UNIVERSITY OF CALIFORNIA
RIVERSIDE

Effects of Astrocyte Specific Swelling on Neuronal Excitability in Elevated Potassium

A Thesis submitted in partial satisfaction
of the requirements for the degree of

Master of Science

in

Neuroscience

by

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June 2018

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Introduction:

Glial cells were long thought to be simply the "glue," or support cells within the brain. However, they greatly outnumber neurons in the brain, and have been shown to carry out many important functions (Kimelberg & Nedergaard, 2010). One of the major types of glial cells are the astrocytes, and much research has been dedicated to understanding their function in recent years. From simply being described as support cells, they have now been recognized as having a wide variety of indispensable functions. Astrocytes play a major role in ion and water homeostasis (Simard & Nedergaard, 2004; Kimelberg, 2004), control of neuronal excitability and firing (Parea et al, 2009; Pannasch & Rouach, 2013), immune responses (Dong & Benveniste, 2001; Farina et al, 2007), and various neurological pathologies (Norton et al, 1992; Verkhratsky et al, 2010). This has opened up a new focus for the importance of astrocytes in normal brain function, and as possible targets for intervention in many pathologies. Overall, astrocytes have been shown to be critical mediators of proper CNS operation, and new studies have highlighted their overall importance.

1.1 Significance of Astrocyte Swelling:

Space within the brain must be tightly regulated to maintain ionic gradients and the space between neural cells. The swelling of brain cells has been shown to be correlated with events such as ischemia and seizures (Hossman, 1976; Unterberg et al, 2004; Olsson et al, 2006; Wetherington et al, 2008), and lead to increases in neuronal

excitability (Traynelis & Dingledine, 1989; Hochman et al, 1995; Schwartzkroin et al, 1998; Lauderdale et al, 2015). Such swelling can occur due to oxygen glucose deprivation (Panickar & Norenburg, 2005; Risher et al, 2009), decreases in extracellular osmolarity (Lauderdale et al, 2015; Murphy et al, 2017), or increases in extracellular potassium (Macvicar et al, 2002; Kimelberg et al, 1989; Pasantes-morales & Schousboe, 1989; Risher et al, 2009). While swelling can lead to cell death (Lipton, 1999; Liang et al, 2007), it can also have other functional consequences when cells are able to recover. Specifically, it has been shown that in conditions which result in cell swelling, there are often strong increases in neuronal excitability (Traynelis & Dingledine, 1989, Dudek et al, 1990, Kilb et al, 2006, Lauderdale et al, 2015). This increase in excitability could possibly lead to a variety of consequences such as the initiation of seizure activity, and as such, further investigation into the mechanisms by which cellular swelling results in such changes is necessary. Additionally, although both astrocytes and neurons have both been shown to swell in a variety of conditions (Somjen, 1999; Borgdorff et al, 2000; Thompson et al, 2006; Risher et al, 2009), astrocytes remove glutamate and other excitatory amino acids from the extracellular space making them an intriguing target for swelling induced excitability changes (Kimelberg et al, 1990; Parpura & Zorec, 2010; Martineau et al, 2013). The development of methods to selectively swell astrocytes remains an important area of research to both better understand cellular swelling dynamics and mechanisms by which astrocytes may exert significant influence on neuronal excitability.

1.2 Astrocytes Maintain Ion Concentrations in the Extracellular Space:

Maintenance of ion concentrations within the extracellular space (ECS) is critical for nervous system health and optimal neuronal function. Astrocytes are key players in maintaining this delicate balance, expressing a plethora of receptors, channels, transporters and pumps that help maintain these gradients. They also maintain the unique ability to transport ions and small molecules through gap junctions, transporting them from areas of high concentration, to those with a low concentration within the astrocyte "syncytium". In so doing, astrocytes ensure that ionic gradients are maintained at optimal levels, and allow for normal functioning of local neurons. In particular, glutamate and potassium are of special interest, as they are released in high concentrations during neuronal synaptic transmission. A buildup of such ions in the extracellular space would have important negative consequences, leading to the deterioration or death of neurons through excess excitability (excitotoxicity). Astrocytes remove these potentially harmful ions and molecules, maintaining the health of neurons in excitable conditions.

1.2.1 **Glutamate Clearance**:

In order to move molecules through gap junctions, astrocytes must first have mechanisms to remove these molecules from the ECS. Since elevated extracellular glutamate can be toxic to neurons, it is especially important that astrocytes have

channels/transporters to sequester it from the ECS. When glutamate is present at extremely high levels, NMDA receptors are activated and allow an influx of calcium. When the influx of calcium is sufficiently elevated, certain enzymes such as calcium calmodulin II, protein kinase C, proteases, and many others become over-activated. Many of these enzymes are involved with cell death signaling, and this over-activation can cause the cells to die (Mark et al, 2001; Arundine & Tymianski, 2003; Dong et al, 2009). For this reason, astrocytes express multiple glutamate transporters such as GLAST and GLT-1 (EAAT1 and EAAT2, respectively, in humans), which are sodium-coupled transporters to move glutamate into the cell. Both transporters move three sodium ions and one hydrogen ion into the cell, and one potassium ion out in order to move one glutamate ion into the cell (Anderson & Swanson, 2000). In this way, astrocytes remove excess glutamate from the ECS during periods of elevated neuronal excitability. Once glutamate is removed, astrocytes convert glutamate into glutamine, and transport it back to neurons to be converted back into glutamate to complete the cycle (Yudkoff et al, 1986; Zielke et al, 1990; Dringen and Hamprecht, 1996). If glutamate concentrations become too high, astrocytes will also convert glutamate into aspartate, lactate, or alanine (McKenna et al, 1996; Yudkoff et al, 2005; McKenna, 2007). In this manner, astrocytes have an efficient system to control extracellular glutamate levels and provide energy to neurons when necessary.

1.2.2 Potassium Uptake and Spatial Buffering:

Astrocytes also exert much control over the amount of extracellular potassium present within the brain. Extracellular potassium builds up during periods of neuronal activity, and can be detrimental if not removed quickly as it is the main ion setting the resting membrane potential of cells due to high resting permeability. Potassium is most concentrated inside cells, whereas sodium is much more concentrated outside the cell membrane. Therefore, a strong increase in the concentration of potassium outside electrically-excitable neurons will depolarize the membrane potential closer to threshold for activation/inactivation of voltage-gated sodium channels which generate action potentials (Walz, 2000; Kofugi & Newman, 2004). As mentioned previously, hyper-excitation can lead to an influx of calcium, which in turn can cause cell death. For this reason, the concentration of extracellular potassium is strongly regulated, with astrocytes taking up the majority of the excess. Astrocytes express a large variety of potassium channels and pumps, in order to accomplish this task. This phenomenon is known as either potassium uptake, or K^+ spatial buffering. In potassium uptake, the influx of potassium is balanced by either the influx of anions or efflux of cations. In this case, potassium is taken up transiently and released later under optimal conditions. Spatial buffering refers to the removal of potassium ions which are then spread through the glial syncytium via gap junctions, and released into areas of lower potassium concentration (Walz, 2000b; Kofuji & Newman, 2004). Gap junctions connect astrocytes, forming the glial syncytium, and are permeable to most molecules up to ~1-1.2 kDa,

including glutamate, glucose, lactate, potassium and much more (Giaume et al, 1997). Astrocytic gap junctions are formed by connexin and/or pannexin hemichannels, that come together to form an open pore (Dermietzel & Spray, 1993; Shestopalov & Panchin, 2008). Gap junctional coupling in astrocytes has been well demonstrated to be an important factor for both glutamate clearance and potassium spatial buffering, which is critical to keeping neurons healthy and reducing the possibility of excitotoxicity (Hansson et al, 2000; Wallraff et al, 2006). Gap junctions are an important mechanism by which astrocytes maintain ionic gradients in the extracellular space.

In order to remove extracellular potassium, astrocytes express an array of potassium channels. In conditions of elevated potassium, most work has implicated Kir 4.1, NKCC1, and the Na^+/K^+ ATPase (Na^+/K^+). Kir 4.1 is a glial-specific potassium channel, and has long been implicated in potassium uptake and spatial buffering (Higashi et al, 2001; Neusch et al, 2006; Butt & Kalsi, 2006). It has been demonstrated that in Kir 4.1 knockdown or knockout mice, there is a strong reduction of spatial buffering with hyperexcitability of neurons (Olsen et al, 2006; Neusch et al, 2006; Kucheryavykh et al, 2007; Djukic et al, 2007). This indicates that Kir 4.1 is important either for the removal of extracellular potassium or its redistribution to areas of lower concentration. Interestingly, work has also been done to show that Kir 4.1 and astrocyte glutamate transporters GLT-1 and GLAST may be functionally coupled (Djukic et al, 2007; Olsen et al 2007). It has been shown that knockdown or knockout of Kir 4.1 leads to reduced astrocyte glutamate uptake, which could also have severe consequences for neuronal

excitability (Djukic et al, 2007; Kucheryavykh et al, 2007). In this manner, Kir 4.1 may not only exhibit a strong capacity to remove potassium from the extracellular space, but also in glutamate clearance. Another protein implicated in removal of extracellular potassium is the sodium, potassium, chloride co-transporter (NKCC1). NKCC1 is normally associated with facilitating the buildup of chloride within cells (Plotkin et al, 1997; Sun & Murali, 1999). However, it has also been heavily implicated in potassium uptake, as block of NKCC1 has been shown to reduce potassium uptake (Kimelberg & Frangakis, 1985; Rose & Ransom, 1996; Macvicar et al, 2002). Most of this research was done in the intact optic nerve or in culture, but this significant role of NKKC1 in K^+ clearance has not been replicated in brain slices (Larsen et al, 2014). Also, the expression of NKCC1 is drastically reduced early in development as the expression of KCC2 increases, perhaps limiting its contribution to early stages in development (Plotkin et al 1997; Lu et al 1999; Vu et al, 2000). This may in part explain its prominent role in cultured cells, which represent an immature or reactive phenotype (Molofsky et al, 2012). Finally, a third option for the clearance of potassium from the extracellular space is the Na^+/K^+ ATPase (Na^+/K^+). The Na^+/K^+ is responsible for the high concentration of potassium and low concentration of sodium within cells compared to outside the cells (Thomas, 1972; Weer and Rakowski, 1984; Stahl, 1986). Due to this function, the Na^+/K^+ has been implicated in potassium clearance in multiple studies (Ransom et al. 2000; D'Ambrosio et al. 2002). In a recent study that demonstrated the significant contribution of the Na^+/K^+ to potassium clearance, it was discussed that neurons and astrocytes express

different isoforms of the Na^+/K^+, and the glial-associated α2β2 isoform may be largely responsible for K^+ clearance (Larsen et al, 2014). While Kir 4.1, NKCC1, and the Na^+/K^+ may all contribute to potassium clearance, the more recent data suggest that in brain slices, the Na^+/K^+ seems to play the largest role.

1.3 Astrocyte Volume Changes and Aquaporin 4 co-localization:

Astrocytes not only take up excess osmolytes from the extracellular space, but also take up water under certain conditions, leading to cellular volume increases (Andrew et al, 2007; Risher et al, 2009). These volume increases can occur in response to a variety of factors, with interesting consequences such as increases in neuronal excitability (Lauderdale et al, 2015). Astrocytes were thought to readily swell due to the expression of the functional water channel aquaporin 4 (AQP4), which is not present on neurons (Nielsen et al, 1997; Nagelhus, 1998). Studies have shown that AQP4 deficient mice exhibit reduced brain edema following ischemic stroke, prolonged seizure duration, and reduced potassium clearance (Manley et al, 2000; Amiry-Moghaddam et al, 2003; Binder et al, 2004). It was also demonstrated that AQP4 co-localized with Kir 4.1, provided an explanation for astrocyte swelling in elevated potassium (Wen et al, 1999; Nagelhus et al, 1999; Nagelhus et al, 2004), although this has recently been called into question (Haj-Yasein et al, 2011; Haj-Yasein et al, 2012). There was additional work demonstrating that in $AQP4^{-/-}$ mice, astrocytes actually increased their volume more than astrocytes from wildtype mice in response to hypoosmolar ACSF, leading to the

idea that AQP4 may be more important for water extrusion than influx (Murphy et al, 2017). More recently, neurons have also been shown to swell in some circumstances, leading to questions about the different mechanisms by which water enters cells, and if this differs between astrocytes and neurons. Overall, cellular volume changes are important for maintaining extracellular ion and neurotransmitter concentrations. However, if continuing unabated, rapid cell volume changes can profoundly impact excitability of neurons.

1.3.1: Conditions of Astrocyte and Neuronal Swelling:

There are many conditions that can lead to the swelling of astrocytes, neurons, or both. It has been shown that in the conditions of oxygen glucose deprivation (OGD), a model of stroke and ischemia, astrocytes and neurons both swell (Werth et al, 1998; Panickar & Norenburg, 2005; Thompson et al, 2006; Risher et al, 2009). Cerebral ischemia can cause cell death in just a few minutes due to sudden loss of oxidative metabolism (Hansen, 1985; Lipton, 1999). Even though astrocytes and neurons both swell to a similar degree in this situation, it appears that astrocytes have the ability to recover, while neurons are much less likely to survive (Risher et al, 2009). In this case, even though both cell types are starved of oxygen and glucose, the recovery by astrocytes suggests that they have additional energy stores and/or a greater flexibility for anaerobic metabolism (Hertz et al, 2007; Sickmann et al, 2009). Another commonly-used model of cellular edema is hypoosmolar challenge. Early work suggested that

hypoosmolar ACSF led to both astrocyte and neuronal swelling (Somjen, 1999; Borgdorff et al, 2000), however this was later refuted by work demonstrating that hypoosmolar conditions only swell astrocytes (Andrew et al, 2007; Risher et al, 2009). More recently, and with new analysis methods, both astrocyte and neuronal swelling is evident within one minute of exposure to hypoosmolar ACSF (Murphy et al, 2017). Cell swelling was apparent with either a seventeen or forty percent reduction in the osmolarity of the ACSF, and suggests there may be common mechanisms of water entry between astrocytes and neurons. Finally, there is also a model to swell astrocytes and neurons using elevated potassium ACSF. This model has been shown to swell astrocytes significantly by many studies (Kimelberg et al, 1989; Pasantes-morales & Schousboe, 1989; Macvicar et al, 2002; Risher et al, 2009). Many studies that demonstrated astrocyte swelling in elevated potassium used extremely high concentrations of potassium (>20 mM), however, normal potassium fluctuations in the brain rarely exceed ceilings of 10-12 mM (Heinemann and Lux, 1977). In fact, at 26 mM potassium ACSF, neurons have also been shown to significantly swell, likely due to initiation of a phenomenon called spreading depression (Risher et al, 2009; Zhou et al, 2010). Neuronal swelling has not yet been demonstrated under physiologically-relevant extracellular potassium concentrations, suggesting a possible model for astrocyte specific swelling.

As summarized above, astrocytes and neurons have both been demonstrated to swell across a variety of conditions, raising the possibility of a shared mechanism for water

influx. However, due to early studies reporting no neuronal swelling in hypoosmolar conditions, it was thought that astrocytes are uniquely susceptible to water influx due to expression of the functional water channel aquaporin 4 (AQP4) (Nagelhus et al, 2004; Saadoun & Papadopoulos, 2010; Wen et al, 1999). Further evidence suggested co-localization of AQP4 and Kir 4.1, providing an explanation for astrocyte induced swelling in conditions of elevated potassium (Wurm et al, 2006; Wen et al, 1999; Nagelhus et al, 1999, 2004). However, this does not account for astrocyte swelling in hypoosmolar conditions (in which extracellular K^+ concentration is diluted), and recent work has shown that in $AQP4^{-/-}$ mice, astrocytes actually swell more compared to wild-type mice in conditions of both hypoosmolarity and elevated potassium (Murphy et al, 2017; Haj-Yasein et al, 2011; Haj-Yasein et al, 2012). This indicates that AQP4 is not the primary influx pathway of water into astrocytes, and supports the findings that both neurons and astrocytes swell in various conditions, perhaps sharing a common, AQP4-independent, mechanism. One possibility is water influx through NKCC1, as this co-transporter has been shown to possess water permeability and is expressed by both astrocytes and neurons (Hamann et al, 2010; Zeuthen & MacAulay, 2012). However, NKCC1 expression is greatly decreased later in development, which likely limits its contribution in the mature brain (Plotkin et al 1997; Lu et al 1999; Vu et al, 2000). Another proposed mechanism includes the electrogenic Na^+/HCO_3^- co-transporter 1 (NBCe1), inhibition of which has been shown to reduce ECS shrinkage in elevated potassium (Larsen & MacAulay, 2017). This finding indicates that swelling occurs partly

due to changes in pH, although further work will have to be done to determine if this is the case in other conditions that result in cell swelling.

1.4 Astrocyte Effects on Neuronal Excitability:

As introduced above, astrocytes have been shown to be much more than support cells in the brain. Much research has been done to determine that astrocytes play an active role in the control of synaptic transmission. Astrocytes are in integral part of the "tripartite" synapse, surrounding synapses and exchanging information with neurons. This is possible as astrocytes have receptors for most neurotransmitters, allowing them to bind neurotransmitters during synaptic transmission causing changes in cellular activity and/or metabolism (Newman, 2003; Porter & McCarthy, 1997). In doing so, astrocytes are able to rapidly respond to changes in excitability, perhaps allowing them to affect neuronal activity pre- and/or postsynaptically. However, in order for this to occur, do astrocytes release substances that can signal back to neurons? Recent work has debated the conditions and mechanisms that govern astrocytic release of glutamate and other potential "gliotransmitters".

1.4.1 Debating Calcium Mediated Gliotransmission:

One of the more popular recent theories regarding astrocyte mediated control of neuronal activity is a concept that has been termed "gliotransmission." Gliotransmission involves increases in astrocyte calcium as a response to neurotransmitters acting on G-protein coupled receptors, which can propagate as a wave throughout a wide field of

astrocytes (Haydon, 2001; Newman, 2003; Perea & Araque, 2005). This elevation in glial calcium was demonstrated to lead to release of glutamate and other gliotransmitters, in a Ca^{2+}-dependent manner (Araque et al, 2000; Montana et al, 2006; Perea & Araque, 2005; Perea & Araque, 2007). In this view, astrocytes have the machinery in place to release transmitters via vesicles, a method previously only prescribed to neurons. However, there are multiple studies that have cast doubt on this view, and suggest that Ca^{2+} mediated gliotransmission does not actually occur under physiological conditions (Fiacco & MacCarthy, 2018).

One study to cast doubt on this phenomenon used an endogenous Gq-GPCR that is not normally expressed in the forebrain, and targeted it to astrocytes. When stimulated, it caused large calcium elevations that were readily recorded, but there were no changes in neuronal synaptic transmission observed via whole-cell patch clamp (Agulhon et al, 2010; Fiacco et al, 2007). If large calcium elevations were responsible for gliotransmission, there should have been evident changes in neuronal excitability. Another piece of evidence recently refuted is that gliotransmission requires calcium waves to propagate throughout the astrocyte population, releasing Ca^{2+} from internal stores via IP3 receptors (Araque et al, 1999; Boitano et al, 1992; Charles et al, 1993; Cornell-Bell et al, 1990). However, the large scale calcium waves seen *in vitro*, have not been observed in intact tissue (Fiacco & MacCarthy, 2004). Also, while $IP3R2^{-/-}$ mice were shown to significantly reduce the amount of calcium produced in astrocytes, there were no evident changes in excitatory synaptic activity or long term potentiation

(Agulhon et al, 2010; Bonder & McCarthy, 2014; Petravicz et al, 2008). Finally, it was found that in intact tissue, expression of vesicular glutamate transporters and other machinery required for vesicular exocytosis is absent in astrocytes (Li et al., 2013; Zhang et al., 2014; Chai et al., 2017). In light of this new evidence, it seems doubtful that astrocytes affect neuronal excitability by gliotransmission, although there are other possible mechanisms for release of excitable molecules by astrocytes.

1.4.2 Reversal of Glutamate Transporters and Release Through Hemichannels:

Another mechanism for glutamate release from astrocytes revolves around the reversal of glutamate transporters. As previously mentioned, astrocytes express the glutamate transporters GLAST and GLT-1 which normally remove glutamate from the extracellular space. There are certain occasions where the transporters reverse, allowing for glutamate from within the astrocyte to spill out into the extracellular space. This has been found to occur in circumstances where the gradient for sodium breaks down due to depletion of ATP, which can occur during ischemia or hypoxia (Anderson & Swanson, 2000; Camacho & Massieu, 2006; Rossi et al, 2000; Takahashi et al, 1997). In such circumstances, the lack of ATP impairs the function of the Na^+/K^+ pump, since this is largely responsible for the sodium gradient on which GLAST and GLT-1 depend, the transporters can actually have the reverse function by pumping glutamate out of astrocytes into the ECS. Glutamate transport reversal has also been demonstrated in

conditions of elevated potassium (Longuemare et al, 1999). Reversal of glutamate transport is unlikely to be a phenomenon that occurs under physiological conditions.

Another possible mechanism for glutamate release is through gap junction hemichannels, which are connexons which do not align with a connexon from an adjoining cell. Multiple studies have demonstrated that glutamate and other molecules can be released through hemichannels in specific conditions (Contreras et al, 2002; Stehberg et al, 2012; Ye et al, 2003). However, much work still needs to be done to determine if hemichannels are capable of opening under normal, or simply pathophysiological conditions. In most studies, opening of hemichannels has been demonstrated only *in vitro* and requires very low external Ca^{2+} and Mg^{2+}, strong membrane depolarization, cytokine release or ischemic conditions (Bennett et al, 2003; Parpura et al, 2003; Retamal et al, 2007). There is also literature stating that some functions attributed to hemichannels may actually be due to other types of channels, as blockers of hemichannels are also known to block anion channels (Eskandari et al, 2002). There has been a movement to set up strict guidelines in order to determine if a given function can be attributed to hemichannels, with the hope to parse out hemichannel function from that of other possible contributors (Spray et al, 2006). While much has been done to determine the effects of open hemichannels, more work needs to be done to determine the specific conditions necessary for hemichannel opening, and to develop additional techniques to specifically target hemichannels so functions are not misattributed.

1.4.3 Glutamate Release Through Volume-Regulated Anion Channels:

Astrocytes maintain ionic gradients and in so doing are constantly generating osmotic gradients for water movement into or out of the cell. Astrocyte swelling has been shown to occur during oxygen glucose deprivation, hypoosmolar conditions, and in elevated extracellular potassium (Kimelberg et al, 1989; MacVicar et al, 2002; Murphy et al, 2017; Risher et al, 2009). Swelling of astrocytes leads to the activation of volume regulate anion channels (VRAC), through which glutamate and a variety of anions and neutral molecules can pass (Mongin & Kimelberg, 2001; Kimelberg, 2005; Abdullaev et al, 2006). It has also been shown that the neurotransmitter ATP is a strong regulator of VRAC, potently increasing its activity and release of glutamate during conditions of cell swelling (Mongin & Kimelberg, 2001). Release of glutamate and excitatory amino acids could possibly lead to excitation of nearby neurons. While this has not been directly demonstrated to occur via VRAC, it has been shown that astrocyte swelling is linked to increases in neuronal excitability (Traynelis & Dingledine, 1989, Dudek et al, 1990, Kilb et al, 2006, Lauderdale et al, 2015). As ionic gradients change during fluctuations in excitability or due to pathology, it becomes increasingly evident that astrocyte swelling may play an important role in regulating the volume of the ECS, extracellular glutamate concentrations, and neuronal excitability. In situations where astrocytes swell for longer periods of time, there may be drastic changes in neuronal excitability as a result.

Recently, the essential pore-forming subunit of VRAC was identified as leucine rich repeat containing protein 8A (LRRC8A), and was shown to be necessary for swelling mediated release of excitatory amino acids from astrocytes (Qiu et al, 2014; Voss et al, 2014; Hyzinski-Garcia et al, 2014). While further studies need to be done to confirm that changes in neuronal excitability during conditions of astrocyte swelling are indeed due to VRAC, the identification of LRRC8A will hopefully lead to the development of specific tools to target this protein in astrocytes.

1.5 Investigating Astrocyte-Specific Swelling Effects on Neuronal Excitability

Given the previous literature suggesting the important roles astrocytes may play in the regulation of neuronal excitability, specifically due to their roles in ion and water homeostasis, corresponding volume changes and release of glutamate through VRAC, we decided to further investigate mechanisms of astrocyte swelling and the eventually effects on neuronal excitability. While many studies have hinted at the importance of astrocyte swelling to neuronal or brain tissue excitability increases, most studies used conditions that have now been shown to nonselectively swell both astrocytes and neurons. We utilized acute hippocampal brain slices, a combination of confocal microscopy and electrophysiology, and a method to selectively swell astrocytes to further determine the role of astrocyte-specific swelling on neuronal excitability.

The Role of Hippocampal Astrocytic Swelling on Neuronal Hyperexcitability in Conditions of Elevated Potassium

Abstract:

Neurotransmitter and ion influx into astrocytes generates osmotic gradients coupled to water movement into the cell, resulting in transient or prolonged fluctuations in cell volume. Increases in cell volume reduce the size of the extracellular space (ECS) and are associated with elevated brain tissue excitability. However, the precise mechanisms at play in coupling astrocyte volume changes to ion movements remain controversial, as does the effect of acute astrocyte volume fluctuations on neuronal function. Here we set out to determine the effects of raised extracellular potassium concentrations (↑$[K^+]_o$) on volume responses of CA1 pyramidal neurons and stratum radiatum astrocytes in the hippocampus. First, we found that elevated $[K^+]_o$ within a physiological range (6.5 and 10.5 mM from a baseline of 2.5 mM) and up to 26 mM produces dose-dependent increases in astrocyte volume, with no effect on neuronal volume. Astrocyte volume increases in elevated $[K^+]_o$ were not dependent on AQP4, Kir4.1, the sodium-bicarbonate cotransporter NBCe1, or the electroneutral cotransporter NKCC1, but were significantly attenuated in 1 mM $BaCl_2$ and by the Na^+/K^+ pump inhibitor ouabain, suggesting that astrocyte volume increases are due to K^+ influx from nonspecific K^+ channels and/or the Na^+/K^+ ATPase. High $[K^+]_o$-induced astrocyte swelling resulted in significant increases in neuronal excitability in the form of NMDA receptor-dependent

slow inward currents (SICs) and mixed AMPA/NMDA mEPSCs. Direct depolarizing effects of high $[K^+]_o$ on neuronal spiking were prevented by application of TTX, and the amount of depolarization was insufficient to activate voltage-gated Ca^{2+} channels, suggesting that changes in neuronal excitability were not due to elevated $[K^+]_o$-related increases in synaptic transmission. Finally, we show that astrocyte-specific swelling in elevated $[K^+]_o$ and effects on neuronal excitability can be completely negated by addition of mannitol, which we found selectively shrinks astrocytes. Overall, our findings suggest that astrocyte-selective volume increases in elevated $[K^+]_o$ conditions are due to activity of the Na^+/K^+ ATPase, which result in astrocyte-specific increases in neuronal excitability independent of direct depolarizing effects of high $[K^+]_o$ on neurons.

Introduction:

Astrocytes play a significant role in maintaining ion and neurotransmitter concentrations in the extracellular space. This includes uptake of glutamate and potassium that is released during synaptic transmission. Potassium influx into astrocytes during neuronal synaptic activity is redistributed through the glial syncytium to areas of lower potassium concentration, a process known as "potassium spatial buffering" (Walz, 2000; Kofuji & Newman, 2004). Potassium influx into astrocytes associated with K^+ spatial buffering generates an osmotic gradient coupled to movement of water into the cell, leading to transient or prolonged fluxes in cellular volume (Pasantes-Morales & Schousboe; Walz, 1992 MacVicar et al, 2002; Risher et al, 2009). Volume changes in astrocytes have been measured in a number of different ways, both directly using fluorescence microscopy (Risher et al, 2009; Benesova et al, 2012) and inferred by recording changes in intracellular ion concentrations (Walz, 1992), light scattering (MacVicar et al, 2001), or assessment of the size or composition of the ECS (Ransom et al. 1985; Traynelis and Dingledine 1988; Binder et al. 2004; Larsen et al, 2014). While the evidence that elevated extracellular K^+ ($[K^+]_o$) leads to volume increases in astrocytes is compelling, the mechanisms involved have remained somewhat controversial.

Astrocytes express an impressive array of potassium channels and cotransporters, including many not expressed in other cell types. One channel that is not only the most highly expressed potassium channel in astrocytes, but also has been identified as

instrumental for spatial buffering is Kir 4.1, an inwardly-rectifying astrocyte specific potassium channel (Djukic et al, 2007; MacAuley & Zeuthen, 2012). Interestingly, Kir 4.1 has been proposed to be functionally linked to the astrocyte specific water channel, AQP4 (Connors et al, 2004; Nagelhus et al, 2004), providing a direct means for coupling ion to water movements. Another possible candidate for coupling K^+ movement into astrocytes is the sodium potassium chloride co-transporter, NKCC1. NKCC1 may contribute to potassium mediated astrocyte swelling in the optic nerve (MacVicar et al, 2001), and plays a clear role in $[K^+]_o$-induced swelling *in vitro* (Su et al, 2002). However, its role in intact tissue is less convincing (Larsen et al, 2014). NKCC1 is especially compelling due to its high purported water permeability (MacAulay et al, 2004). Another consideration is the sodium potassium pump (Na^+/K^+). This pump is responsible for pumping potassium into cells and sodium out, against their respective concentration gradients. It has also been determined that the α2β2 isoform, which may be highly expressed by astrocytes, is most sensitive to changes in extracellular potassium (Larsen et al, 2014), providing an important route for K^+ influx into astrocytes.

Cellular swelling results in a transient decrease in the volume of the extracellular space, positively influencing neuronal excitability and a requirement for generating ictal (or seizure-like) discharges (Traynelis & Dingledine, 1989; Kilb et al, 2006; Shahar et al, 2009; Lauderdale et al, 2015). While cellular volume increases have a strong influence on neuronal and brain tissue excitability, the precise contribution of astrocyte swelling has not been clearly defined. In elevated $[K^+]_o$ conditions (8.5 mM), neurons generate

synchronous ictal bursting activity that requires NMDA receptor activation and reduction of the ECS (Traynelis and Dingledine, 1988). In these conditions, although direct cell volume measurements were not taken, it seems very likely that the reduction of the ECS is due to astrocyte swelling tied to influx of K^+ (Traynelis and Dingledine, 1989). Therefore, astrocyte swelling may directly influence neuronal excitability, contributing directly to seizure generation. However, because epileptiform activity is by definition synchronous neuronal bursting, it is also difficult to dissociate possible influences of changes in astrocyte volume from the direct effects of elevated $[K^+]_o$ on neuronal spiking.

In this study, we utilized acute mouse hippocampal slices, confocal microscopy and whole-cell patch clamp to assess neuron and astrocyte volume changes associated with elevated $[K^+]_o$. In addition, we examined the possible mechanisms involved and assessed the impact of astrocyte swelling on neuronal excitability. Slices were exposed to normal ACSF, as well as elevated potassium ACSF at concentrations of 6.5, 10.5, or 26 mM. In these conditions, astrocytes dose-dependently increased their volume, but neurons showed little volume change at all. Even at 26 mM, neurons steadfastly maintained their volume, suggesting little potassium permeability. Astrocyte volume increases in 10.5 mM $[K^+]_o$ were not due to AQP4, Kir4.1, NBCe1, or NKCC1, but were reduced significantly in 1 mM $BaCl_2$ and by the Na^+/K^+ ATPase inhibitor ouabain. Our findings are consistent with previous studies citing the importance of the Na^+/K^+ pump on astrocyte volume increases associated with influx of K^+ (Larsen et al., 2014). Lastly, by selectively

reversing astrocyte swelling in high $[K^+]_o$, we show that astrocyte swelling dramatically influences excitability of CA1 pyramidal neurons independently of direct depolarizing effects of elevated K^+ on neuronal spiking or voltage-gated Ca^{2+} channel activation. These results highlight the importance of the Na^+/K^+ pump in astrocyte swelling in elevated potassium, and point to a strong relationship between astrocyte volume changes and neuronal excitability.

Materials and Methods:

All experiments were performed in accordance with National Institutes of Health guidelines for the care and use of laboratory animals, and approved by the Institutional Animal Care and Use Committee at the University of California, Riverside.

Slice Preparation:

Hippocampal slices were prepared from C57Bl/6J mice aging from 15-21 days of age as described previously (Xie et al., 2014). In experiments where neurons were imaged, Thy1- enhanced green fluorescent protein (eGFP; JacksonLaboratories, BarHarbor, ME, USA) were used. These mice were of a C57Bl/6J background, and there were no obvious differences in phenotype compared to wild-type mice (Ma et al., 1997; Feng et al., 2000). Animals were anesthetized under isoflurane, followed by a rapid decapitation. The brains were removed quickly, and set into petri dishes containing oxygenated (Carbogen, 95% oxygen and 5% carbon dioxide) ice-cold slicing buffer containing either (in mM): 125 NaCl, 2.5 KCl, 3.8 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 25 glucose, and 1.3 ascorbic acid, or alternatively, we used a sucrose based slicing buffer containing (mM): 87 NaCl, 75 Sucrose, 10 Glucose, 1.25 NaH_2PO_4, 2.5 KCl, 25 $NaHCO_3$, 1.3 Ascorbic Acid, 0.5 $CaCl_2$, 7 $MgCl_2$, 2 Pyruvate, and 3.5 MOPS. In this case, the slicing buffer also contained 100 µM kynurenic acid and was partially frozen, to a “slushy” consistency, before the brain was placed into it. Parasaggital slices were then cut using a Leica VT1200S vibratome, at 350 µM thick, before being transferred to a recovery chamber.

The recovery chamber contained “normal” ACSF solution which was comprised of (in mM): 125 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 15 glucose, for 45 minutes at 36°C, followed by a 15 minute recovery period at room temperature before being used in experiments.

When imaging astrocytes, sulforhodamine-101 (SR-101; Sigma-Aldrich, St. Louis, MO, USA) was used for staining purposes, as described previously (Schnell et al., 2015; Murphy et al., 2017). In this case, slices rested in an incubation chamber containing 1 µM SR-101 and a modified ACSF consisting of (in mM): 1.3 ascorbic acid, 125 NaCl, 15 glucose, 1.25 H_2PO_4, 2.5 KCl, 26 $NaHCO_3$, 0.5 $CaCl_2$, and 6 $MgCl_2$. After remaining in this chamber for 35-45 minutes at 36°C, slices were transferred to another chamber containing the same modified ACSF, but without SR-101 at 36°C for an additional 10 minutes. Slices were transferred to standard ACSF at room temperature for 15 minutes before use in experiments.

Solutions and Pharmacology:

For our elevated potassium conditions, we used either 6.5 mM, 10.5 mM, or 26 mM K^+ ACSF, progressing from a moderate challenge (6.5mM K^+), to near ceiling levels reached during seizure (10.5 mM), to a concentration more closely linked to spreading depression models (26 mM K^+). In elevated $[K^+]_o$ ACSF, solutions were kept iso-osmolar by reducing an equal amount of NaCl. In some experiments, KCl was elevated with no corresponding change to the NaCl concentration to deliberately compare effects of

solution hyperosmolarity vs. effects of elevated $[K^+]_o$ on cell volume. Cell volume measurements were performed in the same conditions as those used to record effects on neuronal excitability (Mg^{2+}-free conditions to remove Mg^{2+} block from NMDA receptors and + 1 µM TTX to block neuronal firing), but separate recordings showed no effect of Mg^{2+} or TTX on astrocyte volume changes in high $[K^+]_o$. For some electrophysiological experiments, 50 µM NBQX was included in addition to TTX in order to block AMPA/Kainate receptor activity (Lauderdale et al, 2015; Murphy et al, 2017).

Whole Cell Patch Clamp Electrophysiology:

Following preparation, slices were transferred to a recording chamber and continuously perfused with oxygenated ACSF at room temperature. In order to visualize slices and individual cells, we used an Olympus BX61 WI upright microscope, a UMPLFLN 10× (N.A. 0.3) and UMPLFLN 60× (N.A. 0.9) water-immersion objectives and DIC optics (Olympus America, Center Valley, PA, USA). Recordings were done using a Multiclamp 700B amplifier and Digidata 1550 digitizer, controlled with pClamp v.10.4 and Multiclamp commander software (Molecular Devices, Sunnyvale, CA, USA). Patch pipettes were pulled from borosilicate glass using a Narishige PC-10 vertical micropipette puller (Narishige, Tokyo, Japan). For neurons, patch pipette resistances ranged from ~3-5mOhms when containing an internal solution of (in mM): 140 K-gluconate, 4 MgCl2, 0.4 EGTA, 4 Mg-ATP, 0.2 Na-GTP, 10 HEPES, and 10 phosphocreatine, pH 7.3 with KOH. Astrocyte pipette resistances ranged from ~7-9 mOhms, filled with a solution containing

(in mM): 130 K-gluconate, 4 MgCl2, 10 HEPES, 10 glucose, 1.185 Mg-ATP, 10.55 phosphocreatine and 0.1315 mg/ml creatine phosphokinase, pH7.3 by KOH.

DIC optics were initially used to identify neurons and astrocytes based on morphology. Location was also an identifying property, as both CA1 and the stratum radiatum are easily located under DIC. Cell identity was confirmed based on the electrophysiological properties of the cells. Neurons typically had a resting membrane potential of ~-60Mv. Using a voltage step protocol, neurons displayed typical voltage-gated sodium and potassium currents at more depolarizing inputs, and were voltage clamped at -70 mV.

Confocal Imaging Experiments:

Throughout our experiments, eGFP was visualized via a 488 argon laser, with a 503-548 bandpass filter, and SR-101 was visualized using a 559 nm semiconductor laser using a 624–724 nm bandpass filter. The laser power used for both cell types ranged from 1.5-2.5%, to minimize possible effects of laser intensity such as photobleaching or phototoxicity. Our experimental design for volume imaging of neurons and astrocytes was performed as described previously (Murphy et al., 2017). Briefly, experiments consisted of a 5-10 minute baseline period, where 3 images were taken and used as the baseline comparison for later time points. This was followed by a 5 minute application of elevated potassium, and an image was taken at every minute. This was followed by a 10 minute wash, during which normal ACSF was applied (or Mg^{+} free ACSF depending on the conditions being tested), where and image was taken every 5 minutes. This was

followed by another 5 minute application of elevated potassium, and finally the last 10 minute wash. In total, 17 images were collected during the experiment. After the baseline images were averaged, later time points were measured as percentages increased above baseline. We determined that a single image was insufficient to encapsulate the entire x-y plane of each cell, so every image was a z-stack taken in 1µm increments at a 3.5x zoom factor. We also cropped to just around the soma for imaging throughout the experiment. These settings ensured a better overall idea of cell swelling, while still allowing for a short enough acquisition time so a stack could be taken each minute during the evoke. As the tissue swelled, cells could drift between stacks, so we did quick scans between each time point to adjust in the x-y directions, and adjust the z plane before taking the z-stack.

Statistical Analysis:

Statistical analysis was carried out using SPSS Statistics 24 software. Repeated measures ANOVA was utilized for all conditions of cell swelling, comparing two or more treatment groups. Outliers were rarely removed, no more than one per data set. Potenital outliers were detected by SPSS boxplots, and studentized residuals following a repeated measures ANOVA. Outliers were only removed in cases where there were severe violations of normality or homogeneity, which were improved upon removal of that cell. Post-hoc tests were performed, and the Bonferroni correction was used for multiple comparisons. In cases where there was a significant interaction between time and

condition, there was a subsequent independent Student's t-test (two groups) or one way ANOVA (3 groups) with Tukey's post-hoc test performed. Each group contained N=6-10 cells (numbers specified in each experiment), unless otherwise noted. Significance values are listed as: *p<.05, **p<.01, and ***p<.001.

Results:

Elevated $[K^+]_o$ ACSF Selectively Swells Astrocytes

We first recorded cellular volume of both CA1 pyramidal neurons and astrocytes within the stratum radiatum (s.r.) in response to 6.5, 10.5 and 26 mM $[K^+]_o$ ACSF. This ACSF was made isoosmolar by removal of NaCl (see Methods) in order to avoid possible hyperosmolar-induced cellular shrinking. Astrocytes were labeled with Sulforhodamine 101 (SR-101) (Schnell et al, 2015), and for imaging neurons, slices were taken from Thy1-eGFP mice (Feng et al., 2000). These mice express eGFP in a small proportion of pyramidal neurons in a random manner, enabling easy visualization of individual cells. The imaging procedure was similar to that used in our previous study (Murphy et al, 2017). Briefly, image stacks were taken through the cell soma, starting with 3 baseline stacks at 1 minute intervals in normal (2.5 mM $[K^+]_o$) ACSF (nACSF), followed by 5 image stacks taken at 1 minute intervals in elevated $[K^+]_o$ ACSF, and ending with 2 image stacks taken in normal ACSF at 5 minute intervals (10 minute wash period in nACSF). This procedure was performed twice per cell (5 min high $[K^+]_o$ application, 10 min. wash, 5 min. high $[K^+]_o$, 10 min wash). Consistent with astrocytic high resting K^+ permeability, and the role of astrocytes in potassium clearance and spatial buffering, we found that astrocytes rapidly swelled within one minute of elevated $[K^+]_o$ application, whereas there was little to no such change in neurons (Figure1). This was consistent even in 26 mM $[K^+]_o$ (Figure 2), which is far beyond normally occurring K^+ ceilings even in

pathological conditions. This may be surprising as extremely high levels of $[K^+]_o$ have frequently been used to induce spreading depression which is associated with neuronal swelling (Zhou et al., 2010). In our conditions we are unlikely to be inducing spreading depression as high $[K^+]_o$ is applied non-directionally through bath application, rather than delivery from a point source. Thus, it appears as though high $[K^+]_o$-induced cell swelling is specific to astrocytes, consistent with their role in ion and water homeostasis.

Astrocyte Swelling in high $[K^+]_o$ is not due to K^+ influx via Kir4.1

Once we determined that $[K^+]_o$-induced swelling is restricted to astrocytes, we attempted to identify the underlying mechanism(s). The astrocyte-selective, inwardly rectifying K^+ channel Kir4.1 has long been considered the predominant K^+ influx pathway coupled to cellular volume changes (Kofugi & Newman, 2004; Nagelhus et al, 2004; Lichter-Konecki et al, 2008; Wetherington et al, 2008). Presumably, water follows the osmotic gradient generated by the influx of K^+ ions into the astrocyte via Kir4.1, currents of which can be readily recorded in astrocytes (Djukic et al, 2007; Devaraju et al, 2013). To determine any contribution of inwardly rectifying potassium channels, which are known to open during conditions of elevated extracellular potassium (Gordon et al., 2007), we compared the astrocyte swelling profile in the presence of 100 µM Ba^{2+}, a selective Kir channel blocker (Rudy, 1988; Hille, 1992) to astrocyte swelling in high $[K^+]_o$ alone. Surprisingly, astrocyte swelling was not affected by application of 100 µM Ba^{2+}

(Figure 3), arguing against a role for Kir4.1 in astrocyte swelling occurring in elevated $[K^+]_o$.

Astrocyte Swelling in high $[K^+]_o$ occurs independently of the water channel AQP4

Aquaporin 4 (AQP4) is a functional water channel expressed in astrocytes, but not in neurons (Nielsen et al, 1997; Nagelhus et al, 1998). AQP4 has been shown to co-localize with Kir4.1, lending support to findings suggesting functional coupling of Kir4.1 to AQP4-dependent water influx (Wen et al, 1999; Nagelhus et al, 1999; Nagelhus et al, 2004). In our previous study, we found that astrocyte swelling was actually *increased* in $AQP4^{-/-}$ mice in hypoosmolar conditions relative to controls, indicating that AQP4 is dispensable for swelling, and perhaps even important for extrusion of water in swelling conditions (Murphy et al, 2017). However, due to the previous work linking AQP4 and Kir4.1, it was possible that AQP4 played a different role in elevated potassium conditions. Although there was no evident contribution of Kir 4.1 in our study, other mechanisms for K^+ influx into astrocytes may be coupled to AQP4 mediated water influx into astrocytes in elevated potassium conditions. In the present study, we found no effect of $AQP4^{-/-}$ on astrocyte swelling in 10.5 mM K^+ (Figure 4). Although there was no significant interaction between astrocyte swelling in wild type vs. $AQP4^{-/-}$ mice, again there was actually a trend towards an *increase* in swelling in the absence of AQP4. This provides more evidence that AQP4 may be more important for limiting astrocyte swelling rather than as a water influx pathway.

The co-transporter NKCC1 and the sodium bicarbonate cotransporter NBCe1 are not responsible for astrocyte swelling in high $[K^+]_o$

Previous work suggests that the electroneutral ion co-transporter NKCC1 plays a major role as an astrocyte K^+ influx pathway coupled to cellular volume changes (Macvicar et al, 2001; Su et al, 2002; Jayakumar & Norenberg, 2010). NKCC1 is especially intriguing not only because of its role as an ion cotransporter, but also because it has been shown to exhibit water permeability (MacAulay & Zeuthen, 2012). Therefore, we decided to see if there was any contribution of NKCC1 to the astrocyte swelling in high $[K^+]_o$ using the NKCC1 inhibitor bumetanide. 10 µM bumetanide had no effect on astrocytic swelling in elevated $[K^+]_o$ (Figure 5), arguing against a role for NKCC1 on astrocyte swelling in elevated extracellular potassium.

Next, we considered the possibility that a flux of bicarbonate ions induced by astrocyte depolarization (Larsen & MacAulay, 2017)could be responsible for high $[K^+]_o$ -induced astrocyte swelling, as this has previously been demonstrated in astrocytes (Florence et al, 2012). In order to determine the contribution of the sodium bicarbonate transporter (NBCe1), we utilized DIDS (300 µM) as an antagonist. This also demonstrated no appreciable effect on astrocyte swelling in our conditions (Figure 6).

Barium is known to nonselectively block K^+ channels at concentrations of 1 mM or higher (Benham et al, 1985; Miller et al, 1987). Therefore, to look for a general role of astrocytic K^+ channels, experiments were performed using 1 mM $BaCl_2$. Co-application

of 1 mM Ba^{2+} in high $[K^+]_o$ significantly reduced astrocyte swelling to about 2% above baseline at five minutes, compared to around 6% in high $[K^+]_o$ alone (Figure 7; After 5 minutes in elevated potassium $p < 0.01$ n =7 cells for $BaCl_2$ group, n=9 cells for control group). As 1 mM $BaCl_2$ is non-selective, it is not possible to narrow down any specific K^+ channel(s) that most significantly contribute to astrocytic volume increases.

Astrocyte swelling in elevated $[K^+]_o$ is reduced by the Na^+/K^+ ATPase inhibitor ouabain

Previous work has reported an important role for the sodium-potassium pump as a K^+ influx pathway in astrocytes (Larsen et al, 2014).The Na^+/K^+ ATPase normally pumps potassium into the cell and sodium outside, against their concentration gradients. It has been shown that the astrocyte-specific α2/β2 subunit isoform of the Na^+/K^+ pump is particularly sensitive to changes in extracellular K^+, which would make it uniquely suited to removing excess extracellular potassium (Larsen et al., 2014). Therefore, pumping of K^+ into astrocytes may be associated with the astrocyte volume increases we observe in high $[K^+]_o$. To test for a role of the Na^+/K^+ ATPase in high $[K^+]_o$-induced astrocyte swelling, experiments were performed using the Na^+/K^+ ATPase inhibitor ouabain. We opted for a concentration of 50 µM, which exhibits a significant yet incomplete block, as higher ouabain concentrations have been shown to negatively affect slice health (Larsen et al., 2015). In the presence of ouabain, astrocyte swelling was significantly reduced to levels very similar to what was observed in 1 mM $BaCl_2$ (Figure 7; After 5 minutes in elevated potassium, $p<0.01$ n=5 for ouabain group, n=9 for control group). These data

suggest that astrocyte swelling in conditions of elevated extracellular potassium is largely due to activity of the NA^+/K^+ ATPase, as even a partial block induced a robust reduction in swelling.

High $[K^+]_o$ increases neuronal excitability when neuronal action potentials are blocked

After demonstrating selective swelling of astrocytes in elevated extracellular potassium, we next attempted to determine effects on neuronal excitability. We used whole-cell patch clamp electrophysiology to record excitatory currents in CA1 pyramidal neurons as described previously (Lauderdale et al., 2015) in normal vs. 10.5 mM $[K^+]_o$ ACSF. In these conditions, Mg^{2+}-free ACSF is used to remove Mg^{2+} block from NMDA receptors, and neuronal action potentials are blocked by addition of TTX to prevent direct depolarizing effects of high $[K^+]_o$ on neuronal spiking. Mixed neuronal AMPA/NMDA mEPSCs were recorded before, during, and after high $[K^+]_o$ application, which was performed twice as previously for cell volume measurements. The overall frequency and amplitude of mEPSCs was elevated in high $[K^+]_o$ compared to the baseline and wash periods (Figure 8). These findings suggest that either elevated $[K^+]_o$ is having direct effects on neuronal synaptic transmission even in the absence of firing, and/or that astrocyte swelling is contributing to the neuronal excitability increases in high $[K^+]_o$.

Astrocytes selectively shrink in hyperosmolar ACSF

In previous work we were surprised to find that application of hypoosmolar ACSF nonselectively swells both neurons and astrocytes (Murphy et al. 2017). Those findings

prompted us to ask if the opposite was also true: That application of *hyper*osmolar ACSF (rACSF) would shrink neurons and astrocytes to a similar extent. To this end, we increased the osmolarity of the ACSF by 40% by addition of mannitol and performed real-time volume measurements of astrocytes and neurons. Unlike the effects of hACSF, rACSF was very selective to astrocytes, producing a significant reduction in astrocyte volume compared to neuronal volume decrease, in a near mirror-image to the volume increases that were observed in hACSF (Figure 9; after 5 minutes in rACSF, $p<.01$, n=5 cells for astrocytes, n=8 cells for neurons). Neurons, on the other hand, steadfastly maintained their volume, only showing a reduction of up to ~1%. The reason for this difference is not clear, but may be due to a more rigid cytoskeleton of neurons compared to astrocytes (Lu et al, 2004; Stiess & Bradke, 2010). Nevertheless, the observation that rACSF selectively shrinks astrocytes provided us with a useful tool to test the effects of selective astrocyte volume reduction on high $[K^+]_o$-induced swelling and effects on neuronal excitability.

Neuronal excitability increases in high $[K^+]_o$ are blocked when astrocyte volume is selectively returned to baseline by addition of mannitol

Finally, we wanted to be able to exert temporal control over astrocyte volume changes while in elevated $[K^+]_o$, to distinguish between effects on neurons due to astrocyte swelling vs. direct effects of elevated $[K^+]_o$. Therefore, instead of return to normal $[K^+]_o$ ACSF after 10.5 $[K^+]_o$ application ("wash"), we added mannitol in the continued presence

of 10.5 mM K^+ in order to reverse the high $[K^+]_o$-induced astrocyte swelling. In these conditions, astrocyte volume recovered to baseline levels even in the continued presence of elevated potassium. Astrocyte volume reduction was reversible upon removal of mannitol, and was repeatable upon a successive application (Figure 10). In this manner, we developed a unique approach to selectively manipulate astrocyte volume in elevated potassium, which allowed us to define the impact of astrocyte swelling on neuronal excitability independently of direct effects of high $[K^+]_o$ on neurons. To our surprise, selectively shrinking astrocytes in high $[K^+]_o$ profoundly reduced neuronal excitability. The following specific effects were observed: 1) Slow inward currents evoked in high $[K^+]_o$ were completely suppressed in high $[K^+]_o$ + mannitol; 2) there was a general decrease in excitability in high $[K^+]_o$ + mannitol as evidenced by a reduction in both the amplitude and frequency of mEPSCs compared to high $[K^+]_o$ alone; 3) application of mannitol produced a positive shift in the holding current, indicative of a hyperpolarizing effect of mannitol on the resting V_m; and 4) residual events observed during the "wash" period in nACSF after application of high $[K^+]_o$ were completely abolished by mannitol (Figure 11). These findings suggest a significant contribution of astrocyte swelling to neuronal excitability increases in high $[K^+]_o$.

Figures

Figure 1.

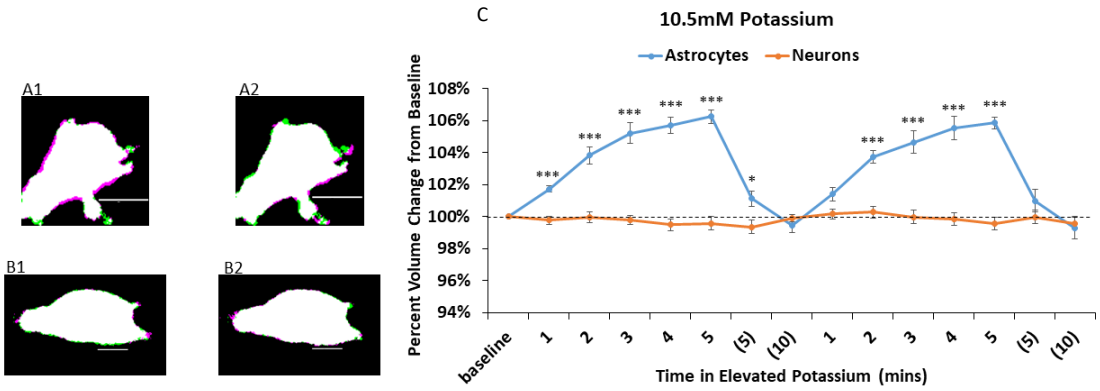


Figure 1: Elevated Potassium Selectively Swells Astrocytes. (A) Representative images of astrocytes labeled with SR-101 5 mins after 10.5 mM potassium ACSF exposure (A1), and after a 10 minute wash period in control (2.5 mM K^+) ACSF (A2). (B) Representative images of neurons labeled with eGFP from Thy-1 mice in the same conditions described for astrocytes. Each image overlays the green baseline image in normal ACSF, with swelling represented by the magenta outline. Astrocytes clearly swelled after 5 minutes of exposure to 10.5 mM K^+ ACSF (A1), and largely returned to baseline volume after a 10 minute wash period in normal ACSF (A2). In contrast, neurons showed little change after 5 minutes of exposure to 10.5 mM K^+ ACSF (B1). Scale bars for images are equal to 5µm. (C) Quantification of astrocyte and neuron soma volume changes upon repeated exposure to 10.5 mM K^+ ACSF as percent change over baseline, with baseline volume set as 100%. Wash periods are labeled as (5) and (10), representing successive 5 and ten minute periods in control 2.5 K^+ mM ACSF, respectively. Astrocytes significantly swelled up to 6% above baseline volume after five minutes in elevated potassium, and recovered to baseline volume after a ten minute wash period in normal ACSF. *p<.05, **p<.01, ***p<.001 between astrocytes (n = 9) and neurons (n = 7) at given time points.

Figure 2.

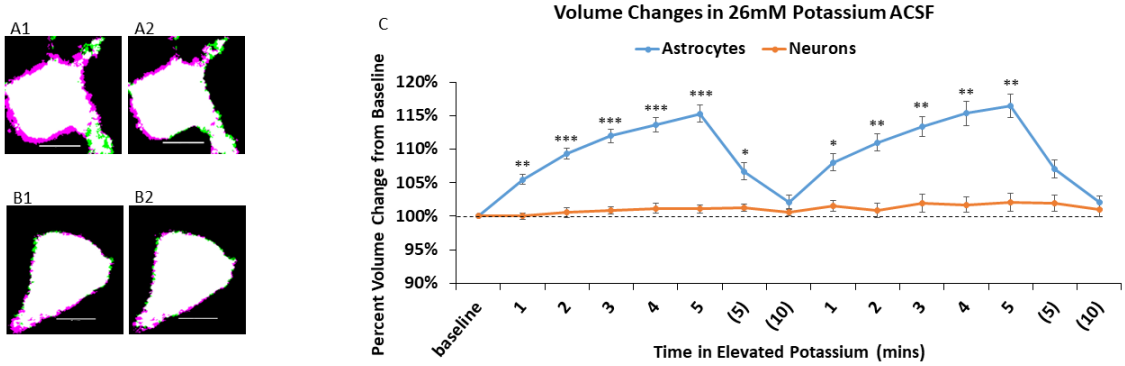


Figure 2: Potassium at 26 mM Swells Astrocytes to 15% Above Baseline Volume, but has Minimal Effect on Neurons. (A) Representative images of an astrocyte five minutes after application of 26 mM K^+ ACSF (A1), and after a ten minute wash period in control (2.5 mM K^+) ACSF (A2). Volume changes are evident after five minutes in elevated potassium, shown as a magenta outline around the cell soma. Astrocyte volume increase returns nearly to baseline after a five minute wash period in control ACSF (A2). (B) Representative images of a neuron at the same points as the astrocyte in (A). There is little observable swelling after five minutes in 26 mM $[K^+]_o$, in contrast to the astrocyte. Scale bars for images are equal to 5µm. (C) Quantitative representation of both astrocyte and neuron volume as percent change from baseline. Astrocytes swell to about ~15-16% above baseline after five minutes in 26 mM $[K^+]_o$, while neurons are affected only minimally, a ~1-2% change above baseline volume. *p<.05, **p<.01, ***p<.001 between astrocytes (n=7) and neurons (n=3) at each time point.

Figure 3.

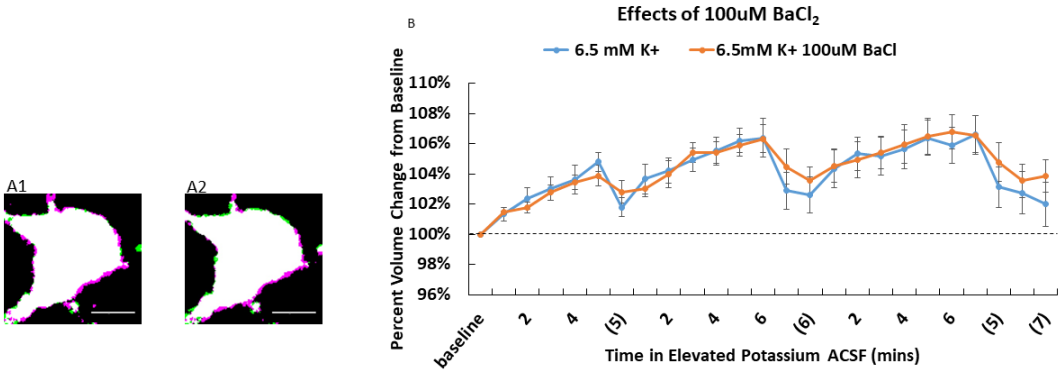


Figure 3: Kir4.1 is not Involved in Astrocyte Swelling in Elevated $[K^+]_o$: (A) Representative image of an astrocyte five minutes after 10.5 mM K^+ ACSF application (A1), and after a five minute wash period in control (2.5 mM K^+) ACSF (A2). $BaCl_2$ (100 µM) was applied 3 minutes prior to 6.5 mM $[K^+]_o$ application, and remained in the ACSF throughout the experiment. The volume change after five minutes in 10.5 mM potassium is readily apparent (A1), as demonstrated by the magenta outline surrounding the soma. Volume increase did not fully recover to baseline during a shortened five minute wash period. Scale bars for images are equal to 5µm. (B) Quantification of cell soma volume changes as percent change from baseline, which is set at 100%. In both conditions, astrocytes swell to ~5-7% above baseline with and without the presence of $BaCl_2$, suggesting that Kir4.1 does not contribute to astrocyte swelling in elevated potassium. There was no statistically significant interaction between these conditions over time. (n=7 for $BaCl_2$ group, n=9 for control).

Figure 4.

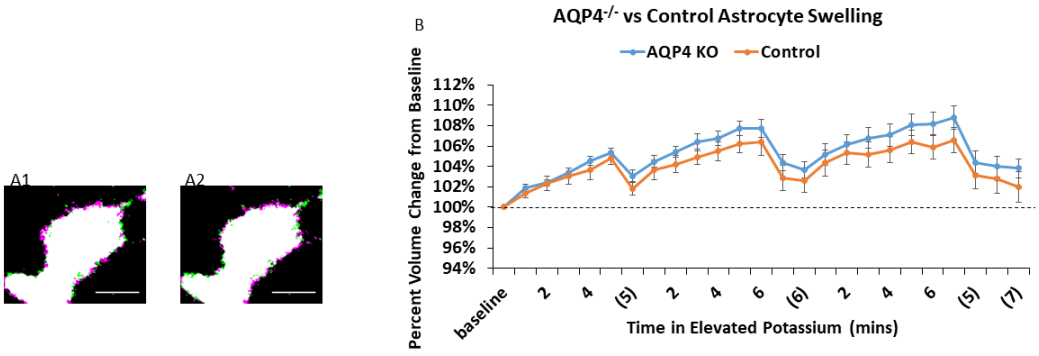


Figure 4: Astrocytes Swell to a Similar Volume in AQP4$^{-/-}$ Mice (A) Representative images of an astrocyte from an AQP4$^{-/-}$ mouse five minutes after application of 6.5 mM K^+ ACSF (A1), and after a five minute wash period in control (2.5 mM K^+) ACSF (A2). Volume changes are evident after five minutes in 6.5 mM potassium, shown as a magenta outline around the cell soma (white) representing baseline volume. The volume increase partially recovered to baseline during a five minute wash period in control ACSF (A2). Scale bars for images are equal to 5µm. (B) Quantification of Astrocyte soma volume changes as percent change over time, with baseline set at 100%. Steady volume increase is evident during application of 6.5 mM $[K^+]_o$ ACSF in both control (red) and AQP4$^{-/-}$ (blue) astrocytes. Overall, there was no significant interaction between the two groups, indicating a lack of a role for AQP4 in astrocyte swelling in elevated potassium. (n=8 for AQP4 KO mice, n=8 for controls).

Figure 5.

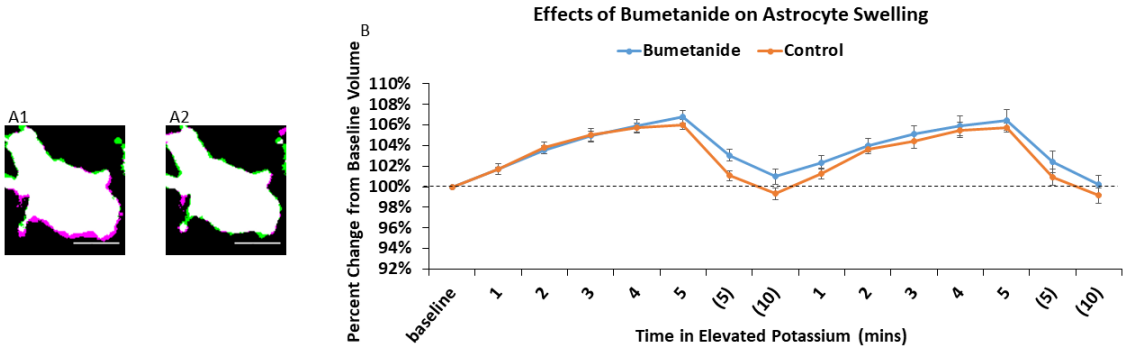


Figure 5: The NKCC1 Inhibitor Bumetanide has no Effect on Astrocyte Swelling (A) Representative images of an astrocyte five minutes after application of 10.5 mM K^+ ACSF (A1), and after a ten minute wash period in control (2.5 mM $[K^+]_o$) ACSF (A2). Bumetanide (10 µM) was applied 3 minutes prior to the first application of 10.5 mM $[K^+]_o$ and remained throughout the experiment. Volume increases are evident after five minutes in elevated potassium, indicated by a magenta outline around the cell soma volume measured at baseline (white). Astrocyte volume recovers to baseline after a five minute wash period in control ACSF (A2). Scale bars for images are equal to 5µm. (B) Quantitative representation of astrocyte volume as percent change over baseline, with baseline set at 100%. Astrocytes swell to ~6-7% above baseline in the presence of bumetanide after five minutes in elevated potassium, while in the absence of bumetanide, they swell to ~6% above baseline. There was no significant interaction between the time and condition, indicating that bumetanide had no significant effect on astrocyte swelling. (n=9 for bumetanide, n=9 for control).

Figure 6.

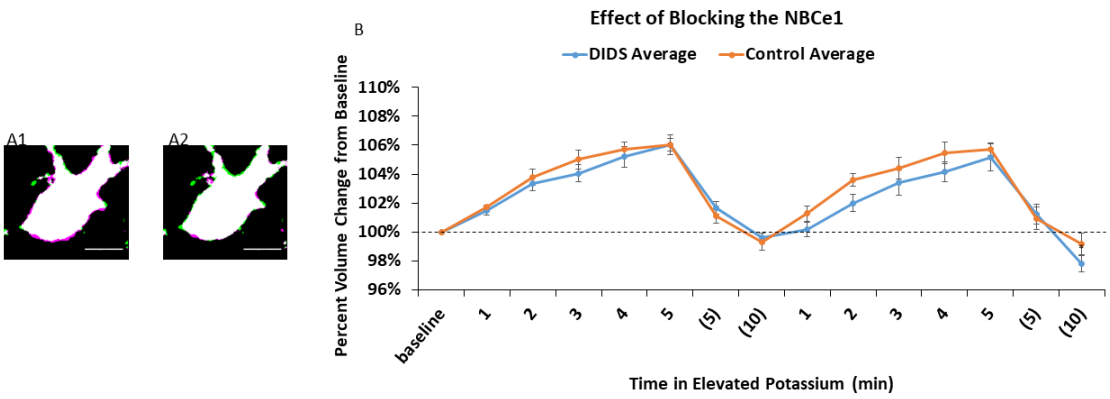


Figure 6: The NBCe1 Antagonist DIDS has no Effect on Astrocyte Swelling (A) Representative images of an astrocyte five minutes after application of 10.5 mM K^+ ACSF (A1), and after a five minute wash period in control ACSF (A2). DIDS (300 µM) was applied 3 minutes prior to the first 10.5 mM $[K^+]_o$ application and remained in the ACSF throughout the experiment. Volume changes are evident after five minutes in elevated potassium, shown as a magenta outline around the cell soma baseline volume (shown in white) in A1. Astrocytes recovered to baseline volume after a five minute wash period in control ACSF (A2). Scale bars for images are equal to 5µm. (B) Quantification of Astrocyte soma volume increase as percent change over time, with baseline set at 100%. Astrocytes swell to ~6% above baseline after five minutes in 10.5 mM K^+ ACSF, and recover to baseline following ten minutes in control ACSF. DIDS does not negate any astrocyte swelling, and there was no significant interaction between time and condition. (n=6 for DIDS, n=9 for control).

Figure 7.

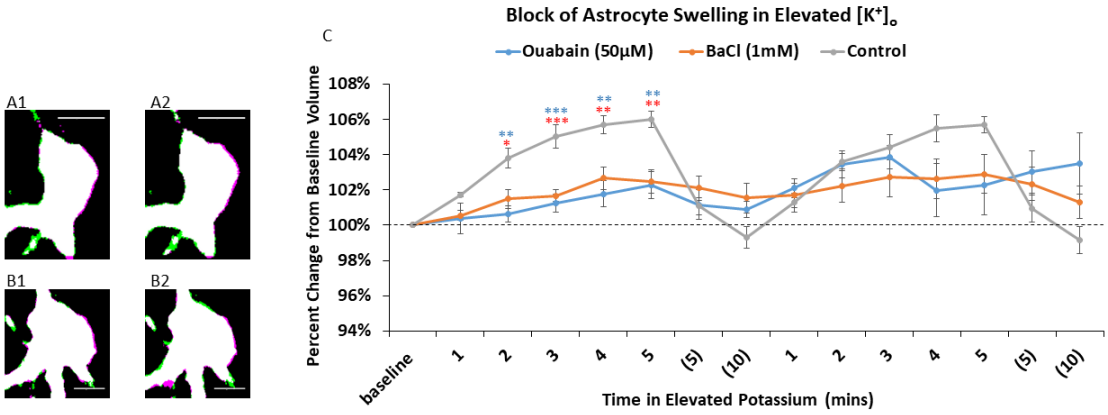


Figure 7: Non-specific Block of K^+ Channels or The Na^+/K^+ Pump Significantly Reduces Astrocyte Swelling (A) Representative images of an astrocyte five minutes after application of 10.5 mM K^+ ACSF with 50 µM ouabain (A1), and after a ten minute wash in control ACSF (A2). Volume changes are minimal after five minutes in elevated potassium, shown as a magenta outline around the cell soma. The small volume increase did not recover fully to baseline after a five minute wash in control ACSF (A2). (B) Representative images of an astrocyte five minutes after application of 10.5 mM K^+ ACSF with 1mM $BaCl_2$ (B1), and after a ten minute wash in control ACSF (B2). Volume changes are similar to what was observed in ouabain, shown as a magenta outline around the cell soma baseline volume (white). Astrocyte volume recovers close to baseline after a ten minute wash period in control ACSF. Scale bars for images are equal to 5µm. (C) Quantification of soma volume for astrocytes in either the control condition (gray), with ouabain (blue), or with $BaCl_2$ (red). Astrocytes swell up to ~6% in the control condition after five minutes in 10.5 mM K^+ ACSF, whereas in the presence of ouabain or $BaCl_2$, the volume increases only up to ~2-3%. *p<.05, **p<.01, ***p<.001. Blue asterisks represent differences between ouabain and control, and orange asterisks represent differences between $BaCl_2$ and control. (n=5 for ouabain, n=7 for $BaCl_2$, n=9 for control).

Figure 8.

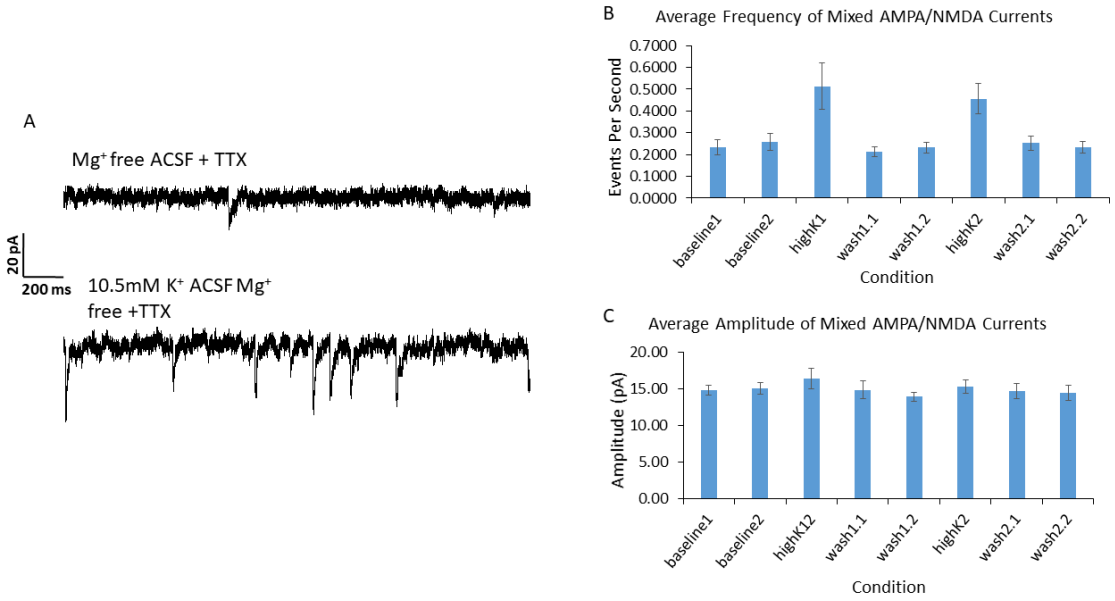


Figure 8: Effects of 10.5 mM K^+ ACSF on Neuronal Excitability in TTX. (A) A representative portion of a trace showing mixed AMPA/NMDA mEPSCs recorded from a CA1 pyramidal neuron during whole-cell patch clamp electrophysiology, voltage clamped at -70 mV. The cell remained in Mg^+-free ACSF + TTX for the entirety of the recording. On top is the baseline recording in Mg^+-free control ACSF + TTX prior to application of 10.5 mM K^+ ACSF. Below, the application of 10.5 mM $[K^+]_o$ produced a noticeable increase in the frequency mEPSCs, suggesting an overall increase in neuronal excitability. (B) Quantification of frequency of mixed AMPA/NMDA mEPSCs as events per second. (C) Quantification of amplitude of mixed AMPA/NMDA mEPSCs. (n=11 full recordings spanning all conditions).

Figure 9.

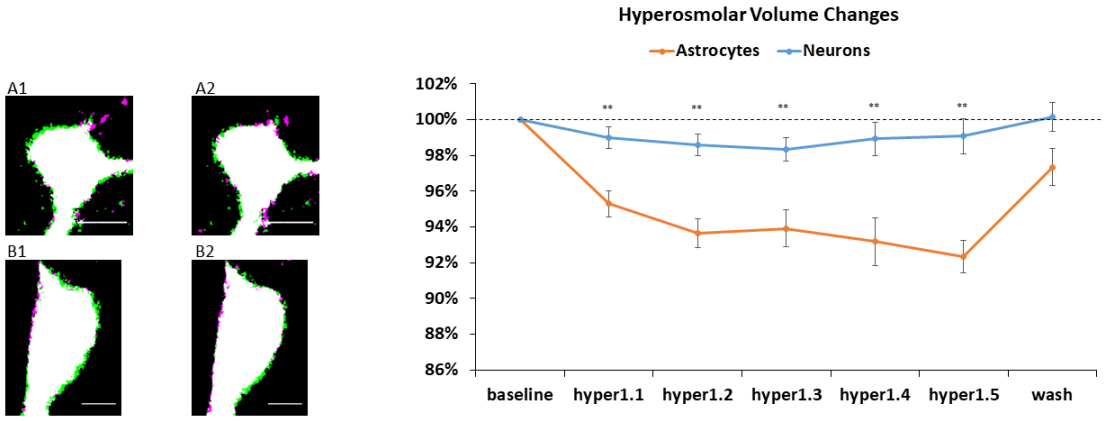


Figure 9: Hyperosmolar ACSF Shrinks Astrocytes Significantly More Than Neurons. (A) Representative images of an astrocyte five minutes after application of 40% hyperosmolar ACSF by addition of mannitol (A1), and after a five minute wash period in normosmolar ACSF (A2). A decrease in cell volume is evident in mannitol (A1), shown as a green outline around the cell soma baseline volume image (white). The cell recovers nearly to baseline volume after a five minute wash period in normosmolar ACSF without mannitol (A2). (B) Representative images of a neuron at the same time points as the astrocyte. There is slight shrinking evident after 5 minutes in mannitol (B1), with volume recovery to baseline after a five minute wash period in normosmolar ACSF (B2). Scale bars for images are equal to 5µm. (C) Quantification of astrocyte and neuron soma volume as percent change over time, with baseline set at 100%. Astrocytes shrink by ~8% below baseline after five minutes, and recover close to baseline following a five minute wash period, whereas neurons shrink by 1-2% over the same period with full recovery to baseline after five minutes in normosmolar ACSF. There was a significant interaction between time and condition.*p<.05, **p<.01, ***p<.001. (n=5 for astrocytes, n=8 for neurons).

Figure 10.

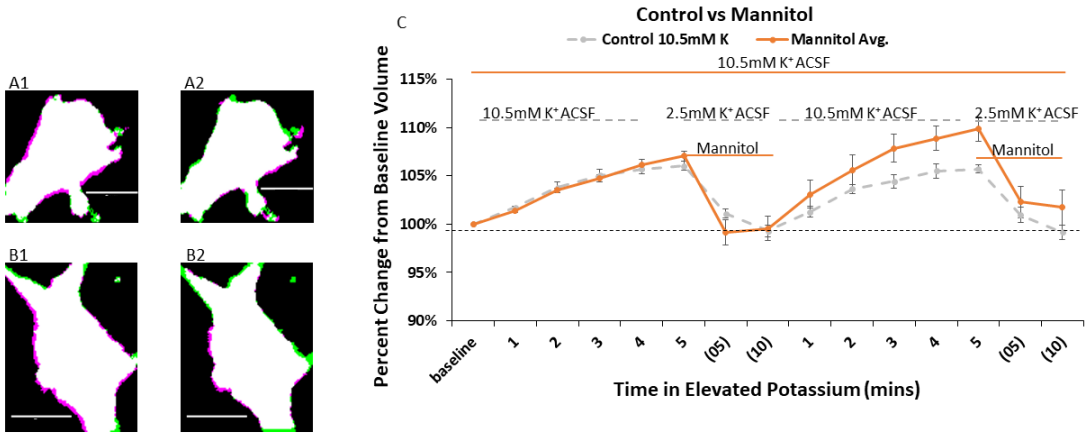
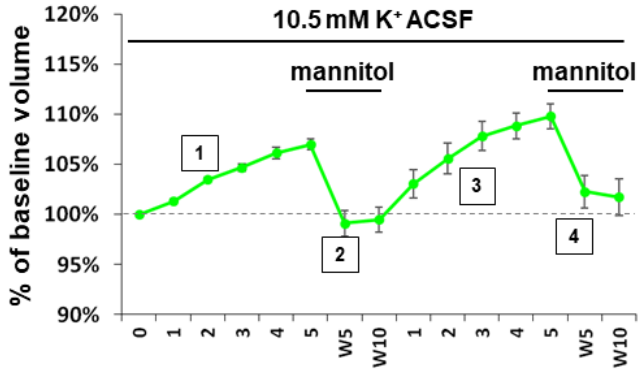


Figure 10: 40% Hyperosmolar ACSF Effectively Negates Elevated $[K^+]_o$-driven Astrocyte Volume Increases. (A) Representative images of an astrocyte five minutes after application of 10.5 mM K^+ ACSF (A1), and after a ten minute wash period in control ACSF (A2). Volume changes are evident after five minutes in elevated potassium, shown as a magenta outline around the baseline cell soma volume (white). (B) Representative images of an astrocyte five minutes after application of 10.5 mM K^+ ACSF (B1), and after ten minutes in 10.5 mM $[K^+]_o$ + 40% hyperosmolar ACSF by mannitol (B2). Mannitol completely reverses the cell volume increases due to high $[K^+]_o$. Scale bars for images are equal to 5µm. (C) Quantification of Astrocyte soma volume as percent change over time, with baseline set at 100%. Mannitol effectively shrinks astrocytes back to baseline volume even in the continued presence of 10.5 mM K^+ ACSF. Gray dotted line shows effect of 10.5 mM $[K^+]_o$ application followed by wash in control (2.5 mM $[K^+]_o$) ACSF for comparison. Overall, there was no significant interaction between the two conditions, although the mannitol group had a tendency to swell more upon the 2nd 10.5 mM K^+ ACSF application when compared to using control ACSF during the wash.

Figure 11.

A


10.5 mM K+ ACSF
mannitol
mannitol
% of baseline volume
120%
115%
110%
105%
100%
95%
90%
1
2
3
4
0
1
2
3
4
5
W5
W10
1
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3
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W5
W10

B

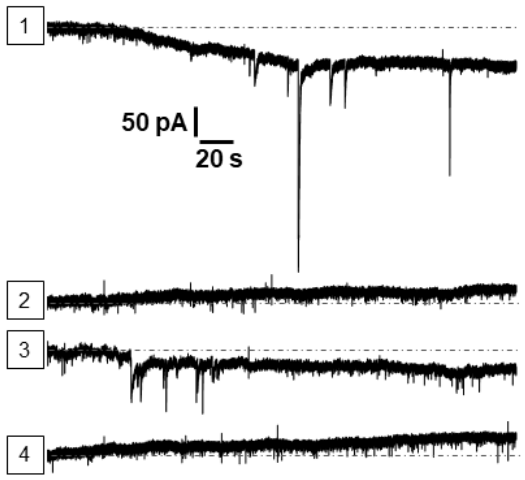
1
50 pA
20 s
2
3
4

Figure 11: Forcing Astrocytes to Shrink to Baseline Volume in the Continued Presence of 10.5 mM $[K^+]_o$ Diminishes Neuronal Excitability. (A) Quantitative representation of astrocyte volume in 10.5 mM K^+ ACSF (Mg^{2+} free with TTX) for thirty minutes, with two 10 min applications of 40% hyperosmolar ACSF by addition of mannitol (as in Fig. 10). Numbers 1-4 are representative periods of the experiment, matched to the same conditions and time periods in the electrophysiology recording in (B). (B) A recording demonstrating mixed AMPA/NMDA inward currents recorded from a CA1 pyramidal neuron during whole cell patch clamp electrophysiology, voltage clamped at -70 mV. TTX was included in the bath to prevent direct depolarizing effects of elevated $[K^+]_o$ on neuronal firing. (1) The first application of 10.5 mM K^+ ACSF evokes large inward currents in neurons. (2) Addition of mannitol to shrink astrocytes back down to baseline volume results in obvious depression of neuronal EPSCs and produces a hyperpolarizing shift in the holding current. (3) Removal of mannitol allows astrocytes to re-swell in 10.5 mM $[K^+]_o$, which corresponds to a return of neuronal EPSCs and depolarization. (4) Re-application of mannitol forces astrocytes to shrink and once again strongly dampens neuronal excitability as evidenced by an absence of EPSCs and a hyperpolarizing shift in the holding current. These findings indicate that the increases in neuronal excitability are tightly linked to astrocyte volume, and not simply a product of direct elevated potassium effects on synaptic transmission.

Discussion:

In these studies, we found that elevated extracellular potassium (6.5, 10.5, or 26 mM) causes rapid (within 1 minute) and significant astrocyte swelling, up to ~6, 7, and 15% above baseline volume respectively after five minutes, while neuronal volume remained completely unchanged. Astrocytes recovered to baseline volume within ten minutes in normal 2.5mM K^+ ACSF, and swelled to a similar extent upon re-application of ACSF with elevated $[K^+]_o$. Using the higher 10.5 mM K^+ concentration, astrocyte volume increases were significantly inhibited, and to a similar extent, by the nonselective K^+ channel blocker $BaCl_2$ and the Na^+/K^+ ATPase inhibitor ouabain. Inhibitors of other astrocytic channels and cotransporters often implicated in astrocyte swelling - including Kir4.1, AQP4, NKCC1, and NBCe1 - had no effect. Application of K^+ in the presence of TTX depolarized neurons, evoked slow inward currents, and produced an increase in mixed AMPA/NMDA mEPSCs, all of which were significantly inhibited when astrocyte swelling was selectively negated in the continued presence of high $[K^+]_o$. Overall, these experiments suggest that the Na^+/K^+ ATPase plays an important role in sequestering extracellular K^+ by pumping it into astrocytes as $[K^+]_o$ rises, producing an inward osmotic gradient and astrocyte swelling. In addition, our findings suggest that astrocyte volume increases significantly contribute to the increased neuronal excitability that occurs in elevated $[K^+]_o$ conditions.

Somewhat surprisingly, even at a concentration of 26 mM, elevated $[K^+]_o$ did not affect neuronal volume at all. It has been shown previously that very high levels of extracellular potassium can cause spreading depression (Gardner-Medwin, 1981; Obrenovitch & Zilkha, 1995; Gorji, 2001), and spreading depression has been shown to lead to neuronal swelling (Takano et al, 2007). The explanation for why we have not observed neuronal swelling in 26mM K^+ is most likely because we are not inducing spreading depression. A few factors may play a role, the first regarding temperature. Many labs induce spreading depression at physiological temperature (Risher et al, 2011; Seidel et al, 2015), but most of our experiments were done at room temperature. However, we did try imaging neurons at 26 mM K^+ at physiological temperature, and still saw very little change in soma volume. There are also multiple publications where spreading depression was induced even at room temperature (Peters et al, 2003; Theis et al, 2003; Zhou et al, 2010), so recording temperature is unlikely to be the cause. Most likely, this has to do with the method of high $[K^+]_o$ application. While we raise the concentration of potassium in the ASCF, then bath apply it to the slice, most studies observing spreading depression have applied potassium more focally. Microfluidic chambers and microinjections have both been used to locally apply increases of potassium to a specific location in the tissue. As spreading depression usually begins in one area, and propagates as a "wave," applying high potassium to a small area of the tissue may facilitate the generation of spreading depression. However, the induction of

spreading depression was not the goal of our studies, and further experiments would be necessary to determine if this is indeed the case.

We found that 1 mM BaCl and 50 µM ouabain exhibited similar effects on astrocyte volume in high $[K^+]_o$. Both of these drugs reduced the swelling of astrocytes to about two percent above baseline after five minutes in 10.5 mM $[K^+]_o$ ACSF, as opposed to nearly six percent in high $[K^+]_o$ alone. Although the effects of 1 mM BaCl are supportive of a role for an unidentified K^+ channel, $BaCl_2$ at this concentration has been reported to nonselectively inhibit the Na^+/K^+ pump (Walz et al, 1984). Because the $BaCl_2$ effects mimicked so closely those obtained using ouabain, our current interpretation is that $BaCl_2$ effects are due to its off target action on the Na^+/K^+ ATPase. It is noteworthy that fifty µM ouabain only induces a partial block of the Na^+/K^+ ATPase. At higher concentrations it would likely reduce potassium-induced swelling in astrocytes in a dose-dependent manner. However, the Na^+/K^+ ATPase is critically important for cell survival by pumping sodium and potassium across the cell membrane against their concentration gradients. It was previously demonstrated that a concentration of 50 µM was sufficient to block the α2 and α3 subunits of the Na^+/K^+ ATPase, while also partially blocking the α1 subunit, while higher concentrations were considered detrimental to slice viability (Larsen et al, 2014). Therefore, we opted for the 50 µM concentration to produce a significant block, while maintaining slice viability.

Potassium induced astrocyte swelling has been attributed to many different channel, pump, and transporter subtypes over the years. Early findings supported a role for NKCC1 in elevated potassium mediated astrocyte swelling (Su et al, 2001; Macvicar et al, 2001; Vázquez-Juárez et al, 2009). In many of these studies, the NKCC inhibitor bumetanide significantly attenuated astrocyte volume increases and reduced swelling-evoked release of osmolytes from astrocytes. However, most of these studies, were done in culture, and in the presence of up to 75 mM potassium. This is in contrast to our results in acute hippocampal slices, and while our elevated potassium models ranged from 6.5 - 26 mM, the majority of experiments were done at 10.5 mM. These key differences could provide an explanation for the differences in contribution of NKCC1 to potassium mediated astrocyte swelling. Our findings are supported by those of Larsen et al. (Larsen et al, 2014), who found that while NKCC1 was important for swelling of cultured astrocytes, it did not contribute to swelling of astrocytes in intact tissue slices.

Another target traditionally associated with potassium buffering and associated volume changes is Kir 4.1. Multiple studies have demonstrated morphological and functional association with aquaporin 4 (AQP4), a glial specific water channel (Wen et al, 1999; Nagelhus et al, 1999, 2004;Manley et al, 2000; Wurm et al, 2006; Strohschein et al. 2011). The working model, which has been supported by some findings, is that influx of potassium through Kir 4.1 leads to corresponding water influx through AQP4, leading to glial swelling. We found that block of Kir 4.1 by 100 µM BaCl had no effect on astrocyte swelling triggered by elevated potassium. Also, using AQP4 knockout mice, we actually

observed a trend toward an *increase* in astrocyte swelling. This is consistent with reports that genetic deletion of Kir 4.1 produced no change in stimulus-evoked shrinkage of the extracellular space (ECS). Deletion of AQP4 actually *increased* ECS shrinkage (Haj-Yasein et al, 2011; Haj-Yasein et al, 2012), supporting a role for AQP4 in water efflux. Finally, it was recently shown that a partial block of the Na^+/K^+ ATPase led to a significant reduction in post-stimulus potassium removal from the extracellular space, whereas block of Kir 4.1 had no effect on post-stimulus potassium removal (Larsen et al, 2014). Our data strongly support the role of the Na^+/K^+ ATPase as the main contributor to potassium influx into astrocytes, and the subsequent astrocyte swelling exhibited in elevated extracellular potassium conditions. We propose that the main role of Kir4.1 and AQP4 is redistribution or removal of K^+ ions and water from the astrocyte either into the vasculature or areas of lower activity, not the influx of K^+ ions and water into astrocytes. This model is supported both by the subcellular polarized distribution of Kir4.1 and AQP4 at astrocytic endfeet and by our data together with the recent findings of Larsen et al (2014) and Murphy et al. (2017).

Finally, an important goal was to try to determine if $[K^+]_o$-induced astrocyte volume changes have any effect on neuronal excitability. We previously used a hypoosmolar model of cerebral edema to examine the acute effects of cell swelling on neuronal activity (Lauderdale et al, 2015; Murphy et al, 2017). Within 30s of cell swelling, a burst of NMDA receptor-mediated slow inward currents occurred, correlating with increased action potentials and burst firing (Lauderdale et al, 2015). However, although the

hypoosmolar model had previously been reported to swell only astrocytes due to selective expression of the water channel AQP4 (Manley et al, 2000; Solenov et al, 2004; Risher et al, 2009), we later found that neurons swell to a similar extent as astrocytes (Murphy et al, 2017). Therefore, it was not possible to define the specific contribution of astrocyte swelling to the neuronal excitability increases we observed. An obvious advantage provided by the high $[K^+]_o$ model is that it swells astrocytes selectively, offering an alternative approach to determine effects of astrocyte swelling on neuronal excitability. This is a particularly challenging endeavor however, given direct effects of elevated $[K^+]_o$ on the neuronal resting potential, shifting neurons closer to, and above, threshold for generating action potentials (Xie et al. 2014). However, a few pieces of evidence support the idea that we have successfully isolated effects of astrocyte swelling on neuronal excitability in our studies. First, recordings were done in TTX, eliminating effects of high $[K^+]_o$ on neuronal firing. Second, we performed Ca^{2+} imaging experiments to test for activation of voltage-gated calcium channels (VGCCs) that might nevertheless be activated by depolarization. Voltage-step protocol demonstrated VGCC activation between the -60 and -40 mV voltage steps. Depolarization from 10.5 mM K^+ in TTX did not evoke Ca^{2+} elevations in neurons, suggesting that in the absence of firing neurons did not depolarize sufficiently to activate VGCCs. Third, selectively reversing astrocyte swelling back down to baseline volume in the *continued* presence of high $[K^+]_o$ completely abrogated effects on neuronal excitability, a strong indication that these effects are driven by astrocyte swelling, not by high $[K^+]_o$ on neurons.

If astrocytes are indeed causing increased neuronal excitability in the presence of elevated potassium, then by what mechanism does it occur? It has been demonstrated that astrocyte swelling can cause the opening of volume regulated anion channels (VRAC), which are permeable to anions and other osmolytes including glutamate (Kimelberg et al, 1990; Rutledge et al, 1996, Mongin & Kimelberg, 2002). Therefore astrocyte swelling in elevated potassium could lead to the release of glutamate and other osmolytes from VRAC, exciting local neurons through NMDA receptor activation. VRAC have been fervently studied in recent years, and it was recently found that leucine-rich repeat containing protein 8A (LRRC8A) is a necessary subunit for VRAC mediated release of glutamate (Hyzinski-García et al, 2014). This makes VRAC, or perhaps more specifically LRRC8A, an interesting target for future studies to determine the contribution of VRAC to astrocyte glutamate release in elevated extracellular potassium. Aside from release via VRAC, it is also possible that astrocyte swelling simply shrinks the extracellular space, elevating ambient glutamate concentrations sufficiently to activate high-affinity NMDA receptors, causing an increase in excitability. It is also possible that both of these mechanisms make significant contributions to the evident changes in neuronal excitability seen in our model, making them intriguing subjects for future study.

Overall this study contributes new evidence that astrocytes exhibit a tremendous influence on neuronal activity. We have shown that (patho)physiologically relevant changes in the concentration of potassium in the extracellular space selectively swells

astrocytes, which may increase the excitability of neurons within the hippocampus. This could have profound implications for a variety of disease models, many of which manifest with changes in potassium levels in the CNS. These changes very likely result in astrocyte volume fluctuations, non-synaptically elevating neuronal excitability which could contribute to the generation of seizure activity and excitotoxicity. Epileptiform bursting observed in previous studies occurred in 8.5 mM $[K^+]_o$, was NMDA receptor-dependent, and required constriction of the extracellular space (Traynelis & Dingledine, 1988). The presence of slow inward currents (SICs), which have been discussed as synchronizing events that raise the excitability of neuronal populations (Wetherington et al, 2008), are particularly intriguing. Although their specific link to seizure activity including interictal or ictal spikes has yet to be elucidated, their TTX insensitivity, NMDA receptor dependency, and slow kinetics have linked them to astrocytes for some time (Perea & Araque, 2005; Carmignoto & Felling, 2006; Fiacco et al, 2007; Lauderdale et al, 2015). In the future, it would be prudent to continue to search for mechanisms to selectively swell astrocytes, in order to isolate astrocyte swelling from conditions that could have additional effects on neurons. It would also be useful to further investigate if swollen astrocytes are indeed the source of glutamate that leads to increased neuronal excitability. Techniques to isolate VRAC activity in astrocytes would be very important, as VRAC is a likely culprit to release glutamate in conditions of swelling. The development of an astrocytic VRAC knockout mouse would be a very advantageous tool to develop. Also, as hemichannels could also be a potential source of glutamate from

astrocytes in conditions favorable for swelling, pharmacological techniques should be employed to determine if there is any contributions of connexin or pannexin hemichannels to the evident increase in neuronal excitability. These techniques would continue to further our understanding of astrocyte volume regulation, and the potential consequences for neuronal excitability.

Conclusions and Future Directions:

Although in this study we have found a model to selectively swell astrocytes, the elevated potassium model leads to other potential confounds. Elevated potassium will also strongly depolarize neurons, and although we used mannitol to parse out the specific effects of astrocyte swelling, mannitol may also produce neuron-specific effects. Although mannitol alone induced astrocyte shrinking by up to ~8% below baseline compared to less than 2% for neurons, it is still possible that even that small amount of neuronal shrinking could be responsible for the dampening of neuronal excitability we observed. The lab is continuing to tinker with the mannitol concentration and the composition of the elevated $[K^+]_o$ ACSF in an effort to minimize these effects. For example, it is possible that a solution made 30% hyperosmolar by mannitol would still result in significant astrocyte shrinking with no effect whatsoever on neurons. In addition to this potential confound, multiple studies have shown that shrinking of neuronal presynaptic terminals can lead to calcium independent exocytosis (Capogna et al, 1996; Rosenmund et al, 1996; Waseem et al, 2008). Since we saw about 1% shrinking of the neuronal soma, it remains possible that there is also significant shrinking of the processes, which could trigger vesicular fusion from neurons due to mannitol acting as a "secretagogue" in this manner. However, there are a few ways to determine if this is indeed the case. If we were to incubate our slices in bafilomycin, we could empty the synaptic vesicles of neurotransmitter (Roseth et al, 1995; Fonnum et al, 1998). In this case, if we used the same conditions and still continued to see increases in neuronal

excitability, this would suggest that there is no secretagogue effect, and would implicate astrocyte volume changes as the source for the change in excitability. Also, we will be analyzing the data looking for changes in the characteristics of slow inward currents (SICs). SICs have been shown to be strongly evoked in swelling conditions such as hypoosmolar ACSF, and are thought to be due to extrasynaptic sources of glutamate (Angulo et al, 2004; Fellin et al, 2004; Fiacco et al, 2007; Lauderdale et al, 2015). An increase in the frequency of slow inward currents would suggest this is not due to mannitol induced shrinking of neuronal processes, but instead to extrasynaptic glutamate sources such as astrocytes. It is important to point out here as well that in our conditions (10.5 mM $[K^+]_o$), application of mannitol *reduces* excitability of neurons very significantly. Therefore, any secretagogue effect that may be occurring by mannitol is minimal compared to the effect of mannitol in dampening neuronal excitability. In fact we may be slightly underestimating the effect of astrocyte shrinking on the neuronal excitability reduction due to mannitol's secretagogue function. Fascinatingly, 30 years ago Traynelis and Dingledine (1989) showed that application of sucrose - known to be a powerful secretagogue - completely blocked NMDA receptor-dependent spontaneously-occurring seizures. This points to the importance of cell and extracellular space volume in regulation of network excitability. Even though it is likely that sucrose is increasing vesicular fusion in neurons, seizures stop due to dilation of the extracellular space.

Even if mannitol is not leading to vesicular release from neurons, elevated potassium will depolarize cells, and could have additional unforeseen consequences. In the future,

it would be prudent to utilize new techniques to specifically target swelling to astrocytes without relying on bath-applied solutions. One possibility to be considered is the utilization of optogenetics. Channel rhodopsin is a non-selective cation channel which becomes activated by blue light, which allows mainly sodium ions and protons into the cell (Nagel et al, 2003; Zhang et al, 2006). If water is following osmotic gradients generated by ions moving into the cell through channels or pumps, it is possible that non-selective cation flow into the cell will lead to the accumulation of water as well. Another possible avenue is halorhodopsin, a chloride pump activated by yellow light that leads to a buildup of chloride within the cell, leading to hyperpolarization (Han & Boyden, 2007; Zhang et al, 2007). It has also been demonstrated that activation of this pump leads to swelling of vesicles due to the influx of chloride (Schobert & Lanyi, 1982). If this is the case, it could be possible to target this channel to astrocytes, and the influx of chloride could potentially lead to volume increases. Finally, archaerhodopsin pumps protons out of the cell, and is activated with a green laser (Han et al, 2011). In this case, we would be interested in the effects of pH on cell swelling. Multiple studies have shown that in conditions of elevated potassium, astrocytes undergo intracellular alkalization via uptake of $HCO3^-$ through the sodium bicarbonate cotransporter e1, known as NBCe1 (Florence et al, 2012; Larsen et al, 2017). The intracellular alkalization induced by the outward pump of protons by archaerhodopsin could also induce astrocyte swelling. Any of these optogenetic tools could be expressed in astrocytes using astrocyte-specific promoters.

Another finding that needs to be investigated further is the strong effect of ouabain and 1 mm $BaCl_2$ on astrocyte swelling in elevated potassium conditions. First, while ouabain is a potent antagonist of the Na^+/K^+ pump, 1 mm $BaCl_2$ is mostly used as a broad-spectrum potassium channel blocker. Although the similarity of the data make it seem as though ouabain and $BaCl_2$ are both affecting the same target, it is impossible to be conclusive without additional testing. One experiment would be to co-apply ouabain and $BaCl_2$ together, and determine if there is an additive effect. If we see an additional block of astrocyte swelling (<2% above baseline), it would be indicative of each agent acting on a separate target. One concern with this approach is that co-application of ouabain and $BaCl_2$ together may be further detrimental to slice health, potentially limiting interpretation of the data. Another interesting question revolves around the significant effect of the Na^+/K^+ pump itself. As this is expressed by both astrocytes and neurons, how is it that this pump is responsible for a significant portion of astrocyte swelling in elevated potassium, but neurons don't swell to any significant degree? One possibility revolves around the different isoforms expressed by each cell type. A recent study demonstrated that the α2β2 isoform combination of the Na^+/K^+ pump displays significant voltage sensitivity when expressed in oocytes, and is glia-specific (Larsen et al, 2014). Larsen et al. (2014) postulate that the sensitivity of this isoform to increases in $[K^+]_o$ may explain its role in sequestration of extracellular K^+ in astrocytes, as opposed to the neuronal isoform which is mainly sensitive to increases in intracellular Na^+. Interestingly, this study also showed that with the application of ouabain, K^+ clearance

was indeed hampered, but the ECS actually shrank more, which they took to mean that astrocyte swelling actually *increased* with the application of ouabain in elevated potassium. However, cell volume change was measured indirectly by recording the resistance of the ECS, not by real-time volume measurements of the cells themselves. It is possible that the addition of ouabain may lead to the swelling of neurons or other cell types in the intact tissue preparation, as in our study we saw decreases in astrocyte volume in elevated potassium by 50 µM ouabain. An easy way to test this would be to directly image neuronal volume using Thy-1-eGFP mice once ouabain is applied, which may help clarify the discrepancy between these studies.

Finally, if astrocyte swelling does induce glutamate release leading to hyperexcitability of neurons, how is the glutamate being released? The most likely, and most well-studied culprit would be VRAC, which has been dubbed a “frenemy” within the brain [cite Mongin paper with that title]. VRAC is stretch sensitive, and has been implicated in the release of glutamate and various other ions and neutral molecules (Mongin & Kimelberg, 2002; Kimelberg, 2005; Abdullaev et al, 2006). Recent work has identified the essential pore-forming subunit of VRAC as LRRC8A (Qiu et al, 2014; Voss et al, 2014). With this new development, we are currently developing a model where VRAC can be conditionally removed in adult astrocytes. If conditional removal of VRAC from astrocytes is confirmed, we could then use the same experimental procedure as used in this study, and look at astrocyte swelling and changes in neuronal excitability in elevated potassium. First we would expect to see that astrocytes, and not neurons, continue to

swell in elevated potassium, although perhaps they may not return to baseline as well during the wash if VRAC is responsible for their ability to release ions to return back to baseline. As we saw an increase in neuronal excitability in elevated potassium that was depressed with the application of mannitol, if this were due to astrocytic release via VRAC, we expect this effect to largely disappear in astrocyte VRAC cKO mice. This model would greatly complement use of pharmacological approaches to inhibit VRAC, such as DCPIB, without off-target effects or inhibition of VRAC on other cell types. These mice could be used in numerous other studies to assess the role of astrocyte swelling on neuronal excitability or excitotoxicity, including epilepsy, stroke, and cerebral edema.

Overall, this study demonstrates that selective astrocyte swelling in elevated $[K^+]_o$ is significantly reduced by the application of 50 μM ouabain, indicating an important role for the Na^+/K^+ pump in astrocyte potassium clearance and associated volume changes. We also show that hyperosmolar ACSF (by the addition of mannitol) shrinks astrocytes by about 8% below baseline, compared to a negligible effect on neurons. Astrocyte swelling in elevated $[K^+]_o$ correlated to an increase in neuronal excitability, and when astrocyte volume was forced to recover to baseline by application of hyperosmolar ACSF in the continued presence of elevated potassium, neuronal excitability was greatly dampened. These results demonstrate the importance of astrocyte swelling in the control of neuronal and brain tissue excitability, while also highlighting the necessity for better and more specific tools to unravel the exact mechanisms involved.

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