopelowitz, E., Norman, V., Wolff, S., & Olveczky, B. (2015). Automated long-term recording and analysis of neural activity in behaving animals. *ELIFE.* doi:10.1101/033266

[13] Silva, A. J. (2017). Miniaturized two-photon microscope: seeing clearer and deeper into the brain. *Light: Science & Applications, 6*(8). doi:10.1038/lsa.2017/104

[14] Ghosh, K. K., Burns, L. D., Cocker, E. D., Nimmerjahn, A., Zic, Y., Gamal, A. E., & Schnitzer, M.J. (2011). Miniaturized integration of a fluorescence microscope. *Nature Methods, 8*(10), 871-878. doi:10.1038/nmeth.1694

[15] UCLA Miniscope. (2017, November 6). Retrieved November 26, 2017, from http://miniscope.org/index.php?title=Main_Page

[16] Zhang, L., Liang, B., Barbera, G., Hawes, S., Zhang, Y., Stump, K., . . . Lin, D. (2018). Miniscope GRIN Lens System for Calcium Imaging of Neuronal Activity from Deep Brain Structures in Behaving Animals. *Current Protocols in Neuroscience,86*(1). doi:10.1002/cpns.56

[17] Zhang, *Current Protocols in Neuroscience*

[18] Zhang, *Current Protocols in Neuroscience*