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Review

Turning down the volume: Astrocyte volume change in the generation and termination of epileptic seizures

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ABSTRACT

Approximately 1% of the global population suffers from epilepsy, a class of disorders characterized by recurrent and unpredictable seizures. Of these cases roughly one-third are refractory to current antiepileptic drugs, which typically target neuronal excitability directly. The events leading to seizure generation and epileptogenesis remain largely unknown, hindering development of new treatments. Some recent experimental models of epilepsy have provided compelling evidence that glial cells, especially astrocytes, could be central to seizure development. One of the proposed mechanisms for astrocyte involvement in seizures is astrocyte swelling, which may promote pathological neuronal firing and synchrony through reduction of the extracellular space and elevated glutamate concentrations. In this review, we discuss the common conditions under which astrocytes swell, the resultant effects on neural excitability, and how seizure development may ultimately be influenced by these effects.

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1. Introduction: epilepsy models and astrocytes

Epilepsy is one of the most common neurological disorders in the world, affecting roughly 65 million people worldwide (Hirtz et al., 2007; Ngugi et al., 2010) and encompassing over 40 different seizure disorders (Berg et al., 2010). Current antiepileptic drugs (AEDs) are inadequate, as most produce negative side effects on cognition (Aldenkamp, 2001; Aldenkamp et al., 2003; Lagae, 2006) and fail to control seizures in approximately 30% of patients (Brodie et al., 2012; Brodie and Dichter, 1996; Kwan and Brodie, 2000). Those with uncontrolled seizures suffer high rates of mortality (Devinsky, 2004a; Laxer et al., 2014; Park et al., 2015) and comorbid neurological disorders (Boylan et al., 2004; Devinsky, 2004b; Kwan and Brodie, 2001). Research on drug-resistant forms of epilepsy, such as temporal lobe epilepsy (TLE), has historically focused almost exclusively on acute and/or long-term changes in neuronal firing and excitability. This approach, however, does not account for non-neuronal cells, which comprise ~50%

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of the brain (Azevedo et al., 2009). A growing body of work suggests that astrocytes, microglia and other glial cell types may play essential roles in the pathology of epilepsy.

Many recently developed epilepsy models have been built upon manipulations of glial cells, especially astrocytes. Astrocytes are a particularly attractive target for epilepsy given their control over neuronal excitability (Hubbard et al., 2013; Wetherington et al., 2008). Key changes contributing to hyperexcitability in epileptic tissue can be directly linked to changes in astrocyte function. For example, extracellular glutamate levels are excessively high in epileptic tissue (Cavus et al., 2005; During and Spencer, 1993), resulting in part from impaired astrocyte glutamate metabolism (Eid et al., 2004) and possibly glutamate transport (Proper et al., 2002). Broberg et al. (2008) found that seizures could be reliably induced by focal intracerebral injection of the astrocyte metabolic inhibitor fluorocitrate (FC). Similarly, the astrocytic enzyme glutamine synthetase (which recycles glutamate into glutamine for transport back to neurons) has been targeted with methionine sulfoximine (MSO) infusions into the hippocampus, producing acute status epilepticus (SE, a prolonged seizure) and gradual development of spontaneous recurrent seizures (SRS) (Albright et al., 2016; Eid et al., 2008; Wang et al., 2009). Serum albumin, which can sometimes enter the brain through a compromised blood brain barrier (BBB) in epileptic tissue, was found to directly bind astrocytic TGF-β receptors and induce reactive gliosis followed by seizures in rats (Ivens et al., 2007; Seiffert et al., 2004). Genetic deletion of β-integrin from astrocytes, which produces reactive gliosis without BBB disruption (Robel et al., 2009) was also sufficient to produce SRS within the first six weeks of life (Robel et al., 2015), indicating reactive gliosis alone may be sufficient for epileptogenesis. These and other findings suggest that astrocytes may be actively involved in the development of recurrent seizures.

A common underlying factor in many models of epilepsy is astrocyte swelling. In the following sections, we will discuss the evidence pointing to a significant role for astrocyte swelling in the development and maintenance of seizures, including the specific observations of tissue volume changes in seizures, physiological and pathological factors affecting astrocyte volume, and finally the consequences of astrocyte swelling in the context of hyperexcitable tissue.

2. Tissue swelling and seizures

Space within the brain is both limited and tightly regulated, and neuronal function depends upon the careful balancing of water, ions and neurotransmitter concentrations in the extracellular space (ECS). Accordingly, losing this balance can be a medical emergency. Acute plasma hypoosmolality, often due to low serum Na^+ levels (hyponatremia) has been known for nearly a century to cause direct swelling of the brain, muscle spasms, generalized seizures, coma or even death (Andrew, 1991; Castilla-Guerra et al., 2006; Rowntree, 1926). The effects of plasma hypoosmolality are most directly induced by accidental overhydration, as might occur in psychogenic polydipsia (compulsive water drinking, a symptom occurring in some cases of schizophrenia), or rapid water intake following dehydration. Multiple disorders which can acutely reduce plasma osmolality are also associated with seizures, including syndrome of inappropriate ADH secretion (SIADH), dialysis disequilibrium syndrome, transurethral resection of the prostate (TURP) syndrome and diabetes mellitus (Andrew, 1991). Ironically, certain antiepileptic drugs may even result in hyponatremia, although generally asymptomatic (Berghuis et al., 2016; Kim et al., 2014; Lu and Wang, 2017; Shepshelovich et al., 2017). Reductions of brain interstitial osmolarity can lead to "cellular" edema (occasionally sub-classified as "osmotic" edema), as water flows into neural cells causing them to swell (Kimelberg, 1995; Thrane et al., 2014). This in turn shrinks the ECS (Andrew and MacVicar, 1994; Chebabo et al., 1995b; Kilb et al., 2006) which is a critical regulator of neuronal excitability. In addition to increasing effective concentrations of extracellular ions and neurotransmitters, ECS reductions bring neurons closer together and increase nonsynaptic, neuron-to-neuron electrical field (ephaptic) interactions, resulting in more synchronous firing and bursting activity (Andrew et al., 1989; Ballyk et al., 1991; Dudek et al., 1986). Unsurprisingly, neuronal excitability is highly sensitive to extracellular shifts in osmolarity (Azouz et al., 1997; Chebabo et al., 1995a; Huang et al., 1997; Lauderdale et al., 2015). Multiple studies have demonstrated that seizures and other epileptiform activity can either be induced by lowering extracellular osmolarity, or abolished by increasing extracellular osmolarity (Dudek et al., 1990; Kilb et al., 2006; Roper et al., 1992; Rosen and Andrew, 1990; Saly and Andrew, 1993; Traynelis and Dingledine, 1989), particularly in regions such as the hippocampal CA1 where the ECS is smaller at baseline (McBain et al., 1990). Similarly, a recent study has found that genetic knockdown of the extracellular matrix glycosaminoglycan hyaluronan (HA) leads to a 40% ECS reduction within the CA1 stratum pyramidale and results in both ictal (seizure, or seizure-like) and interictal (inter-seizure) epileptiform activity. The epileptiform activity is subsequently abolished in hyperosmolar conditions known to cause cell shrinkage and increase the volume of the ECS (Arranz et al., 2014). Indeed, pre-seizure ECS constriction has been observed in multiple models of epilepsy (Binder et al., 2004b; Broberg et al., 2008; Traynelis and Dingledine, 1989). Tissue swelling may therefore represent both an important treatment target, and an important early warning sign, for seizures (Binder and Haut, 2013).

2.1. Water channels and brain volume

In general, brain water content is thought to be controlled by astrocytes due to their selective expression of the water channel aquaporin-4 (AQP4) (Nagelhus et al., 2004; Nielsen et al., 1997). AQP4 expression is particularly enriched at astrocytic endfeet adjoining cerebral vasculature, providing tight control of water fluxes both into and out of the brain (Amiry-Moghaddam et al., 2003a; Nagelhus et al., 2004; Nielsen et al., 1997). Consequently, the role of AQP4 in neurological disease reflects the role of water movements in said disease. For example, AQP4-dependent water movement through astrocytes is essential for clearance of vasogenic edema (plasma ultrafiltrate which leaks into and accumulates in the ECS). The same water permeability can exacerbate cellular/cytotoxic (intracellular) edema, making AQP4 a liability in cases of stroke (Katada et al., 2014), liver failure (Rama Rao et al., 2014) or other disorders which cause cell swelling in the brain (Papadopoulos and Verkman, 2007). Genetic deletion studies have provided evidence that AQP4 may be more than a simple, passive water channel, and is most likely not the sole mechanism for astrocyte water permeability. In one study, knockout of the anchoring protein α-syntrophin, which removes perivascular AQP4 (Amiry-Moghaddam et al., 2004), significantly inhibited swelling in severe (~33%) hypoosmolar conditions, but had no effect on swelling in mild (~17%) hypoosmolar conditions (Anderova et al., 2014). With a full knockout of $AQP4^{-/-}$, another study found nearly the opposite effect; little or no change in volume in 30% hypoosmolar solution, but significant reduction in volume at 20% (Thrane et al., 2011).

During synaptic transmission, AQP4 may be important for the activity-dependent fluxes of water which occur alongside potassium uptake and buffering in astrocytes. Increases in extracellular potassium, whether activity-induced or bath-applied, cause astrocyte swelling and a reduction in size of the ECS (Andrew and MacVicar, 1994; Dietzel et al., 1980; Holthoff and Witte, 1996; Risher et al., 2009; Walz and Hinks, 1985). Loss or mislocalization of AQP4 channels is reported to impair astrocyte $[K^+]_o$ clearance and buffering (Amiry-Moghaddam et al., 2003b; Binder et al., 2006; Eid et al., 2005; Haj-Yasein et al., 2015; Strohschein et al., 2011). Suppression of activity-dependent cell swelling has also been reported in $AQP4^{-/-}$ mice (Kitaura et al., 2009), but these data are inconsistent with more recent work in which AQP4 deletion led to an increase in activity-induced tissue swelling (Haj-Yasein et al., 2012). These disparate findings may result from regional astrocyte heterogeneity or methodological differences, but they also suggest that the

precise role of AQP4 in activity-driven glial swelling may be more complex than initially anticipated.

In contrast to astrocytes, most CNS neurons do not express any known functional water channels (Papadopoulos and Verkman, 2013) and are considered resistant to osmotically-driven volume changes (Andrew et al., 2007; Caspi et al., 2009). This is perhaps due to their functional roles in synaptic transmission as opposed to clearance of neurotransmitters and ions. This does not imply, however, that neuronal volume is static. For example, neuronal somata will rapidly swell in excitotoxic conditions, commonly occur in stroke or traumatic brain injury (Andrew et al., 2007; Choi, 1992; Liang et al., 2007; Risher et al., 2009). Excessive entry of Na^+, often as a result of overactive ionotropic glutamate receptors, depolarizes neurons and opens a voltage-gated Cl^- channel. Both ions increase intracellular osmolarity, drawing water into and swelling the neuron (Choi, 1992; Lee et al., 1999; Rungta et al., 2015). In light of these data, the reported lack of neuronal sensitivity to hypoosmolar conditions in some studies (Andrew et al., 2007; Caspi et al., 2009) is rather curious, since there is little reason to believe that osmotic swelling of neurons would occur exclusively in excitotoxic conditions. In fact, several studies of neurons in culture have reported varying degrees of neuronal swelling in hypoosmolar conditions (Aitken et al., 1998; Borgdorff et al., 2000; Boss et al., 2013; Somjen, 1999). Disparate conditions between culture *vs*. intact tissue settings may in part account for these differences.

2.2. Potassium uptake and glial swelling

Three general mechanisms contribute to potassium uptake and redistribution ("spatial buffering") by astrocytes: The Na^+/K^+-ATPase (NKA), the inwardly-rectifying K^+ channel 4.1 ($K_{ir}4.1$), and the $Na^+/K^+/2Cl^-$ cotransporter 1 (NKCC1). The individual contributions of each to basal $[K^+]_o$ regulation, K^+ uptake and activity-dependent astrocyte swelling have been debated for some time (Kofuji and Newman, 2004; Larsen et al., 2014; MacAulay and Zeuthen, 2012; Walz, 2000). Many authors have found evidence that the astrocytic NKA isoforms are partially (if not predominantly) responsible for the rapid clearance of extracellular K^+ which enters the extracellular space following neuronal firing (D'Ambrosio et al., 2002; Larsen et al., 2014; Ransom et al., 2000; Xiong and Stringer, 2000). NKA is also required to maintain physiologically low $[K^+]_o$ concentrations (D'Ambrosio et al., 2002; Larsen et al., 2014; Ransom et al., 2000), which is in line with the traditional role NKA plays in establishing transmembrane gradients of Na^+ and K^+. NKCC1 may supplement K^+ uptake under conditions of especially high $[K^+]_o$ (Larsen et al., 2014; Walz and Hertz, 1982; Walz and Hertz, 1984). $K_{ir}4.1$, the most abundant K^+ channel expressed by astrocytes, is generally considered essential for the passive K^+ conductance in astrocytes (Chever et al., 2010; Meeks and Mennerick, 2007; Neusch et al., 2006; Seifert et al., 2009; Tang et al., 2009). Passive movement of K^+ through $K_{ir}4.1$ channels may be particularly important for spatial buffering (Djukic et al., 2007; Haj-Yasein et al., 2011; Kofuji and Newman, 2004).

$K_{ir}4.1$ and the Na^+/K^+-ATPase have both been shown to physically (and possibly functionally) interact with AQP4 (Illarionova et al., 2010; Nagelhus et al., 2004; Strohschein et al., 2011). $K_{ir}4.1$ has been particularly well-studied in this context. Initial reports of $K_{ir}4.1$ colocalizing with AQP4 in astrocyte endfeet provided the first indication of a functional interaction (Connors et al., 2004; Nagelhus et al., 2004). These data have since received support from studies in which AQP4 was either deleted, or mislocalized away from astrocyte endfeet through deletion of the scaffolding protein α-syntrophin (to which both AQP4 and $K_{ir}4.1$ bind). In either case, K^+ clearance following synaptic activity was impaired (Amiry-Moghaddam et al., 2003b; Binder et al., 2006; Haj-Yasein et al., 2015; Strohschein et al., 2011). The exact nature of the functional interaction between $K_{ir}4.1$ and AQP4 remains debated however, as some studies have found no impairment of glial Ba^{2+}-sensitive ($K_{ir}4.1$) currents and no change in $K_{ir}4.1$ distribution or expression levels in $AQP4^{-/-}$ mice (Binder et al., 2006; Ruiz-Ederra et al., 2007; Zhang and Verkman, 2008). It has been suggested that AQP4 deletion may indirectly alter activity-driven K^+ elevations as a consequence of impaired ECS volume regulation (Haj-Yasein et al., 2015; Haj-Yasein et al., 2012; Jin et al., 2013). Additionally, AQP4 deletion may impair the function of NKA. The NKA was recently shown to be capable of forming a macromolecular complex with AQP4 alongside the metabotropic glutamate receptor mGluR5, which may also regulate AQP4 permeability (Illarionova et al., 2010). Group I mGluRs (such as mGluR5) or $K_{ir}4.1$ channels may indirectly increase or decrease astrocyte water permeability through phosphorylation or dephosphorylation, respectively, of AQP4 channels (Gunnarson et al., 2008; Song and Gunnarson, 2012), although these data are controversial (Assentoft et al., 2013; Assentoft et al., 2014). Lack of a direct functional interaction between NKA or $K_{ir}4.1$ and AQP4 could explain why Larsen and colleagues found neither K^+ uptake pathway to be directly responsible for stimulus-induced shrinkage of hippocampal tissue (Larsen et al., 2014).

Similar to $K_{ir}4.1$ and NKA, NKCC1 has been implicated in $[K^+]_o$-induced glial swelling (MacVicar et al., 2002; Su et al., 2002a; Su et al., 2002b). NKCC1 may be capable of transporting water directly rather than through interactions with AQP4 (Hamann et al., 2010). The extent to which NKCC1 directly participates in stimulus-induced $[K^+]_o$ clearance and glial swelling, however, is debatable and may be overshadowed by NKA (Larsen et al., 2014). NKCC1 activity also increases $[Na^+]_i$ and $[Cl^-]_i$ in glial cells, which may be important for processes dependent upon these ions. For example, NKCC1-dependent increases in $[Na^+]_i$ help offset the acute $[Na^+]_i$ reductions caused by NKA activity following stimulus-induced $[K^+]_o$ elevations (Rose and Ransom, 1996). This suggests a potential indirect, if not direct, role for NKCC1 in $[K^+]_o$ clearance and glial swelling.

2.3. Astrocyte swelling in seizures and epilepsy

Evidence supporting a role for astrocyte swelling in epilepsy has been provided through specific targeting of AQP4 or $K_{ir}4.1$ in various seizure models. In a series of informative studies, Binder and colleagues used PTZ injections (Binder et al., 2004a; Binder et al., 2004b) or hippocampal electrical stimulation (Binder et al., 2006) to evoke acute seizures in wild-type and $AQP4^{-/-}$ mice. As discussed above, ECS in wild-type animals was observed to shrink just prior to a seizure (Binder et al., 2004b). However, in $AQP4^{-/-}$ animals, baseline ECS was significantly larger and attenuated ECS shrinkage (Binder et al., 2004b). $AQP4^{-/-}$ animals required a correspondingly higher electrical stimulation or PTZ dose to reach seizure threshold (Binder et al., 2004a; Binder et al., 2006), but also experienced seizures nearly 3 times the duration of wild-type animals (2006). The increased seizure threshold may reflect both the significant increase in baseline ECS and the reduced tissue swelling observed in $AQP4^{-/-}$ animals (Binder et al., 2004b). Impaired K^+ buffering was hypothesized as the cause for prolonged seizure duration, as $AQP4^{-/-}$ mice have noticeably slower K^+ decay kinetics (Binder et al., 2006; Strohschein et al., 2011). It is interesting to note that, despite this impaired K^+ clearance (or perhaps as a result of it), $AQP4^{-/-}$ astrocytes are coupled to a greater degree through gap junctions than their wild-type counterparts (Strohschein et al., 2011). Loss of astrocyte gap junctional coupling is associated with impaired $[K^+]_o$ buffering, seizures and neuronal death (Bedner et al., 2015; Wallraff et al., 2006). Conversely, increased gap junctional coupling between $AQP4^{-/-}$ astrocytes may reflect a compensatory measure to offset reduced K_{ir} and/or NKA activity by increasing the K^+ redistribution capacity in the syncytium.

A key finding in both human epileptic tissue and animal models of epilepsy is the presence of reactive astrogliosis, a complex array of changes in the morphology, protein expression patterns and function of astrocytes following neural injury (Hubbard et al., 2013; Sofroniew, 2009). Two of these changes are particularly relevant to astrocyte

swelling and the development of epilepsy: 1.) $K_{ir}4.1$ currents in astrocytes are consistently smaller (Hinterkeuser et al., 2000; Kivi et al., 2000), with reduced K_{ir} expression particularly at vascular endfeet (Heuser et al., 2012); and 2.) AQP4 expression is drastically altered and polarization at the vascular endfeet is lost, paralleling the loss of $K_{ir}4.1$ (Alvestad et al., 2013; Eid et al., 2005; Hubbard et al., 2016). Together, these reactive changes impair potassium clearance and spatial buffering (Amiry-Moghaddam et al., 2003b; Bedner et al., 2015; Bordey and Sontheimer, 1998; Kivi et al., 2000), and could augment cellular swelling, although this has not yet been directly measured (Hubbard et al., 2013; Wetherington et al., 2008). Several animal models of epilepsy have demonstrated that reactive changes in astrocytes likely precede development of seizures. For example, in kainic acid models of SRS, AQP4 was severely downregulated 1 day after SE and mislocalized weeks before the development of seizures (Alvestad et al., 2013; Hubbard et al., 2016; Lee et al., 2012). Using a β1-integrin deletion from astrocytes, Robel et al. (2015, 2009) provided evidence that reactive changes in astrocytes including AQP4 and dystrophin mislocalization, astrocyte hypertrophy and loss of K^+ buffering are sufficient to induce recurrent seizures. In the albumin seizure model, serum albumin directly taken up by astrocytic TGF-β receptors produced astrogliosis a few days before seizures developed (David et al., 2009; Ivens et al., 2007). Interestingly, while reduced K^+ buffering efficacy in the albumin model correlated directly with reduced $K_{ir}4.1$ expression (Ivens et al., 2007), no such reduction in $K_{ir}4.1$ expression was found in the $\beta1^{-/-}$ model (Robel et al., 2015). These data suggest that the loss of K^+ buffering may not necessarily be a direct consequence of $K_{ir}4.1$ expression changes, but rather a functional change resulting from the loss of perivascular AQP4 channels (Amiry-Moghaddam et al., 2003b). The changes occurring in reactive astrocytes may create a "perfect storm" of epileptogenic conditions: Impaired K^+ buffering increases neuronal excitability, augmenting local glutamate concentrations and further raising extracellular K^+ to promote synchronization; loss of $K_{ir}4.1$ and AQP4 at perivascular endfeet exacerbates astrocyte swelling which can no longer be effectively relieved through water efflux into the bloodstream (Hubbard et al., 2013; Wetherington et al., 2008), further augmenting neurotransmitter and ion concentrations while also increasing ephaptic interactions between neurons.

3. VRAC and volume regulation

Most cell types, including neural cells, exhibit some form of volume regulation. Cells swollen or shrunken by osmotic stress will, over time, return to their original volume even in the continued presence of the osmotic stressor. These two processes are correspondingly termed regulatory volume decrease (RVD) and regulatory volume increase (RVI) (Hoffmann et al., 2009). As described above, astrocyte swelling may be a catalyst for inducing and/or augmenting seizures, so the following discussion will focus specifically on RVD subsequent to cell swelling. RVD has been repeatedly observed in cultured astrocytes swollen by hypoosmolar or high K^+ media (Eriksson et al., 1992; Kimelberg and Frangakis, 1985; Olson et al., 1995; Vitarella et al., 1994). Disagreement exists as to whether astrocytic RVD occurs in intact tissue, as various groups have reported astrocytic swelling in brain slices without RVD (Andrew et al., 1997; Hirrlinger et al., 2008; Risher et al., 2009). Others have observed swelling with an RVD, but only at certain hypoosmolar doses (Thrane et al., 2011), and even swelling which does not recover following removal of the hypoosmolar stimulus (Anderova et al., 2014). These data (with the possible exception of the latter study) may reflect the ability of intact tissue to maintain a relatively constant volume with more gradual changes in osmolarity, referred to as "isovolumetric" volume regulation or IVR (Franco et al., 2000). The mechanisms involved in IVR are likely similar to those in RVD (Pasantes-Morales and Cruz-Rangel, 2010). It is possible that RVD does not occur in some brain slice preparations due to depletion of osmotically-active substances ("osmolytes") within the slice (Kreisman and Olson, 2003). It must also be noted that RVD may depend on the method used to reduce extracellular osmolarity (Andrew et al., 1997). Emerging evidence suggests that astrocyte swelling itself may provide the stimulus necessary for RVD to occur. In Müller glia, an "astrocyte-like" glial cell type in the retina, rapid water influx through AQP4 produces membrane stretch, which then activates nearby stretch-activated TRPV4 channels to permit Ca^{2+} entry to promote RVD (Iuso and Križaj, 2016; Jo et al., 2015). While the requirement of TRPV4 opening and Ca^{2+} influx in astrocytic RVD has recently been questioned (Mola et al., 2016), these findings suggest that rapid water influx is an important trigger for subsequent astrocyte volume regulation (Benfenati et al., 2011; Mola et al., 2016).

In general terms, RVD occurs following either active transport or opening of volume-sensitive channels resulting in efflux of K^+, Cl^-, organic osmolytes and water from the cell (Chamberlin and Strange, 1989; Pasantes-Morales et al., 2006). While the exact transporters and channels involved remain unclear, it is generally agreed that the "volume-regulated anion channel" (VRAC) is one of the essential components of astrocytic RVD (Benfenati and Ferroni, 2010). Until recently, VRAC were identified pharmacologically, and by their unique swelling-activated currents known as $I_{Cl,Swell}$ (Akita and Okada, 2014). In 2014 the likely pore-forming subunits comprising VRAC were finally identified as belonging to the leucine-rich repeat containing protein 8 (LRRC8) family, with LRRC8A being required for VRAC currents and other members (B-E) modifying their kinetics (Hyzinski-García et al., 2014; Qiu et al., 2014; Voss et al., 2014). For clarity and consistency with the large body of prior literature (Jentsch et al., 2016), this multimer will be referred to by the term "VRAC" for the remainder of this section.

VRAC are particularly relevant for the study of epilepsy. Their permeability to not only small inorganic anions such as F^- and Cl^-, but also to large anionic excitatory amino acids (EAAs) including taurine, glutamate and aspartate (Akita and Okada, 2014), suggests that VRAC are a pathway through which astrocytic EAAs might be released back into the ECS. Indeed, many studies have demonstrated that cultured astrocytes release EAAs primarily through VRAC following swelling in high-K^+ or hypoosmolar conditions (Abdullaev et al., 2006; Kimelberg et al., 1990; Kimelberg et al., 1995; Rutledge et al., 1998). In the middle cerebral artery occlusion (MCAO) model of stroke, the VRAC antagonist DCPIB was found to significantly improve behavioral outcome and reduce infarct volume and extracellular glutamate levels (Zhang et al., 2008). These findings suggest that astrocytic swelling in ischemia could lead to glutamate release through VRAC and directly contribute to excitotoxic tissue death (Risher et al., 2009; Zhang et al., 2008). VRAC may not be simple, "stretch-activated" channels, despite being activated consistently by cell swelling. Dilution of intracellular ionic strength, which can occur due to water influx in some cell-swelling conditions, may be sufficient for their activation (Syeda et al., 2016; Voets et al., 1999). Similarly, VRAC activity can be potentiated independently of cell volume (discussed further below).

Importantly, VRAC in the CNS are probably not limited to astrocytes. Neurons also appear to express VRAC, with similar activation in hypoosmolar or excitotoxic conditions but differing from astrocytic VRAC in specific pharmacology (Inoue et al., 2005; Inoue et al., 2007; Inoue and Okada, 2007; Zhang et al., 2011). Differentiating between neuronal *vs.* astrocytic VRAC is complicated, however, as there are currently no antagonists specific for neuronal VRAC and some of the most commonly-used VRAC antagonists (NPPB, DIDS, etc) have rather broad nonspecific effects (Evanko et al., 2004). For example, DIDS is a broad-spectrum Cl^- channel blocker, and was recently found to block the neuronal voltage-gated Cl^- channel SLC26A11 which opens following massive cytotoxic Na^+ influx such as occurs during stroke (Rungta et al., 2015). The question of whether neurons can swell and/or release glutamate through VRAC will require more specific tools. Regardless of whether neuronal swelling contributes to increased tissue excitability,

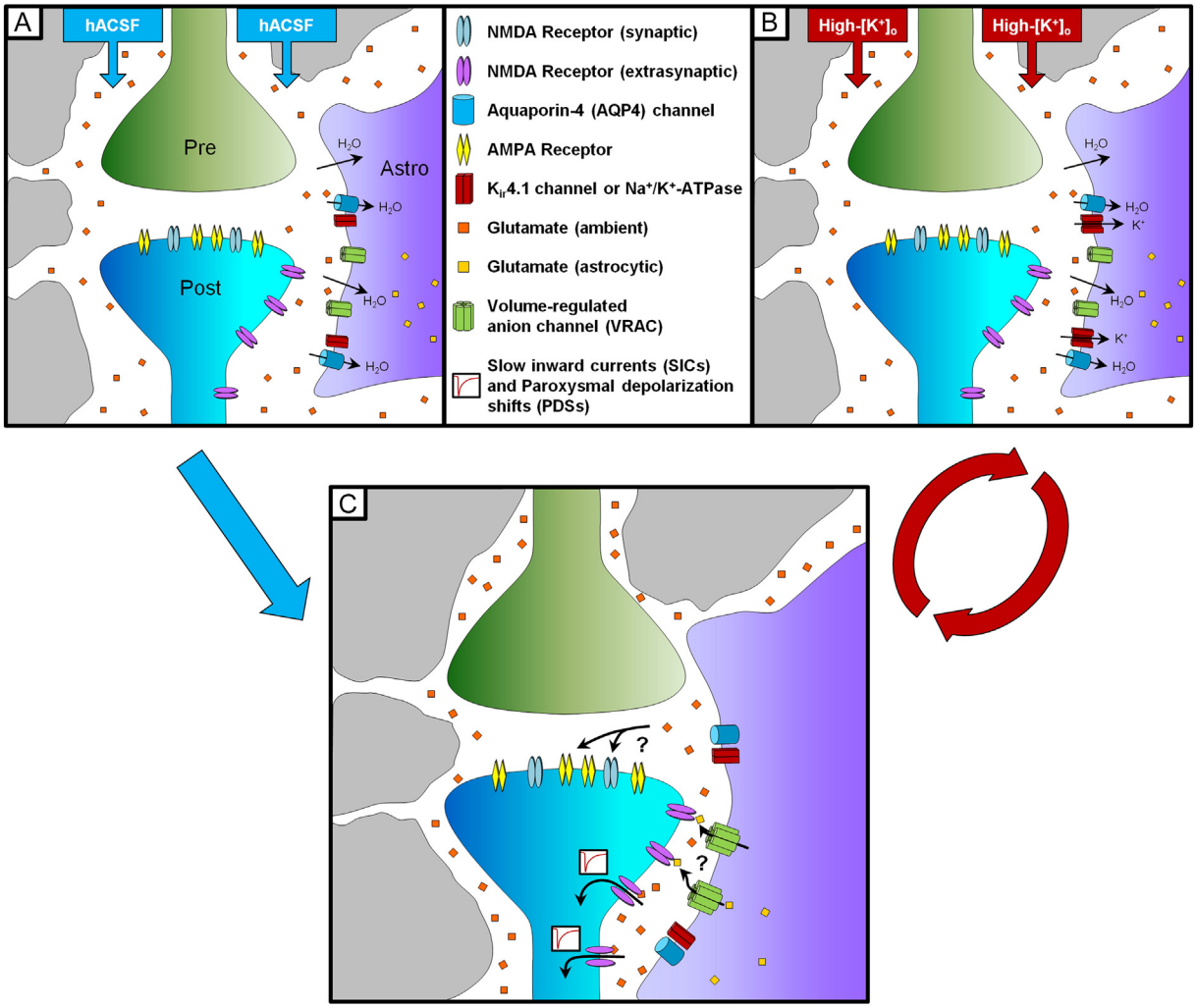

Fig. 1. Hypothesized role of astrocyte swelling in two different models of neuronal hyperexcitability. In a hypoosmolar model of neuronal hyperexcitability (A), astrocytes swell through direct water influx. In a high-$[K^+]_o$ model (B), astrocyte swelling is caused by initial influx of K^+ followed by osmotically-obligated water directly across the membrane or through AQP4 channels. In both cases (C), astrocyte swelling leads to a decrease in the extracellular space (which increases local glutamate concentrations and enhances neuronal excitability). In addition, astrocyte swelling may lead to activation of additional glutamate release pathways, such as volume-regulated anion channels (VRAC). The elevated glutamate during astrocyte swelling then results in NMDA receptor-dependent locally-synchronous neuronal depolarization. Neuronal firing results in a positive feedback loop as glutamate and additional K^+ are released into the ECS, causing additional astrocyte swelling, opening of VRAC, and further increases in neuronal excitability. As ephaptic interactions cause neuronal firing to become synchronized, this system is driven from locally-synchronous activity (SICs) to global hypersynchrony (PDSs) and finally into ictal discharges.
(Modified from Lauderdale et al. (2015): "Osmotic edema rapidly increases neuronal excitability through activation of NMDA receptor-dependent slow inward currents in juvenile and adult hippocampus". *ASN Neuro* 7(5): 1–21. DOI: 10.1177/1759091415605115. Available from SAGE Publications under a Creative Commons Attribution 3.0 license at: http://asn.sagepub.com/content/7/5/1759091415605115.)

glutamate release through astrocytic VRAC is a compelling avenue for future study based on the ample evidence for the role of these channels in glutamate release from astrocytes *in vitro*.

3.1. Astrocytic glutamate and neuronal excitability

There is little doubt that astrocytic glutamate can influence neuronal activity. In quiescent conditions, astrocytes are thought to maintain the so-called "ambient glutamate" concentration in the extracellular space, possibly through release by VRAC or a related channel (Cavelier and Attwell, 2005; Le Meur et al., 2007). In hypoosmolar conditions, NMDA receptor-dependent slow inward currents (SICs) have also been observed in hippocampal CA1 neurons even when neuronal firing is blocked and vesicles depleted of neurotransmitter (Fiacco et al., 2007; Lauderdale et al., 2015), suggesting volume-dependent glutamate release from a nonsynaptic source. Astrocytic glutamate released by a Ca^{2+}-dependent process has also been reported to induce SICs. In these studies, stimuli which evoked Ca^{2+} elevations in astrocytes including direct mechanical stimulation, group 1 metabotropic glutamate receptor (mGluR) agonists, or simply uncaging Ca^{2+} within the astrocyte were sufficient to induce SICs in nearby neurons (Angulo et al., 2004; Fellin et al., 2006; Fellin et al., 2004; Tian et al., 2005). These findings seem at odds with hypoosmolar-induced SICs, which persist when vesicles are emptied using bafilomycin and in transgenic mice in which activity-driven astrocyte Ca^{2+} elevations are significantly depressed (Fiacco et al., 2007). More puzzlingly, one group found that neuronal SICs were produced following astrocytic Ca^{2+} elevations induced by activation of PAR-1, but not $P2Y_1$, receptors (Shigetomi et al., 2008). Given these conflicting studies, it is quite interesting to note that VRAC, apart from their typical Ca^{2+}-independent mode of activation, can also be potentiated by several mechanisms which elevate intracellular Ca^{2+}, including src kinase activation by peroxynitrite (Haskew et al., 2002), protein kinase C (PKC) activation from B2 bradykinin receptor activity (Akita and Okada, 2011; Liu et al., 2009), and P2Y ATP receptor activation (Mongin and Kimelberg, 2002; Mongin and Kimelberg, 2005; Takano et al., 2005) Takano et al. (2005) also found that P2Y receptor activation induced a transient astrocyte volume increase that was necessary for VRAC to open. Taken together, these studies raise the

possibility that most or all astrocytic glutamate leading to SICs is released through VRAC.

The unique properties of SICs are particularly interesting in the context of epilepsy. SICs evoked by astrocyte Ca^{2+} increases are synchronized between neurons located within ~100 μm, occurring in up to 12 neurons simultaneously (Angulo et al., 2004; Fellin et al., 2004). This property supports their astrocytic origin, since a single astrocyte within the hippocampal CA1 area can cover nearly 66,000 μm^3 of neuropil and ensheathe over 100,000 synapses (Bushong et al., 2002). Astrocytic glutamate release may thus be capable of synchronizing the activity of local neuron populations. It is unclear to what degree these locally-synchronous events can contribute to the much larger, synchronous activity characterizing interictal and ictal discharges. Hypoosmolar conditions augment hippocampal tissue excitability, at least in part, through rapid reduction of the extracellular space accompanied almost simultaneously by SIC generation in CA1 neurons (Fiacco et al., 2007; Lauderdale et al., 2015). SICs have also been observed in multiple slice seizure models, and it has been debated whether SICs themselves are a type of epileptiform activity (Fellin et al., 2006; Tian et al., 2005). Current evidence suggests that SICs are distinct from epileptiform currents, the latter being dependent upon synaptic transmission and synchronous over large distances, while the former are locally synchronous and nonsynaptic (Fellin et al., 2006; Fellin et al., 2004; Wetherington et al., 2008). SICs are more likely to modulate tissue excitability and thereby play a role in the "build up" to seizure activity during the period of cell swelling and reduction of the ECS that immediately precedes seizures. Small groups of neurons synchronized by SIC activity, for example, may gradually fuse into larger "hypersynchronous" groups that characterize interictal or ictal events (Bikson et al., 2003; Jiruska et al., 2010). Release of glutamate through astrocytic VRAC may be sufficient to turn interictal spikes into full ictal discharges, or exacerbate and lengthen ictal bursts in seizure-prone tissue (Ding et al., 2007; Fellin et al., 2006).

4. Conclusions

Astrocytes exert tight control over neuronal excitability through a number of mechanisms including glutamate uptake, $[K^+]_o$ buffering, and brain water regulation. These same mechanisms, however, may also be actively involved in seizure generation. High astrocyte water permeability facilitates cellular edema in hypoosmolar, high-$[K^+]_o$ and other pathological conditions. Intracellular glutamate in astrocytes can be released from volume-regulated channels that open during swelling, increasing local glutamate concentrations and potentially binding directly to neuronal glutamate receptors. Extracellular concentrations of glutamate, as well as K^+ and other ions, would also be augmented by astrocyte swelling and subsequent reduction of the ECS. These mechanisms combined would elevate neuronal excitability through increased ephaptic interactions, depolarized membrane potentials, and increased excitatory currents (Fig. 1). Seizures are preceded by brain parenchyma swelling in both humans and animal models and abolished by increases in extracellular osmolarity, supporting the idea that astrocyte swelling may promote seizure generation. Moreover, reactive astrogliosis is common to epileptic tissue, and includes changes in AQP4 expression and K^+ buffering which may directly impact astrocyte volume regulation (Hubbard and Binder, 2016).

5. Future directions

The role of astrocyte/tissue volume changes in seizure generation remains largely unexplored, and many key questions remain to be answered. First and foremost, do astrocytes swell prior to a seizure *in vivo*? Multiple studies have demonstrated that astrocytes can swell in hyperexcitable environments, and others still have shown that tissue swelling precedes seizure generation. To date, however, astrocyte volume prior to and during preictal, ictal and postictal periods *in vivo* has not been directly measured. Simultaneous astrocyte imaging and EEG recordings, in multiple seizure models, will be essential to closing this gap. Second, what is the role of AQP4 in astrocyte swelling *in vivo*? Conventional wisdom holds that AQP4 channels facilitate the majority of water movement across the astrocyte membrane, but this is far from the only possible mechanism of astrocyte swelling (Kimelberg, 2005). Are different astrocyte swelling mechanisms active under different seizure paradigms (*i.e.* high $[K^+]_o$ *vs.* hypoosmolarity)? Imaging astrocytes from $AQP4^{-/-}$ animals during seizure will be an important first step in answering these questions. Answers to the above questions will guide approaches to a more central one: To what degree does astrocyte swelling facilitate seizure generation? The threshold requirements (such as osmotic pressure) for astrocyte swelling *in vivo* have not been firmly established, nor has it been established whether the same threshold is capable of eliciting seizures. Moreover, astrocytes may not be the only cells swelling during the preictal period. Volume measurements of neurons and other cell types will be essential, especially if AQP4-independent astrocyte swelling is possible. Lastly, the effect of inhibiting astrocyte swelling on seizures *in vivo* is currently unknown. The findings from these immediate questions will be invaluable for understanding the role of astrocyte swelling in epilepsy.

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