THE ROLE OF THE VOLUME REGULATED ANION CHANNEL (VRAC) IN ASTROCYTE VOLUME REGULATION AND NEURONAL EXCITABILITY

By

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Abstract

Currently, in the field of Neuroscience, the mechanisms behind neuron excitability are a sought-out topic. Further understanding neuron excitability can lead to advancements in areas such as learning, consciousness, and neuron regeneration. Our lab seeks to determine the role a specific membrane protein (VRAC) may have on astrocyte volume regulation and thus neuron excitability in conditions of induced swelling via high extracellular potassium (Jentsch et al., 2016 and Yang et al., 2019).

Acknowledgments

The novel coronavirus pandemic that began in 2019 drastically altered the course of my senior thesis. While the topic and the goal have remained the same, the approach was modified. Since undergraduates were restricted from entering the lab, Dr. Todd Fiacco and I decided to investigate the new literature regarding VRAC and incorporate it with the preliminary data that a fellow graduate student was kind enough to share with me.

That being said, I would like to kindly thank Dr. Fiacco for his cooperation and guidance as we progressed with this project. I would also like to thank Erin Walch, who is currently a PhD candidate in the Fiacco lab, for her generosity in sharing the aforementioned data. Lastly, I would like to thank Dr. Stephanie Dingwall from the Biochemistry Department for guiding me through the Capstone Project and helping me meet the standards of the Honors Department.

Background

Neurons, since their discovery, have predominately been the main area of focus of neuroscientists as they are the pathway behind the transfer of information, called synaptic transmission. However, astrocytes, a type of glial or support cell in the brain, have been receiving increasing attention due to their ability to modulate and control neuronal excitability. Astrocytes are electrically silent cells, meaning they do not fire action potentials by themselves. However, they impact the surrounding neurons and their likelihood to propagate signals via the uptake and spatial buffering of potassium ions, clearance of glutamate (the primary excitatory transmitter in the brain), and regulation of the volume of the extracellular space (ECS). Without these critical supportive roles, the nervous system would be hyperexcitable and not be able to function properly (Andrew et al., 2007). Irregular osmotic conditions disrupt the normal flow of ions leading to alterations of the ECS and changes in concentration of excitable neurotransmitters (MacAulay et al., 2004). In addition, astrocytes have been identified to have volume regulated anion channels (VRAC) on their membranes that may or may not have an influential role in the excitability of the surrounding neurons (Pedersen et al., 2015).

We hypothesize that these VRAC channels in astrocyte membranes are causing increased neuronal and brain tissue excitability under the conditions of tissue swelling. In high extracellular potassium conditions, we see that these astrocytes take up the extra potassium ions, causing water to osmotically flow into the cell (Risher et al., 2009 and Murphy et al., 2017a). In this induced swelling, we believe that the cell releases glutamate, the primary excitatory neurotransmitter in the brain, through these VRAC into the ECS to have water osmotically follow out and restore the cell volume. Because of this increased glutamate concentration in the ECS in combination with the reduced ECS size, the neurons bind this glutamate, causing accidental neuron excitability (Cavelier

et al., 2005). That being said, for our objective, we aim to identify the mechanism behind astrocytic swelling *in situ* and *in vivo* using a new approach to develop initial data for this topic in the field of neuroscience.

Significance

Previous studies have shown that a reduction of the cerebrospinal fluid (CSF) osmolarity produced in the brain prompts tissue swelling and elevates neuron excitability (Traynelis and Dingledine 1989). This mechanism can trigger epilepsy, seizures, and even strokes. All of which are medical emergencies. The significance of this research lies in the concept that understanding the VRAC mechanism of glutamate release from astrocytes can lead to new treatment opportunities for these conditions. Future work examining the conditions of astrocyte swelling will assist in the treatment of a variety of neurological disorders.

Design and Limitations

I will discuss the experimental procedure that I was planning on performing before the novel coronavirus pandemic. The obtained data that will be incorporated with the literature review is from Erin Walch's experiments in the lab.

It is still unknown the contributions of astrocyte swelling and the causal release of glutamate in the mechanism behind brain tissue excitability. We will use mice with astrocyte specific VRAC cKO (knockout) to test this. As a result, we aim to determine the effects on the brain tissue post-removal of these channels, and also to attempt to understand the gradual development of epilepsy in the brain tissue (epileptogenesis). Currently, the presumption is that VRAC cKO will lead to a decrease in *in vivo* excitability along with the inhibition of

epileptogenesis. We will use traditional approaches that involve *in vivo* hippocampal stimulation, intrahippocampal kainic acid administration, and electrophysiological recordings. Since we know that there is a strong correlation between cell volume fluctuation and neuron excitability, we can use the removal of the VRAC to predetermine some outcomes (Qiu et al., 2014). If we see that the astrocytes swell less, then we would argue that this phenomenon is neuroprotective. However, if we knock out the VRAC that normally regulates cell volume, then we would anticipate a greater amount of swelling, and thus more neuron excitability.

The main focus of my project will be to determine how the removal of VRAC from astrocytes affects volume change in hypoosmolar (reduced osmolarity) conditions. Using confocal microscopy, I will image astrocyte volume changes, in real-time, of both the control wild type mice and the experimental VRAC cKO mice (Murphy et al., 2017a).

In the first part of my experiment, I will start by taking images of the astrocytes of the control wild type mice in hypoosmolar and isosmotic conditions. Astrocytes will be fluorescently labeled using sulforhodamine-101 dye and imaged on a confocal microscope. From there, I will approximate the volume of these cells based on the confocal microscopic images taken to estimate the swelling induced in the astrocytes respective osmotic condition. These are established procedures in the Fiacco lab (Murphy et al., 2017b). After the control measurements have been made, I will move on to the experimental measures using the VRAC cKO mice. The first step would include the physical knockout of the volume regulated anion channels using transgenic approaches. After this has been done, the same tests will be done on the new mice. I will take the astrocytes of the VRAC cKO mice and expose them to baths of hypoosmolar and isosmotic solutions (similar to that of the control wild type mice). Again, in real-time, I will record images of these astrocytes to capture the swelling induced by these various osmotic conditions and see if

it varies from the control. Once we have the data, we will plot it on a time vs. relative volume (%) graph to visualize the trend(s) compared to the initial measured volume. If we see a variation from the control in each respective osmotic condition, then we can further test the claim that the VRAC affects astrocyte swelling and thus neuronal excitability.

There are two major barriers preventing progress to understand the role of VRAC and how it affects neuron excitability. One is that the current models of cellular edema (swelling) are applied to all of the tissue, thus causing a change in a variety of cell types and not just astrocytes (Murphy et al., 2017b). The other is that cell volume is very difficult to regulate as a certain cell needs different volumes and is constantly changing for various functions such as mitigation, apoptosis, and signaling (Pedersen et al., 2015).

Data

In this section, I will dive into two published research papers and our lab's preliminary data that discuss various components of the VRAC. The former will discuss the effects of VRAC cKO during ischemic conditions and also the age-related changes of VRAC, while the latter will demonstrate how our findings stand compare to the literature.

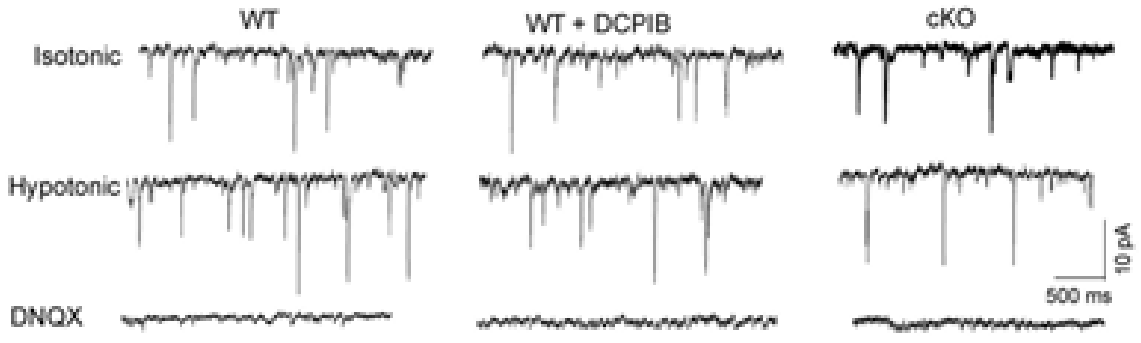
"LRRC8A-Dependent Volume-Regulated Anion Channels Contribute to Ischemia-Induced Brain Injury and Glutamatergic Input to Hippocampal Neurons" (Zhou, et al., 2020)

The VRAC throughout the body is a channel that is composed of many subunits; however, one is of particular interest in this experiment. The leucine rich repeat containing protein 8A (LRRC8A) has been postulated to be the pore forming subunit of this channel (Jentsch et al., 2016). Therefore, we can knock out (KO) the function of this channel by removing this subunit's coding

segment. We are interested in doing this to see if this LRRC8A subunit has a role in ischemia-induced glutamate release and brain injury. From the Fiacco lab, we know that astrocyte swelling can result in increased neuronal excitability most likely secondary to the reduced extracellular volume and the glutamate efflux by the VRAC. However, we want to investigate if ischemic brain injury can cause neuronal VRAC to have a related role. The Zhou group chose to use hippocampal tissue in their mice as this tissue is peri-infarcted (ischemic penumbra) with the potential to be salvageable and a target for neuroprotective interventions.

There are several main findings from this paper. The first finding is that their KO mice die around 7-8 weeks of age in both males and females. This helps to visualize the crucial role of VRAC in various biological processes, not just neurological, such as cell migration. The second main finding was that oxygen deprivation significantly increased the amplitude of the current in VRAC in the hippocampal CA1 neurons in the wild type, but not in the LRRC8A KO mice. Hypotonic solution did not have an effect on the current in the KO mice (Figure 1). Current flow helps to indicate the activity level of the VRAC. It is established that hypotonic solution causes the cell to swell, leading to VRAC opening and releasing anions, and this release is determinable via current amplitude. Since the pore-forming subunit of VRAC has been removed (LRRC8A), the channel has been rendered inactive and thus no current flows.

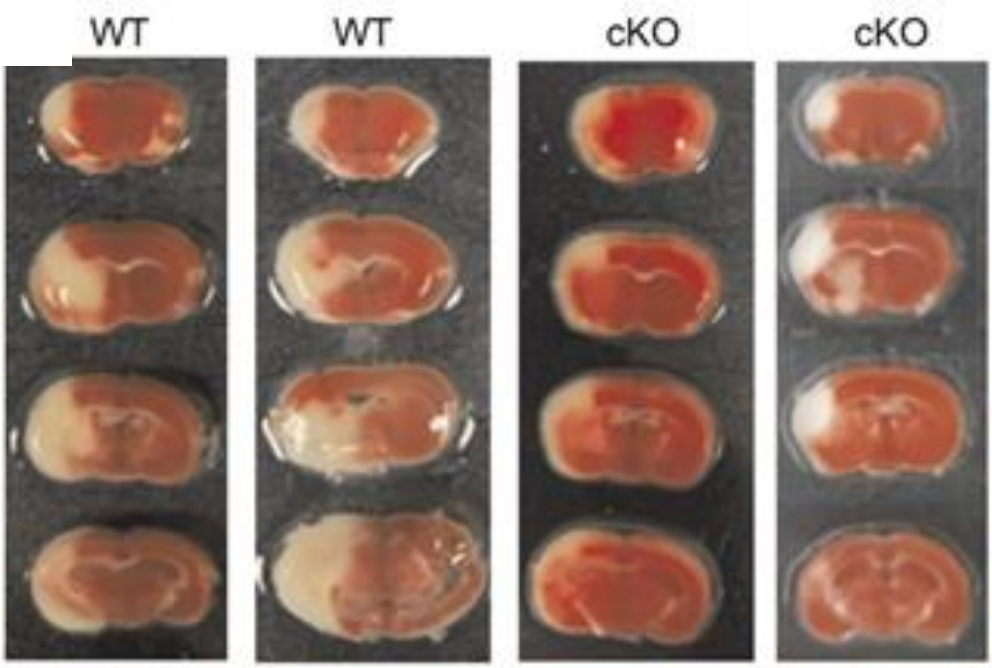
Figure 1



Further evaluation of the data shows that there is no difference between the wild-type and KO in isotonic conditions even with DCPIB acting as a VRAC channel blocker in the WT. The application of DNQX (AMPA receptor blocker) eliminates the glutamatergic sEPSCs altogether (Figure 1).

Next, we see that the brain infarct volume (amount of cell death because of ischemia) was significantly lower in the KO mice than in the WT. In other words, LRRC8A dependent VRAC can contribute and even worsen, when compared to the knockout mice, the volume of ischemic brain injury and the other associated deficits.

Figure 2



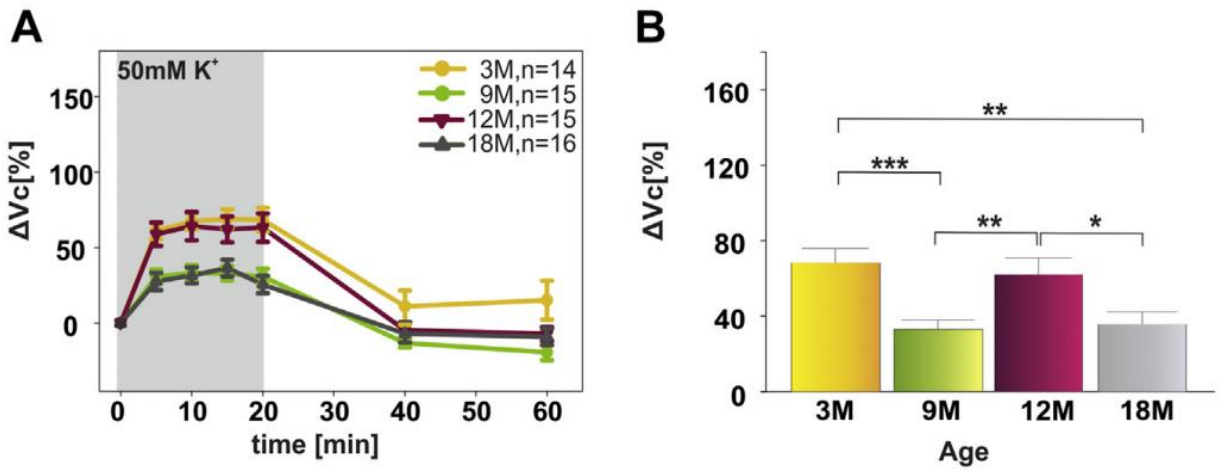
On the left, we see 8 different slices of the WT condition, and on the right are eight different slices of the KO condition. These slices were stained with 2,3,5-triphenyltetrazolium, a stain that allows us to visualize the infarct volume in each condition (Figure 2).

"High Potassium Exposure Reveals the Altered Ability of Astrocytes to Regulate their Volume in the Aged Hippocampus of GFAP/EGFP Mice" (Kolenicova, et al., 2020)

It is important to be cognizant that cells do age within the body, and their function may deteriorate over time. Within the central nervous system, various cells undergo multiple changes that will lead to their eventual demise. One of these cells is the astrocyte. Therefore, it is important to see what effect aging may have on their ability to regulate their volume in response to a pathological stimulus. The loss of normal function may be the beginning and/or progression of various neurological diseases. This paper investigates this potential deterioration by identifying the expression and function of transport proteins on the aging astrocytic membrane, along with how this may be related to the astrocyte's ability to regulate the extracellular space when triggered with high potassium.

Interestingly, the Kolenicova group report that swelling in the younger hippocampal astrocytes (three-months and twelve-months) was greater than that of the older astrocytes (nine-months and eighteen-months) in the high potassium conditions (Figure 3). There is no clear explanation of the awkward age-related position of the nine-month or twelve-month condition that was reported. Overall, the proposed mechanism is thought to be a result of homeostatic functions such as an increase in potassium/glutamate uptake or a poor clearance.

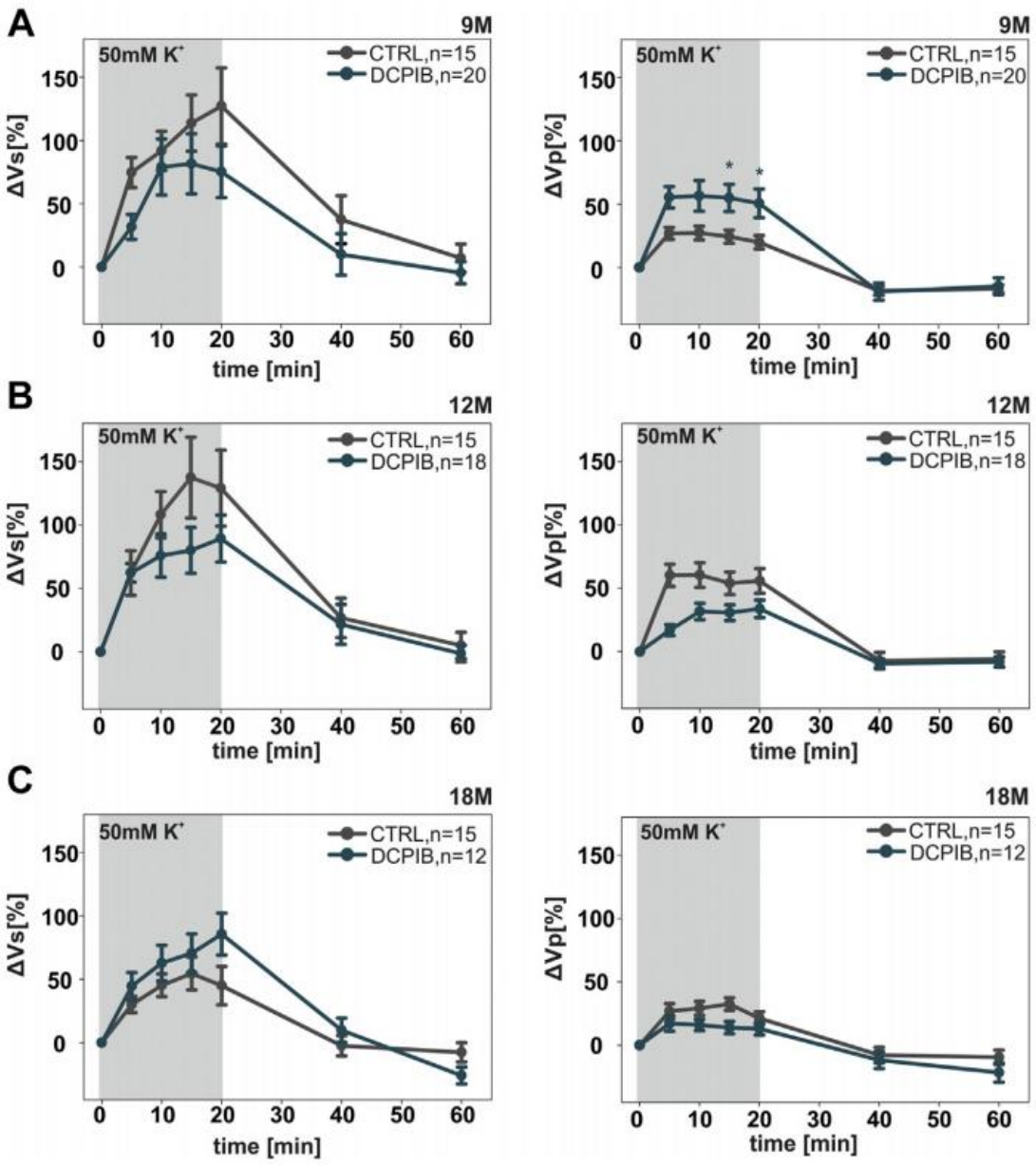
Figure 3



In Figure 3a we see the total astrocyte volume as a function of time, with the percent change statistical significance demonstrated in Figure 3b. It is important to note that astrocyte processes and their soma (cell body) have different amounts of swelling depending on the age; however, this swelling was not statistically significant, and the aforementioned trend still holds. Additionally, volume decrease during the washout phase (white area of Figure 3a) was evident in all astrocytes with no age-dependent differences.

That being said, VRAC activity appears to be the highest in astrocytes around nine-months and then strongly reduces afterward. Again, this seems to be resultant of a maturation process that occurs in these hippocampal slices (Figure 4).

Figure 4



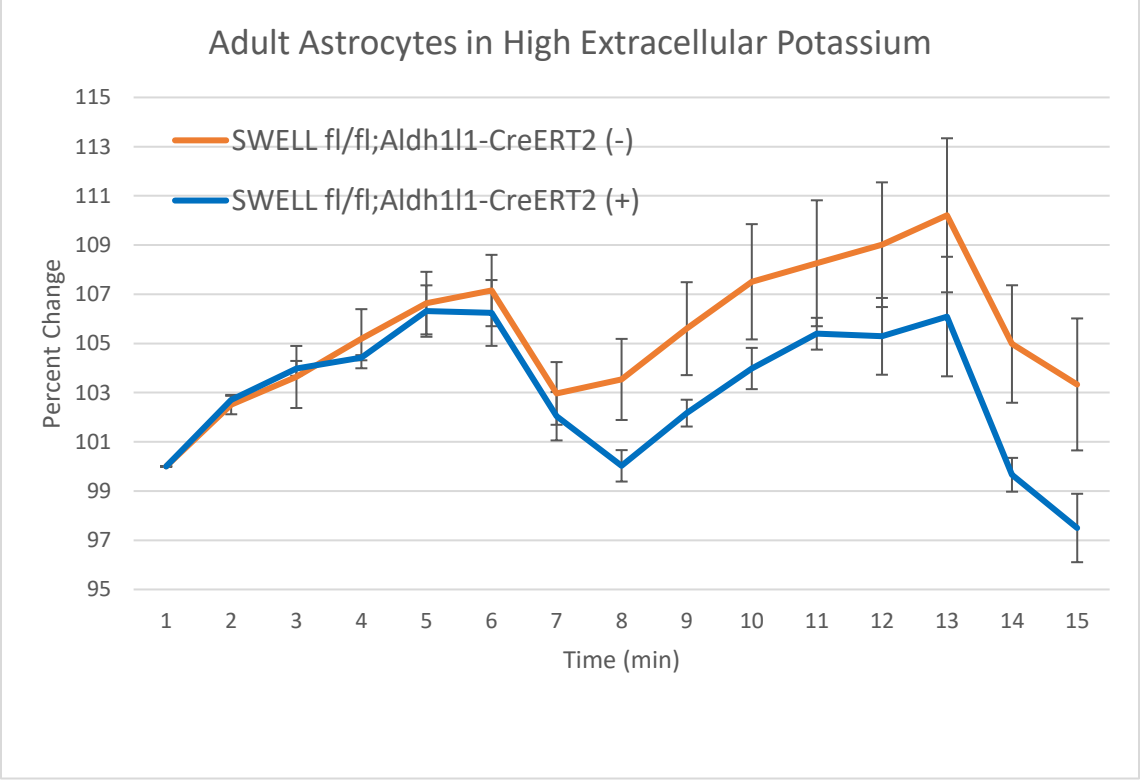
The graphs on the left represent the volume changes that happen in the soma, while the right represents the volume changes in the processes, each broken down by age. DCPIB was used as an inhibitor to evaluate the relative function level of VRAC at each stage. Afterward, it is apparent that inhibiting VRAC with DCPIB had no effect on astrocyte soma volume at any age, the processes at twelve-months, or the processes at eighteen-months. There was only a significant change in the processes of the nine-month mice (Figure 4).

Erin Walch's VRAC cKO Data from the Fiacco Lab Using the Established Procedures

In this experiment, Erin Walch, a PhD candidate in the Fiacco Lab compared the effects of VRAC cKO on astrocyte total volume in two conditions: high extracellular potassium and hypoosmolar conditions. Both of these conditions are known to increase astrocyte volume. This comparison will be done using SWELL fl/fl; Ald-Cre + (VRAC cKO specifically for astrocytes only using the Cre-Lox promoter targeting system) vs SWELL fl/fl; Ald-Cre – (control).

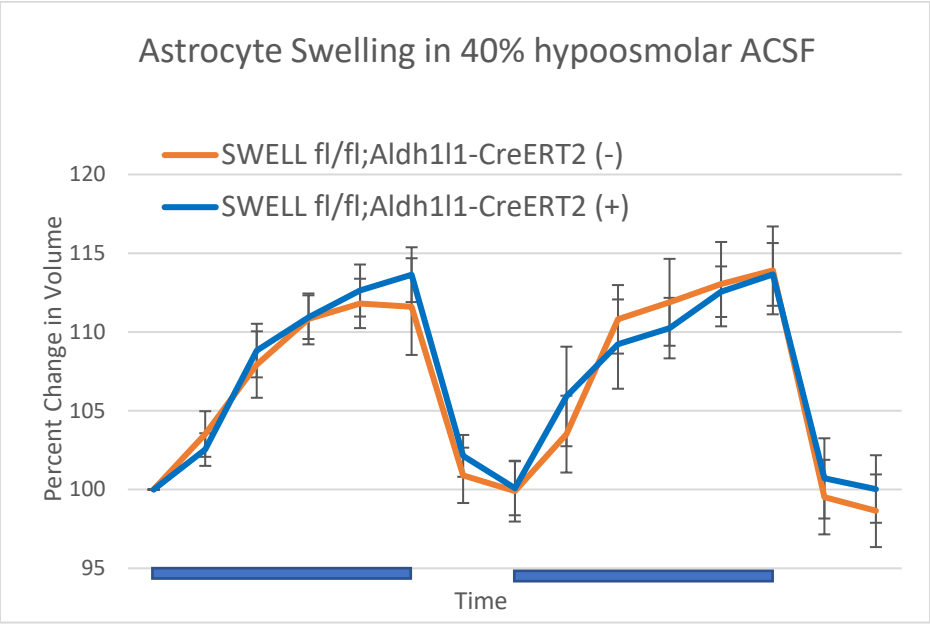
In this first condition, under high extracellular potassium (10.5 mM K+), it was found that VRAC cKO has a small effect on the swelling of astrocytes. Moreover, it appears that this effect is at its greatest in magnitude on the second application of the high potassium aCSF after the first application has been washed out (Figure 5).

Figure 5



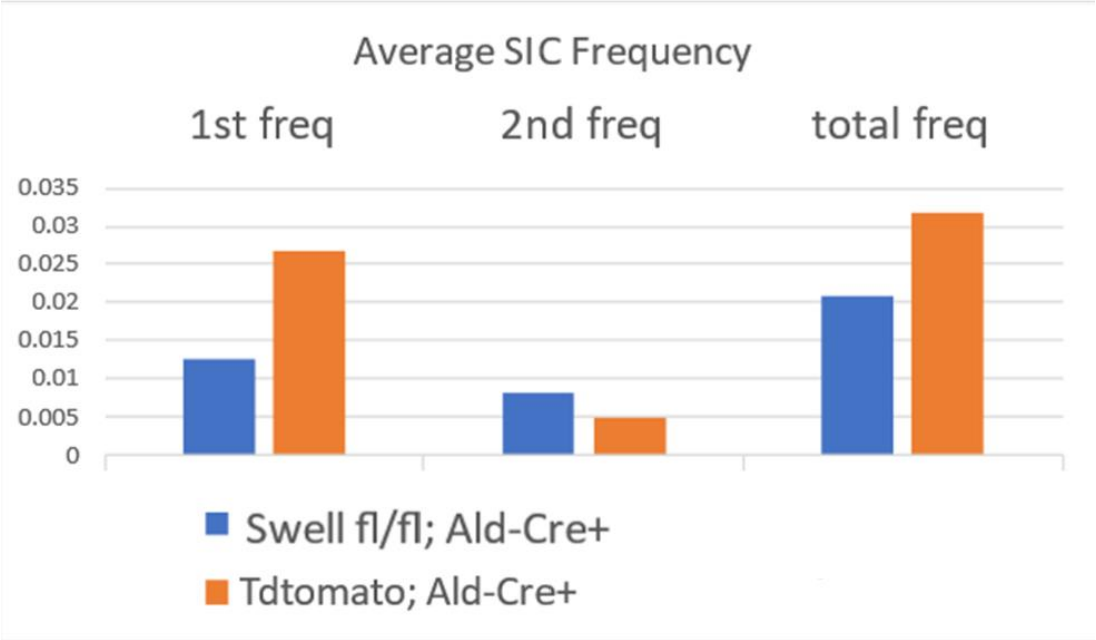
On the other hand, a significant effect is not present regarding astrocyte swelling in 40% hypoosmolar (aCSF) conditions. This trend holds for both applications of the hypoosmolar conditions (Figure 6).

Figure 6



Further experiments were performed to investigate the potential outcome of VRAC cKO on neuronal excitability considering that glutamate is released from swollen astrocytes and neurons. This released glutamate will bind to NMDA receptors to induce a cation current that will depolarize the postsynaptic membrane and thus potentially increase neuronal excitability. To test this, Erin recorded neuron activity during tissue swelling (hypoosmolar aCSF) in both the control SWELLfl/fl;Ald-Cre- mice and the knockout mice SWELLf/f;Ald-Cre+. This was done using a protocol similar to the volume recordings. This protocol consisted of a 10-minute baseline, 5-minute hypoosmolar application, and then a 10-minute wash for volume recovery before repeating. Overall, it appears that the removal of VRAC from astrocytes does indeed seem to have a small blunting effect on neuronal excitability as shown by the SIC (slow inward current) in conditions of tissue swelling (Figure 7).

Figure 7



Discussion

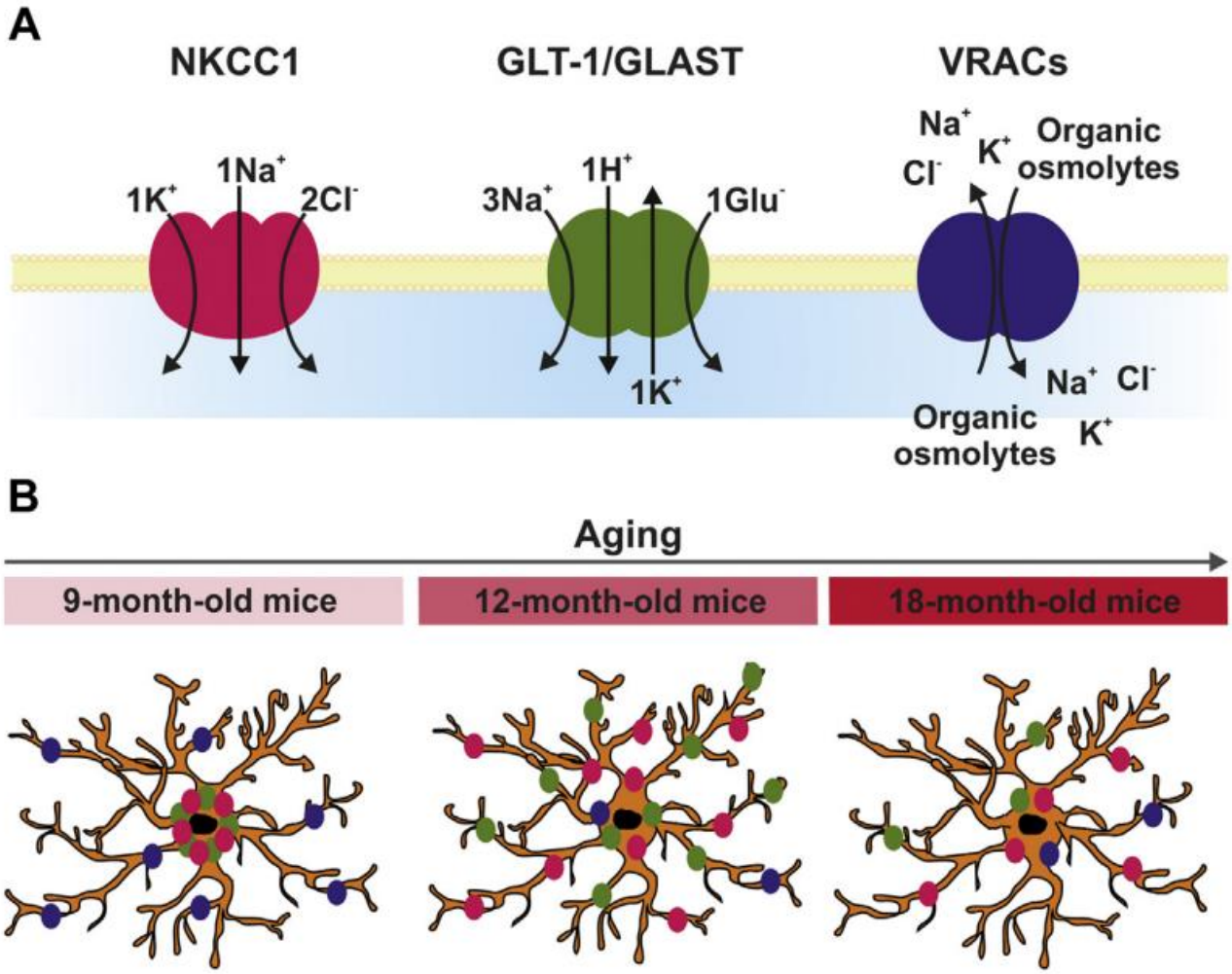
"LRRC8A-Dependent Volume-Regulated Anion Channels Contribute to Ischemia-Induced Brain Injury and Glutamatergic Input to Hippocampal Neurons" (Zhou, et al., 2020)

As a result of this data, we can see that there is some relationship between cerebral ischemia and the neuronal dependent LRRC8A (Figure 2). Ischemia within the brain can likely increase the activity of the neuronal VRAC due to cell swelling (cellular edema). That being said, it is also likely that these VRAC can contribute an increased glutamate input to the mice hippocampal neurons, which leads to further neuronal damage.

"High Potassium Exposure Reveals the Altered Ability of Astrocytes to Regulate their Volume in the Aged Hippocampus of GFAP/EGFP Mice" (Kolenicova, et al., 2020)

The Kolenicova group proposes that over time the amount of VRAC channels decrease. This proposal is in conjunction with the earlier finding that VRAC peaked in function at nine-months in the processes of astrocytes, and then deteriorated after that (Figure 4). Since the soma did not have a significant volume change during the KO conditions at any age, it is postulated that there is a low density of VRAC in the soma compared to the processes (Figure 8b).

Figure 8



They also propose that other channels on the astrocyte membrane, sodium-potassium-chloride cotransporter (NKCC1) and glutamate transporters (GLT-1/GLAST) undergo age-related changes as well (Figure 8a). Specifically, these channels increase in number up to a certain age, and then decrease afterward, further contributing to this maturation process. These membrane channel changes over time in summation is believed to explain why the astrocyte has a more difficult time regulating its volume at certain ages.

As previously mentioned, astrocytes have a harder time regulating their volume as they age in response to a pathological stimulus, such as high extracellular potassium concentrations. This effect seems to be a cause of a maturation process that occurs on the membrane of astrocytes regarding various channels and their ability to uptake and clear potassium and glutamate. For VRAC, it appears that peak function in the astrocyte processes is at nine-months (Figure 4). Overall, it seems that this is a result of the age-related spatial redistribution of various channels over the course of an astrocyte's lifespan.

Erin Walch's VRAC cKO Data from the Fiacco Lab Using the Established Procedures

The astrocyte volume change for the VRAC cKO mice has a different effect in high extracellular potassium conditions than in hypoosmolar conditions (Figures 5 and 6). This suggests that there is likely a mechanistic difference of astrocyte swelling under both of these stimuli. It is uncertain, however, if water enters the cell via a discrete mechanism, or if multiple pathways can cause swelling. These different pathways may be the result of different channels or transporters that are active during a specific condition, which leads to astrocyte swelling. From our data, since there does not appear to a significant volume change in 40% hypoosmolar aCSF, it is unlikely that VRAC is involved in this pathway.

However, despite the lack of significant volume change in 40% hypoosmolar conditions, it is likely that VRAC cKO of these mice does have a blunting effect on neuronal excitability. In the first application of the hypoosmolar aCSF, there was less activity in the knockout mice than in the control mice (Figure 7). This suggests that VRAC is no longer able to release glutamate, which provides less excitability to the surrounding neurons when compared to the control mice that are capable of releasing glutamate through the VRAC channel. It is also possible that astrocytic glutamate concentration does not have ample time to recover and thus the overall activity of the cell is decreased. This would make visualizing the effects of the VRAC channel more difficult to appreciate. That being said, the second application is a little more confusing. The trend for the first application is reversed, but there may be the possibility of no significant change if the error bars are considered. Between these two applications, the "total" portion still shows that VRAC cKO mice are a bit less excitable when compared to the control mice under conditions of hypoosmolar tissue swelling (Figure 7).

Conclusion

With this abundance of information, it is necessary to come full circle to interpret all of it. We are interested in investigating the role that these VRAC on the membranes of both astrocytes and neurons may have in astrocyte volume regulation and neuronal excitability. This was done by comparing the effects of astrocyte VRAC cKO mice vs control mice in conditions that induce tissue swelling. The data from Erin Walch suggests that astrocyte VRAC cKO does have a blunting effect on neuronal excitability, but only during the first application of the stimulus (Figure 7). Furthermore, this data also suggests that there are most likely multiple mechanisms of astrocyte

swelling depending on the stimulus (Figures 5 and 6). These varying mechanisms may involve VRAC or they may not, which could be the focus of any future experiments.

A portion of the literature does provide insight into this. The proposed aging hypothesis from the Kolenicova group notes that VRAC is mainly on the processes of the astrocyte, and not the soma (Figure 8). As aging continues, this composition changes and deteriorates, and so does the efficiency and productivity of astrocytes when prompted with a swelling stimulus. Because our experimental data regarding astrocytes is mostly taken from the soma, there is potential for some misleading data. If VRAC is truly dense only in the processes as the Kolenicova group proposes, then this could be a likely explanation for the insignificant volume changes of the 40% hypoosmolar condition. However, if recording astrocyte volume changes from the processes in both the knockout and the control mice in hypoosmolar conditions shows no significant difference, then this suggests that the aging hypothesis from the Kolenicova group needs to be reevaluated.

Special Requirements

Due to the nature of the project, there are a few requirements and modules that need to be completed before beginning this project. Since we are working with mice, it is necessary to obtain animal handling clearance via the Institutional Animal Care and Use Committee (IACUC) to use proper treatment techniques. On top of that, since I am in a lab, it is necessary to abide by the University's guidelines and complete the online safety modules for various scenarios that apply to our lab such as: how to deal with chemical exposure, fire prevention/safety, laser safety, and general lab safety. In addition, I may need to apply for some mini grants that are offered through various organizations either on or off campus to fund my research. Lastly, since my faculty mentor already has IRB approval, there is no need for me to apply for them.

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