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## Cell Volume Control in Healthy Brain and Neuropathologies

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### Abstract

Regulation of cellular volume is a critical homeostatic process that is intimately linked to ionic and osmotic balance in the brain tissue. Because the brain is encased in the rigid skull and has a very complex cellular architecture, even minute changes in the volume of extracellular and intracellular compartments have a very strong impact on tissue excitability and function. The failure of cell volume control is a major feature of several neuropathologies, such as hyponatremia, stroke, epilepsy, hyperammonemia, and others. There is strong evidence that such dysregulation, especially uncontrolled cell swelling, plays a major role in adverse pathological outcomes. To protect themselves, brain cells utilize a variety of mechanisms to maintain their optimal volume, primarily by releasing or taking in ions and small organic molecules through diverse volume-sensitive ion channels and transporters. In principle, the mechanisms of cell volume regulation are not unique to the brain and share many commonalities with other tissues. However, because ions and some organic osmolytes (e.g., major amino acid neurotransmitters) have a strong impact on neuronal excitability, cell volume regulation in the brain is a surprisingly treacherous process, which may cause more harm than good. This topical review covers the established and emerging information in this rapidly developing area of physiology.

### 12.1 COMMON AND UNIQUE ASPECTS OF OSMOTIC BALANCE WITHIN THE CNS

Much like any other cell type in the human and animal body, brain cells are subjected to the actions of osmotic forces. The underlying mechanisms and principles are discussed in detail in the first chapter of this book (Delpire & Gagnon, 2018) and, therefore, are only briefly recapitulated here.

Prototypical lipid membranes of animal cells have high passive permeability for water, but in practical terms are essentially impermeant to ions and polar extra- and intracellular solutes. Owing to the semi-permeability of the plasmalemma, water moves freely in and out of the cell as dictated by osmotic gradients set by membrane-impermeant molecules (Finkelstein, 1987). Any net accumulation or loss of solutes and metabolites is immediately followed by

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water flow and ensuing changes in hydrostatic pressure. Due to the absence of a rigid cell wall, animal cells are poorly equipped to withstand hydrostatic forces. Therefore, they swell or shrink, and cope with changes in cell volume and ionic homeostasis using several distinct strategies.

Moderate degrees of cell shrinkage produce relatively limited strain on cell architecture and, in the short-term, are accommodated by folding of the cell membrane. Moderate degrees of cell swelling are more problematic because the plasma membrane has a very limited stretch capacity and is tethered to extensive cytoskeletal networks. Nevertheless, upon encountering an acute osmotic challenge, many cell types can rapidly increase their volume up to several-fold using extensive membrane reserves and, in extreme cases, via exocytotic recruitment of intracellular membranes (Morris & Homann, 2001; Groulx, Boudreault, Orlov, & Grygorczyk, 2006). Movement of water and changes in cell volume dissipate osmotic gradients on the scale of seconds to several minutes, which is then followed by cell volume regulation.

Osmotic changes in cell volume are typically compensated via the act of active volume regulation. Cell volume control is a vital homeostatic property of living organisms, which emerged at early stages of evolution and is characteristic for organisms belonging to all branches of the phylogenetic tree (Chamberlin & Strange, 1989). The process of volume restoration is much slower than the initial osmotic swelling or shrinkage. Depending on conditions and cell type, it takes minutes to hours to complete. In principle, osmotic imbalances are counteracted via the accumulation or loss of small solute molecules. Osmolyte fluxes are mediated by diverse “volume-sensitive” membrane transporters and ion channels, the majority of which have been studied at length. Unfortunately, we know very little about how cells actually sense changes in their volume [the nature of cell volume sensor(s)] and have insufficient knowledge of how cell volume sensing is transduced to changes in solute transport [the nature of downstream intracellular signals]. For detailed discussion of these topics, the reader can be addressed to several comprehensive reviews (Lang et al., 1998; Wehner, Olsen, Tinel, Kinne-Saffran, & Kinne, 2003; Mongin & Orlov, 2001; Hoffmann, Lambert, & Pedersen, 2009).

### 12.1.1 Common mechanisms of regulatory volume decrease (RVD)

As schematically depicted in Fig. 12.1A, osmotic cell swelling is counteracted via the process of RVD. In general, volume recovery in swollen cells is mediated by the coupled efflux of cytosolic  $K^+$  and the inorganic anions,  $Cl^-$  and bicarbonate ( $HCO_3^-$ ).  $K^+$  is the main intracellular osmolyte, but its movement must be accompanied by anions to preserve electroneutrality and sustain the electrochemical driving force for  $K^+$ . In most cell types, RVD is dominated by  $K^+$  and  $Cl^-$  channels, which are activated independently but are coupled at the functional level via changes in the membrane potential. Among the diverse  $K^+$  channels, many subtypes show sensitivity to cell swelling, including the two-pore domain TREKs (products of the *KCNK1* and *KCNK2* genes), TASKs (*KCNK3*, *5* and *9*), and TRAAK (*KCNK4*), the voltage-sensitive Kv1.1 and Kv1.3 (*KCNA1* and *KCNA3*), Kv4.1 and Kv4.3 (*KCND1* and *KCND3*), Kv7.1, 4 and 5 (*KCNQ1*, *KCNQ4*, and *KCNQ5*), and the  $Ca^{2+}$ - and voltage-sensitive BK (*KCNMA1*) and IK1 (*KCNN4*) [reviewed in (Lang et al.,

1998; Wehner et al., 2003; Hoffmann et al., 2009)]. It is important to note that due to the dominant nature of  $K^+$  permeability in most mammalian cells,  $K^+$  channels need not be cell-volume sensitive to contribute to cell volume control.

In contrast to  $K^+$  conductance, the typical basal  $Cl^-$  permeability of the plasma membrane is low and must be augmented during RVD. This is accomplished via activation of a volume-sensitive chloride current ( $I_{Cl,vol}$  or  $I_{Cl,swell}$ ), which was initially characterized in the pioneer work done by the groups of Y. Okada, K. Strange, and B. Nilius. Because the biophysical properties of  $I_{Cl,vol}$  are similar in different cell types, this led to the hypothesis that they are mediated by the same ion channel or a group of similar channels. The putative  $I_{Cl,vol}$  channel was christened volume-regulated anion channel (VRAC), volume-sensitive outwardly rectifying  $Cl^-$  channel (VSOR), or volume-sensitive organic osmolyte-anion channel (VSOAC) (Strange, Emma, & Jackson, 1996; Okada, 1997; Nilius et al., 1997). In this review we will use the acronym VRAC. Despite detailed studies of  $I_{Cl,vol}$  currents in many cell types, the molecular identity of VRAC remained an enigma until a few years ago. In 2014, the laboratories of A. Patapoutian and T.J. Jentsch independently identified VRAC as a heteromeric complex of proteins from the family of leucine-rich repeat-containing 8 (LRRC8) (Qiu et al., 2014; Voss et al., 2014). The functional properties and physiological roles for the LRRC8-containing VRAC are discussed in several specialized reviews (Stauber, 2015; Pedersen, Okada, & Nilius, 2016; Mongin, 2016; Jentsch, 2016) and for the CNS tissue are additionally covered in **sections** 12.2 and 12.8.

One of the properties of VRAC, which is reflected in their alternative name VSOAC, is the ability to conduct not only inorganic anions ( $Cl^-$ ,  $HCO_3^-$ , and others), but also small, negatively charged and neutral organic molecules. The first evidence that  $Cl^-$  channels, which were later identified as VRAC, can constitute a route for transmembrane amino acid fluxes was collected in MDCK cells by U. Banderali and G. Roy, who measured anion currents mediated by aspartic acid, glutamic acid, and the amino sulfonic acid taurine in excised membrane patches (Banderali & Roy, 1992). Subsequent whole-cell electrophysiology studies measured amino acid currents in a variety of cell types subjected to hypoosmotic swelling (Jackson & Strange, 1993; Jackson, Morrison, & Strange, 1994; Strange et al., 1996; Manolopoulos, Voets, Declercq, Droogmans, & Nilius, 1997). Although the relative permeability of VRAC to organic osmolytes is a fraction of that for  $Cl^-$  (in the order of 0.05–0.3), the loss of organic molecules is strongly favored by their electrochemical gradients and may play a significant role in RVD. In some cell types, the loss of organic osmolytes accounts for about 50% of the total osmolyte loss during RVD, but such contribution is likely cell type specific [reviewed in (Wehner et al., 2003)]. What is essential in the context of the present discussion is that some of the VRAC-permeable osmolytes, such as glutamate, aspartate, taurine, and GABA, are major neurotransmitters in the CNS. Therefore, release of these molecules exerts a dramatic impact on neural functions, irrespectively of their relative contribution to RVD [see (Mongin, 2016) and **sections** 12.2 and 12.8].

Besides channel-mediated ion fluxes, in many cell types, RVD is supplemented by KCl loss via the electroneutral KCl cotransporters (KCC1–4, encoded by *SLC12A4–6*). Discussion of the physiological roles of KCC and their contributions to cell volume control can be found

in specialized reviews (Lauf & Adragna, 2000; Gamba, 2005; Kahle et al., 2015; Kahle et al., 2008), and in a brain-specific context in section 12.2.

### 12.1.2 Generic mechanisms of regulatory volume increase (RVI)

Following osmotic or metabolic shrinkage, cells recover their volume through the process of RVI (see Fig. 12.1A). The two main ion transporters which drive RVI are the electroneutral  $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$  co-transporter NKCC1 (gene product of *SLC12A2*) and the ubiquitous isoform of the  $\text{Na}^+/\text{H}^+$ -exchanger NHE1 (*SLC9A1*). Through their combined work, NKCC1 and NHE1 mediate the net accumulation of NaCl and KCl and drive the osmotically obligated movement of water inside the cell (Lang et al., 1998; Wehner et al., 2003; Mongin & Orlov, 2001; Hoffmann et al., 2009). Activity of the shrinkage-activated NHE1 (and other cell type-specific NHEs) causes alkalization of the cytosol and stimulates the pH-sensitive anion exchangers (AE), which are not directly regulated by cell volume changes. Ubiquitous AE2 (*SLC4A2*) and the brain- and heart-enriched AE3 (*SLC4A3*) take  $\text{Cl}^-$  inside the cell in exchange for  $\text{HCO}_3^-$  out, and in such a way contribute to the accumulation of intracellular  $\text{Cl}^-$  and removal of alkaline equivalents (Alper, 1991; O'Neill, 1999). In contrast to NHEs, shrinkage-activated NKCC1 works “autonomously”, but its efficacy in RVI depends on the preservation of the combined inwardly-directed gradient for [ $\text{Na}^+$  plus  $\text{K}^+$  plus  $\text{Cl}^-$ ]. In certain cell types, hyperosmolar media strongly activate NKCC1, but this is not accompanied by RVI due to an insufficient electrochemical driving force for ionic movement. In contrast, the same cells can effectively regulate their volume in response to isosmotic cell shrinkage (e.g., caused by the receptor-mediated loss of intracellular electrolytes or the metabolic decrease of osmolyte content) or as a result of the so-called “RVI after RVD” protocol (when cells are returned to isosmotic medium after prolonged adaptation to hypoosmotic conditions) (O'Neill, 1999).

A relatively rare, but thermodynamically-effective, mechanism of RVI, which has been identified in only a few cell types, involves the opening of hypertonicity-induced non-selective cation channels (HICCs). HICCs mediate the rapid accumulation of monovalent cations, primarily  $\text{Na}^+$ , and very fast RVI (Bohmer et al., 2000; Wehner, Shimizu, Sabirov, & Okada, 2003; Wehner et al., 2006; Koos et al., 2018). The molecular composition of HICCs remains rather obscure, but depending on the cell type they may incorporate heteromeric assemblies of the transient receptor potential (TRP) channels from the melastatin family (e.g., TRPM2 and TRPM5), and/or one or several types of the “epithelial”  $\text{Na}^+$  channels from the degenerin family (ENaCa through  $\delta$ , *SCNN1A-D*) (Bondarava, Li, Endl, & Wehner, 2009; Koos et al., 2018).

The long-term adaptation to hyperosmotic conditions involves the intracellular accumulation of small, nonionic organic molecules, which have the collective name ‘idiogenic’ or ‘compatible’ osmolytes. The compatible osmolytes are beneficial for cell volume control cells because, unlike ions, they do not modify ionic strength and composition in the cytosol, and in such a way preserve the optimal conditions for intracellular enzymatic reactions. The relevant compounds include amino acids (e.g., glutamine and alanine), amino acid derivatives (e.g., the aminosulfonic acid taurine), methylamines (betaine and glycerophosphorylcholine), and polyalcohols (sorbitol and *myo*-inositol). Changes in the

cytosolic levels of compatible osmolytes are in part due to the increase in expression of Na<sup>+</sup>-dependent membrane transporters (such as SMIT for *myo*-inositol, BGT1 for betaine, and TauT for taurine), which lead to net osmolyte accumulation. Alternatively, there are increases in the expression of certain synthetic enzymes (aldose reductase for sorbitol, phospholipase for glycerophosphorylcholine, etc.) that lead to the net increase in osmolyte content inside of the cell. In all cases, the main driving mechanism for the long-term adaptation to hyperosmolarity are changes in gene expression, which are initiated by the osmosensitive transcription factor TonEBP, also known as OREBR and NFAT5 (Burg, Ferraris, & Dmitrieva, 2007; Hoffmann et al., 2009). As in the case of RVD, changes in transmembrane fluxes of certain organic osmolytes can have a dramatic impact on brain functions.

### 12.1.3 Donnan cell swelling

Besides the above-mentioned mechanisms responsible for “emergency” cell volume control (RVD and RVI), it is very important to recognize that under normotonic steady-state conditions all animal cells, including cells in the brain, are also constantly subjected to a persistent osmotic challenge. Due to the high content of impermeant organic macromolecules in the cytosol and intracellular organelles which create significant osmotic (or colloid) load, cells continuously accumulate water. The resulting osmotic behavior, which is often referred to as Donnan swelling or the Gibbs-Donnan effect, was theoretically predicted at the turn of the 20<sup>th</sup> century by J.W. Gibbs and experimentally tested by F.G. Donnan [reviewed in (Macknight & Leaf, 1977)]. Animal cells are capable of resisting Donnan swelling via mechanisms which were first recognized in the studies by E.B. Wilson (Wilson, 1954) and A. Leaf (Leaf, 1956). These investigators found that metabolically compromised cells and tissue slices steadily accumulate water, Na<sup>+</sup>, and Cl<sup>-</sup>, suggesting that cell volume maintenance involves an energy-dependent process. The subsequent work led to the development of the “double Donnan” or “pump-and-leak” model (Macknight & Leaf, 1977; Lang et al., 1998; Hoffmann et al., 2009). This model proposes that Donnan cell swelling is counteracted by the osmotic and electrical work of the Na<sup>+</sup>,K<sup>+</sup>-pump, which sets the negative membrane potential and drives the passive extrusion of intracellular Cl<sup>-</sup> (Fig. 12.1A). The low intracellular [Cl<sup>-</sup>] compensates for the presence of the net-negatively charged macromolecules (e.g., proteins and polynucleotides) and numerous organic metabolites [see discussion in (Lang et al., 1998; Hoffmann et al., 2009) and the “neurocentric” view in (Wilson & Mongin, 2019)].

### 12.1.4 The peculiarities of cell volume regulation in the brain

Several unique features of brain physiology strongly influence the execution and efficacy of cell volume control within the CNS. For the purposes of our discussion, it is helpful to briefly mention some of the relevant factors upfront:

*The brain is contained within a rigid skull, which dramatically limits to what extent the CNS tissue and its functional components can expand or shrink (see Fig. 12.1B).* In the context of fluid-electrolyte homeostasis, the brain can be divided into several distinct compartments [reviewed in (Strange, 1992; Kimelberg, 2004; Thrane, Rangroo Thrane, & Nedergaard, 2014)]. (i) An intracellular partition is filled with the intracellular fluid (**ICF**). Within CNS,

there are four major functionally distinct classes of cells: neurons, astrocytes, oligodendrocytes, and microglia; additionally, ciliated ependymal cells line the brain ventricles and spinal cord canal. (ii) The interstitial extracellular fluid (**ECF**) bathes all brain cells and creates a milieu for their physiological activities. (iii) The cerebrospinal fluid (**CSF**) is produced by the specialized epithelial cells of the choroid plexus in the brain ventricles; it serves as a precursor for ECF. CSF is moved through the ventricles into the subarachnoid space by positive hydrostatic pressure generated by the choroid plexus and with the assistance of ependymal cells. From there it is pushed via the periarterial spaces deep into the brain, where it is slowly equilibrated with ECF. The movement and equilibration of CSF and ECF is mediated by the glymphatic system, the idea of which has been largely shaped by M. Nedergaard and co-workers (Iliff et al., 2012; Iliff et al., 2013; Thrane et al., 2014). The combination of CSF plus ECF represents the extracellular space of the brain. (iv) Finally, the segregated blood compartment contains vessels of various diameters and circulating blood cells, which assist in delivering oxygen and nutrients to the brain tissue. All hydrophilic components (ions and nutrients) are transported inside the brain in a highly regulated manner via either the choroid plexus or the endothelial blood-brain barrier (BBB). The main message here is that in the brain, any change in the volume of an individual fluid compartment can happen only at the expense of its counterparts, unlike other tissues which can expand in a relatively uniform fashion (see Fig. 12.1C).

*The total volume of the ECF is very small, which puts significant constraints on cell volume regulation.* Due to the restricted transport of electrolytes across the BBB, brain tissue contains a rather small pool of extracellular osmolytes. For the sake of argument, let us simplistically assume that the extracellular space occupies 20%, and the intracellular space 80%, of the total brain volume. A moderate, 10% increase in volume of the cellular compartment (swelling) will occur at the expense of the extracellular space and reduce the latter from the initial 20 to 12% of the total brain volume (or nearly two-fold). This on its own would concurrently increase the concentration of extracellular ions and signaling molecules and modify their diffusion parameters. Now, consider that swollen brain cells engage in the process of RVD (see section 12.1.1) and release 10% of their cytosolic  $K^+$  content. Based on the intracellular  $[K^+]$  in the range of 130–140 mM, and taking into account the above-mentioned volume ratios, we should expect a rise in the extracellular  $[K^+]$  to well above 50 mM. Needless to say, this would dramatically diminish the driving force for RVD and be incompatible with brain function. This overly-simplistic example can explain why cell volume regulation in the brain is likely significantly less effective than in other tissues, but also disproportionately impacts tissue functions.

*The morphological complexity of brain cells may allow for “local” fluctuations in the volume of their subcellular compartments.* As an extreme example, we can consider projection neurons, which have exceptionally long and voluminous processes (both axonal and dendritic). The volume of the neuronal cell body represents <1% and in many cases as little as 0.1% of the total cell volume (Morfini, Stenoien, & Brady, 2006). Therefore, local ionic fluxes in the distal processes (such as during action potentials or pathological depolarization) are likely to change local volume independently of the cell body.

*Release of typical cellular osmolytes (both inorganic and organic) has a strong physiological impact on brain cells.* Since the brain is an excitable tissue, changes in the intracellular and the extracellular levels of  $K^+$ ,  $Na^+$ , and  $Cl^-$  not only contribute to cell volume control, but also dramatically modify neuronal excitability (see example above). Furthermore, many of the “classical” compatible organic osmolytes are important signaling molecules in the brain. For instance, the most abundant cytosolic amino acid and organic osmolyte, glutamate, is the main excitatory neurotransmitter in the brain playing a signaling role in 90% of the excitatory synapses (Meldrum, 2000). Another highly enriched cytosolic molecule, taurine, is an endogenous ligand for the two most important inhibitory receptors, GABA and glycine receptor-channels (Albrecht & Schousboe, 2005).

## 12.2. RVD IN THE CNS CELLS

Even in the face of the abovementioned limitations and caveats, brain cells are capable of regulating their volume much like the majority of cells in other tissues. The idea of active cell volume control within the brain was initially proposed based on early *in vivo* observations of the compensatory loss of inorganic ions and organic osmolytes from brain tissue in response to systemic hyponatremia (Yannet, 1940; Melton & Nattie, 1983; Thurston & Hauhart, 1987; Melton, Patlak, Pettigrew, & Cserr, 1987; Verbalis & Gullans, 1991). These latter studies were matched with numerous clinical and basic science findings that reductions in local or systemic osmolarity (hyponatremia) lead to the modification of neuronal excitability and the development of life-threatening neurological deficits, which tend to resolve over time as the brain adapts [see (Andrew, 1991; Adrogue & Madias, 2000; Sterns, 2015) and section 12.6.1 for references and further discussion].

### 12.2.1 Neuronal RVD and its ionic mechanisms

To the best of our knowledge, the first direct observation of neuronal RVD was made in cultures of dorsal root ganglion (DRG) cells by H. Horie and colleagues (Horie, Ikuta, Takenaka, & Ito, 1989). Most of the subsequent early mechanistic studies were done in cultures of cerebellar granular cells by the group of H. Pasantes-Morales. They used a combination of Coulter counter and radiotracer assays to quantify RVD and osmolyte fluxes. Once subjected to hypoosmotic media, DRG and cerebellar neurons rapidly swell and then completely or partially regulate their volume to the same or a new steady-state level within 15 min (Horie et al., 1989; Pasantes-Morales, Maar, & Moran, 1993; Pasantes-Morales, Chacon, Murray, & Moran, 1994). The RVD process is driven by the loss of cytosolic  $K^+$ , because disruption of the transmembrane  $K^+$  gradient with either high  $[K^+]_o$  or high  $[Rb^+]_o$  media completely blocks compensatory volume decrease (Pasantes-Morales et al., 1993). As in many other cells,  $K^+$  permeability is the rate-limiting step in this process because the  $K^+$  ionophore gramicidin dramatically increases the rate of RVD (Pasantes-Morales et al., 1994). Nevertheless,  $K^+$  fluxes have to be supplemented by the efflux of  $Cl^-$  and other organic and inorganic osmolytes, the functional importance of which is underscored by the fact that RVD is strongly inhibited by  $Cl^-$  channel blockers or by adding high millimolar levels of extracellular amino acids (Pasantes-Morales et al., 1993; Pasantes-Morales et al., 1994).

The initial observations of neuronal swelling and RVD have been reproduced in many neuronal cell types, including acutely isolated peripheral sympathetic neurons (Leaney, Marsh, & Brown, 1997), cerebellar granular cells (Patel, Lauritzen, Lazdunski, & Honore, 1998), cultured cortical neurons (Inoue, Mori, Morishima, & Okada, 2005), and the neuroblastoma N1E-115 cell line (Falke & Misler, 1989; Altamirano, Brodwick, & Alvarez-Leefmans, 1998). In these latter studies, cell volume dynamics in the substrate-attached cells was assessed using phase microscopy, or indirectly, based on the changes in intracellular calcein fluorescence. Several publications have explored the biophysical properties and possible mechanisms of activation of volume-sensitive  $\text{Cl}^-$  channels and  $^{125}\text{I}^-$  efflux in neuroblastoma cell lines (Basavappa et al., 1995; Bond, Basavappa, Christensen, & Strange, 1999; Cheema, Pettigrew, & Fisher, 2007) and primary neuronal cells (Leaney et al., 1997; Inoue et al., 2005). The conclusions of these early reports were that (i) neurons respond to hypoosmotic gradients with predictable osmotic swelling, (ii) in the majority of cases, neurons are capable of active cell volume control, (iii) neuronal RVD is driven by volume-sensitive  $\text{K}^+$  and  $\text{Cl}^-$  fluxes. However, the molecular identities of swelling-activated  $\text{K}^+$  and  $\text{Cl}^-$  channels in neuronal cells remain poorly unexplored. They are inferred based on gene expression profiles and functional data obtained in other cell types. Due to the high background  $\text{K}^+$  conductance and diverse expression, many types of  $\text{K}^+$  channels may assist in neuronal RVD, and these need not be necessarily cell-volume sensitive. In contrast, since the resting  $\text{Cl}^-$  conductance is low, RVD is only possible with significant increases in  $\text{Cl}^-$  permeability, which are mediated by VRAC. Brain VRAC channels are assembled from the proteins belonging to the LRRC8 family, but, with the exception of one study in primary sensory neurons (Wang et al., 2017), this yet to be directly tested in diverse neuronal cells [see section 12.8 and review (Mongin, 2016)].

Besides the functionally coupled  $\text{K}^+$  and  $\text{Cl}^-$  channels, neuronal RVD can be additionally powered by one or more electroneutral  $\text{K}^+, \text{Cl}^-$  cotransporters. The “classical” KCC1, KCC3, and KCC4 are silent under basal conditions but are activated by increases in cell volume, while the neuron-specific KCC2 is basally active and additionally stimulated by cell swelling (Lauf & Adragna, 2000; Gamba, 2005). The contribution of KCCs to neuronal cell volume control has been suggested in the past but never definitively tested (Kahle et al., 2015; Wilson & Mongin, 2019). In one study, cultured hippocampal pyramidal neurons from KCC3-null mice showed a lack of RVD in hypoosmotic media (Boettger et al., 2003). Also, one of the phenotypic features of the KCC3-knockout animals is the enlarged diameter of peripheral axons, possibly due to defective cell volume regulation (Byun & Delpire, 2007).

The views on the osmotic behavior of neuronal cells have been strongly influenced by a number of studies coming from the groups of R.D. Andrew, G.G. Somjen, and S.A. Kirov, who found that pyramidal neurons in the hippocampus and cortex (acutely isolated or in brain slices), do not swell in response to moderate hypoosmotic challenge or swell with a significant delay (Somjen, Faas, Vreugdenhil, & Wadman, 1993; Aitken et al., 1998; Andrew, Labron, Boehnke, Carnduff, & Kirov, 2007; Caspi, Benninger, & Yaari, 2009). Interestingly, these publications also reported that the same neuronal cells readily increase their volume in response to spreading depolarizations or ischemia-like conditions. Another study performed side-by-side comparison of acutely isolated hippocampal pyramidal cells and the hypothalamic magnocellular neurons and found that only magnocellular neurons



swell to the predicted degree, while pyramidal cells do not behave as typical osmometers (Zhang & Bourque, 2003). Furthermore, intravital work in transgenic animals expressing EGFP in neuronal cells, registered no swelling of dendritic processes in response to hypoosmotic gradients as large as  $-100$  mOsm (Steffensen, Sword, Croom, Kirov, & MacAulay, 2015). Overall, these findings have been explained by the very low water permeability of neuronal membranes due to the lack of aquaporins (Andrew et al., 2007; Caspi et al., 2009), and/or by the paradoxical osmotic shutdown of the major ion permeability pathways in swollen pyramidal cells (Somjen et al., 1993). Although this narrative has become very dominant, is hard to say if low water permeability is a general property of pyramidal neurons, or the result of select experimental conditions. The idea of osmotic resistance of cortical and hippocampal pyramidal cells has been recently challenged by the work of T. Fiacco and co-workers, who found in brain slices that pyramidal neurons swell and shrink to the same degree as nearby “water-permeable” astrocytes (Murphy et al., 2017). Additionally, the already mentioned KCC3 study in hippocampal pyramidal cells exposed to hypoosmotic conditions showed complete RVD within 20 minutes (Boettger et al., 2003). In brain slices, the ability to regulate cell volume under hypoosmotic conditions has been attributed to a dependence on intracellular taurine content, as it was regained in slices preincubated with taurine-containing media (Kreisman & Olson, 2003). Another already mentioned factor (see section 12.1.4) are the limitations imposed by small extracellular space *in vivo*, which may reduce the driving force of RVD.

As briefly outlined in the introduction (section 12.1.4), brain cells, specifically neurons, have such an elaborate morphology that the distant parts of the cell are likely to experience “local” hydration gradients and require “decentralized” mechanisms of cell volume control. Neuronal hyperexcitation is associated with significant fluxes of ions and water. Dendrites, which are endowed with a high density of postsynaptic ionotropic receptors, have especially high propensity to swell. This phenomenon is known as dendritic beading or the formation of synaptic varicosities; which is particularly pronounced under pathological conditions [reviewed in (Fiala, Spacek, & Harris, 2002; Greenwood & Connolly, 2007)]. Consequently, an important question is; are neuronal processes capable of “local” volume control? The few studies that have explored this topic suggest an affirmative answer. For instance, swollen synaptosomes (isolated presynaptic nerve endings) completely recover their volume within 2 minutes after exposure to hypoosmotic media (Babila et al., 1990). Within the same time frame, swollen synaptosomes strongly upregulate their  $\text{Ca}^{2+}$  uptake via activation of unidentified cation channels (Mongin, Aksentsev, Orlov, & Konev, 1997). Hypoosmolarity-induced  $\text{Ca}^{2+}$  influx may explain the swelling-induced exocytosis of neurotransmitters, however, other mechanisms of release have also been suggested (Tuz, Pena-Segura, Franco, & Pasantes-Morales, 2004; Waseem, Rakovich, Lavrukevich, Konev, & Fedorovich, 2005; Tuz & Pasantes-Morales, 2005). In cortical neurons, acute exposure to glutamate or NMDA causes rapid (within 10 min), swelling of their somata and formation of dendritic varicosities (Inoue & Okada, 2007). For both compartments, this process is completely  $[\text{Na}^+]_o$ -dependent, but also inhibited by non-selective VRAC blockers. Once NMDA-receptor agonists are removed, swelling of the cell body and dendritic varicosities is resolved within 20–60 min, and the latter volume recovery is also sensitive to VRAC inhibitors (Inoue & Okada, 2007). These data suggest that in neuronal processes, VRAC contributes to both

cellular swelling and compensatory RVD during and after treatment with excitotoxins. Collectively, studies in synaptosomes and neuronal cultures suggest that in neuronal cells, cell volume-sensitive ion channels are distributed throughout their distal cell processes, in both the dendritic and the axonal compartments, and mediate cell volume control. The  $\text{Cl}^-$  movement in local volume control appears to be largely mediated by VRAC.

### 12.2.2 Astrocytic RVD and swelling-activated ion channels

Although neurons represent the main cell type in the CNS, they are significantly outnumbered by several classes of non-neuronal cells – neuroglia. Among neuroglia, astrocytes are the most numerous and functionally complex, and the most studied in the context of cell volume regulation (Haydon & Carmignoto, 2006; Mongin & Kimelberg, 2009; Sofroniew & Vinters, 2010; Verkhratsky & Nedergaard, 2018). The keen interest in astrocytic cell volume homeostasis and its dysregulation was prompted by the very early knowledge that many diverse factors that lead to brain edema – such as toxins, hypoxia and metabolic inhibition, the  $\text{Na}^+, \text{K}^+$ -pump blocker ouabain, and brain traumas – all trigger profound swelling of astrocytes and corresponding reductions in the extracellular space [e.g., (Cornog, Gonatas, & Feerman, 1967; Bakay & Lee, 1968; Bourke & Nelson, 1972; Baethmann & Van Harreveld, 1973; Bakay, Lee, Lee, & Peng, 1977)]. The pathological significance of these and other relevant findings is discussed in section 12.6, while the potential reasons for preferential swelling of astrocytes are considered in section 12.5. The first observations of RVD in glial cells have been made in the C6 glioma cell line by the laboratory of A. Baethmann (Kempinski, Chaussy, Gross, Zimmer, & Baethmann, 1983), and primary astrocyte cultures by H.K. Kimelberg and co-workers (Kimelberg & Frangakis, 1985; O'Connor, Kimelberg, Keese, & Giaever, 1993) and J.E. Olson *et al.* (Olson, Sankar, Holtzman, James, & Fleischhacker, 1986). The bulk of the subsequent mechanistic studies were carried out by the laboratories of H. Pasantes-Morales (Sanchez-Olea, Pena, Moran, & Pasantes-Morales, 1993; Pasantes-Morales, Murray, Lilja, & Moran, 1994; Pasantes-Morales, Murray, Sanchez-Olea, & Moran, 1994) and M.D. Norenberg (Bender, Neary, Blicharska, Norenberg, & Norenberg, 1992; Bender, Neary, & Norenberg, 1993).

Much like in neuronal cells, astrocytic RVD is mediated by the loss of  $\text{K}^+$  and  $\text{Cl}^-$  via functionally coupled  $\text{K}^+$  and  $\text{Cl}^-$  channels. These were initially explored using radiotracer assays and pharmacological inhibitors (Sanchez-Olea *et al.*, 1993; Sanchez-Olea, Moran, Martinez, & Pasantes-Morales, 1993; Vitarella, DiRisio, Kimelberg, & Aschner, 1994; Pasantes-Morales *et al.*, 1994). The biophysical properties and regulation of swelling-activated ion channels have been further explored using an electrophysiological approach. Surprisingly, the molecular nature and properties of swelling-activated glial  $\text{K}^+$  channels received little attention, perhaps because the membrane conductance in swollen cells is dominated by  $\text{Cl}^-$  channels. Yet, it is known that two types of channels dominate  $\text{K}^+$  permeability in astroglia: the volume-insensitive, inward-rectifier channels, such as Kir4.1 (*KCNJ10*), and the swelling-activated two-pore domain TWIK-1 and TREK-1 channels [reviewed in (Kofuji & Newman, 2004; Olsen *et al.*, 2015); but see the alternative opinion on Kir4.1 volume sensitivity in (Soe, MacAulay, & Klaerke, 2009)]. In contrast, numerous laboratories studied the biophysical properties of astroglial VRAC as a pathway for  $\text{Cl}^-$  release (Jackson & Strange, 1993; Jackson *et al.*, 1994; Olson & Li, 1997; Parkerson &

Sontheimer, 2004; Crepel, Panenka, Kelly, & MacVicar, 1998; Abdullaev, Rudkouskaya, Schools, Kimelberg, & Mongin, 2006; Liu, Tashmukhamedov, Inoue, Okada, & Sabirov, 2006; Minieri, Pivonkova, Harantova, Anderova, & Ferroni, 2015). VRAC properties and functions in astrocytes and other CNS cells are summarized in two recent comprehensive reviews (Akita & Okada, 2014; Mongin, 2016). Here, we present for illustrative purposes our own electrophysiological recordings of typical VRAC currents in primary astrocytes (Fig. 12.2A–B). Besides VRAC, other types of swelling activated  $\text{Cl}^-$  channels, such as the voltage gated  $\text{ClC-2}$  channels and maxi- $\text{Cl}^-$  channels, also exist and are expressed in astrocytes. These may provide additional minor contributions to RVD (Parkerson & Sontheimer, 2004; Liu et al., 2006). The molecular nature of astrocytic VRAC has been uncovered only recently (Hydzinski-Garcia, Rudkouskaya, & Mongin, 2014; Schober, Wilson, & Mongin, 2017) and is further discussed in section 12.8.

As already discussed, besides conducting inorganic anions, VRAC is permeable to a variety of organic osmolytes. The first publications on glial swelling-activated release of organic osmolytes putatively linked this process to VRAC based on sensitivity to diverse chloride channel blockers (Pasantes Morales & Schousboe, 1988; Pasantes-Morales, Moran, & Schousboe, 1990; Martin, Madelian, Seligmann, & Shain, 1990; Kimelberg, Goderie, Higman, Pang, & Waniewski, 1990; Strange & Morrison, 1992; Strange, Morrison, Shrode, & Putnam, 1993). The release of compatible osmolytes was initially considered a solely beneficial process, assisting in cell volume control. However, H.K. Kimelberg introduced a very counterintuitive at the time idea on the potentially harmful aspects of such release (Kimelberg et al., 1990). Because two cytosolic amino acids, glutamate and aspartate, are the excitatory neurotransmitters, their release can be toxic and drive tissue damage in a variety of brain pathologies. This aspect of VRAC activity has been the subject of several comprehensive reviews (Kimelberg & Mongin, 1998; Kimelberg, 2005; Mongin, 2007; Mongin, 2016) and is further discussed in sections 12.6 and 12.7. The link between organic osmolyte release and VRAC has been extensively tested in glial cells in several electrophysiological studies, which directly measured amino acid currents (Jackson & Strange, 1993; Roy, 1995; Olson & Li, 1997; Abdullaev et al., 2006; Liu et al., 2006). Here, we present our own data on the swelling-activated glutamate release in cultured astrocytes, and the microdialysis study of the VRAC-mediated glutamate release in the brain cortex *in vivo* (Fig. 12.2C, D). Interestingly, our recent molecular biology work strongly suggests that there are at least two different heteromeric VRAC complexes, which have preferential selectivity for uncharged and charged organic osmolytes [see (Schober et al., 2017) and additional discussion in section 12.8].

Another important idea related to the functional consequences of cell volume control in the CNS is VRAC regulation by G-protein-coupled receptors (GPCRs). In the brain, the degree of cell swelling under physiological conditions is estimated to be in the order of 2–5% of “normal” cell volume. This is unlikely to be sufficient to open VRAC to any significant degree. However, we and others found that astroglial VRAC can be potently modulated by GPCR signaling. E.g., in astrocytes, activation of purinergic P2Y receptors causes partial,  $\text{Ca}^{2+}$ -dependent stimulation of VRAC, even when cells are not swollen (Mongin & Kimelberg, 2002; Darby, Kuzmiski, Panenka, Feighan, & MacVicar, 2003; Takano et al., 2005; Mongin & Kimelberg, 2005; Akita, Fedorovich, & Okada, 2011). ATP and ADP act at

P2Y<sub>1</sub>, P2Y<sub>2</sub> and/or P2Y<sub>4</sub> receptors, prompt the release of intracellular Ca<sup>2+</sup>, activation of classical PKC  $\alpha$  and  $\beta$ I, and additionally Ca<sup>2+</sup>/calmodulin-dependent PK II (Mongin & Kimelberg, 2005; Rudkouskaya, Chernoguz, Haskew-Layton, & Mongin, 2008; Akita et al., 2011). Interestingly, pharmacological inhibitors and molecular biology tools that block the GPCR-driven VRAC opening have little to no effect on VRAC activation by hypoosmotic media. Based on our work, we proposed a model in which purinergic signaling activates or modulates channel activity via phosphorylation of a small fraction of VRAC channels that are already open or “primed” for opening. Although ATP effects are measurable in non-swollen cells, the main, larger pool of VRAC molecules remains inaccessible for purinergic stimulation. The concurrent cell swelling, even as small as 5%, recruits from the “silent”, volume-sensitive pool of VRAC, and acts synergistically with the purinergic receptors (Mongin & Kimelberg, 2005).

Following our findings on the purinergic modulation of VRAC activity in astrocytes, several other groups uncovered very similar synergistic effects of cell swelling and diverse GPCR receptors, linked to either Ca<sup>2+</sup> or cAMP signaling, on VRAC-like Cl<sup>-</sup> currents and organic osmolyte release. For astrocytes and astrocytic cell lines, the reader can be referred to the following significant studies: (Rosso, Peteri-Brunback, Poujeol, Hussy, & Mienville, 2004; Cheema, Ward, & Fisher, 2005; Ramos-Mandujano, Vazquez-Juarez, Hernandez-Benitez, & Pasantes-Morales, 2007; Liu, Akita, Shimizu, Sabirov, & Okada, 2009; Akita & Okada, 2011). Findings in non-CNS cells are covered by three comprehensive reviews (Fisher, Cheema, Foster, & Heacock, 2008; Franco, Panayiotidis, & de La Paz, 2008; Fisher, Heacock, Keep, & Foster, 2010). Recently, to definitively discriminate between VRAC and other potential mechanisms of ATP-stimulated amino acid release (such as vesicular release and others), we demonstrated that ATP-stimulated glutamate and taurine release is abolished by knocking down the essential VRAC subunit LRRC8A (Hydzinski-Garcia et al., 2014).

### 12.3. RVI IN THE CNS CELLS

Severe systemic hyperosmolarity, such as increases from ~290 to 410–420 mOsm, places mammals (studied mostly in rodent models) in danger of death (Arieff, Guisado, & Lazarowitz, 1977; Gullans & Verbalis, 1993). Milder elevations in systemic osmolarity are typically associated with behavior abnormalities, such as restlessness and increased startle response (Arieff et al., 1977; Gullans & Verbalis, 1993). Numerous early studies, most notably by H.F. Cserr and colleagues, found that in response to systemic hypertonic perturbations the brain as an organ shrinks but then effectively adapts, and regulates its volume via the accumulation of osmolytes and osmotically obligated water on the scale of a few hours to days [e.g., (Cserr, DePasquale, & Patlak, 1987; DePasquale, Patlak, & Cserr, 1989; Cserr et al., 1991)}(Gullans & Verbalis, 1993)]. The measurable tissue volume regulation occurs as rapidly as 90 min after hypertonic insult and is associated with the uptake of Na<sup>+</sup>, Cl<sup>-</sup>, and K<sup>+</sup>, with the bulk of these ions accumulating in the intracellular rather than extracellular compartment (Cserr, DePasquale, & Patlak, 1987; Cserr et al., 1991). These results strongly suggest that brain cells undergo successful RVI *in vivo*. The secondary phase of adaptation to chronic hyperosmotic conditions involves the accumulation of idiogenic (compatible) organic osmolytes, which slowly replace the excess of inorganic

ions within one or two weeks [see for example (Chan & Fishman, 1979; Lohr, McReynolds, Grimaldi, & Acara, 1988) and review (Gullans & Verbalis, 1993)].

In contrast to the whole brain, studies in cultured glial and neuronal cells found no evidence of RVI when cells have been exposed to hyperosmotic media for 10–90 min (Kimelberg & Frangakis, 1985; Horie et al., 1989; O'Connor et al., 1993; Zhang & Bourque, 2003). Similarly, pyramidal neurons in brain slices prepared from either neocortex or hippocampus readily shrunk under hyperosmotic conditions but did not demonstrate RVI, at least during incubation times under 1 h (Andrew et al., 2007). To the best of our knowledge, the only two known reports of RVI in brain cells come from the studies of Kempfski et al. in a C6 glioma cell line (Kempfski et al., 1983) and D. Sun and colleagues in immature rat neurons (Schomberg et al., 2003). The former work found complete cell volume recovery (RVI) in C6 cells incubated in hyperosmotic media for a full three hours, but only if medium osmolarity was increased by adding non-ionic compounds (mannitol). When osmolarity was increased with NaCl, RVI was not observed (Kempfski et al., 1983). The latter publication (Schomberg et al., 2003) reported relatively fast (within 20 min) and effective (60%) volume recovery in immature rat neurons exposed to osmolarity increased by adding sucrose. Additionally, murine astrocytes also demonstrated a trend for RVI upon exposure to sucrose-containing hyperosmotic media, but this process was efficacious only when cells were co-stimulated with adrenergic agonists (Song, Xu, Hertz, & Peng, 2015). Overall, it appears that cultured neuronal and glial cells do not have transmembrane ionic gradients and ion transporter levels which would favor RVI (see section 12.1.2).

Due to the lack of RVI in model experiments, and because hypertonicity represents a relatively rare and not life-threatening phenomenon in the CNS, there have been surprisingly few studies exploring volume-sensitive ion transporters in brain cells. We could recall only one paper on hypertonic activation of the  $\text{Na}^+/\text{H}^+$  exchanger in rodent astrocytes (Shrode, Klein, O'Neill, & Putnam, 1995). NKCC1 stimulation under hyperosmotic conditions has been reported in the already mentioned study in immature rat neurons (Schomberg et al., 2003). In contrast, there are numerous studies linking NHE transporters and NKCC1 to adverse outcomes in various neuropathologies, particularly stroke [reviewed in (Mongin, 2007; Kintner, Wang, & Sun, 2007)].

## 12.4 CELL VOLUME CHANGES DURING NORMAL BRAIN FUNCTIONING

Brain tissue exists in an environment of near constant osmolarity, which is accomplished via the tight control of systemic water-electrolyte homeostasis and is due to restrictions of electrolyte transport across the blood-brain barrier [reviewed in (Strange, 1992; Bourque & Olié, 1997; Kimelberg, 2004)]. Nevertheless, brain cells experience constant fluctuations of their volume due to ionic and osmolyte fluxes resulting from electrical activities, reuptake and recycling of neurotransmitter molecules, and dynamic changes in tissue metabolism. The first study directly demonstrating nerve swelling upon electrical stimulation was performed in isolated nerve fibers of invertebrates (Iwasa, Tasaki, & Gibbons, 1980). This pioneer work was followed by numerous observations in higher organisms. One approach, which has been widely utilized in brain slices, is based on detecting variations in light scattering or the intrinsic optical signal (IOS) of the tissue, which indirectly reflect changes

in cell volume (Lipton, 1973; Andrew & MacVicar, 1994; Aitken, Fayuk, Somjen, & Turner, 1999). Using IOS signals, several groups demonstrated that electrical stimulation of cortical and hippocampal brain slices causes cellular swelling (MacVicar & Hochman, 1991; Andrew & MacVicar, 1994; Holthoff & Witte, 1996).

An alternative technique allowing for the measurement of interstitial volume is dynamic monitoring of the extracellular concentrations of poorly membrane permeant cations, such as tetramethylammonium (TMA<sup>+</sup>) or tetraethylammonium (TEA<sup>+</sup>) [reviewed in (Nicholson & Sykova, 1998; Sykova, 2004; Sykova & Nicholson, 2008)]. The relevant chemical probes are introduced into the CNS electrophoretically, and then detected with ion-selective electrodes. The rate of diffusion of TMA<sup>+</sup>/TEA<sup>+</sup> from the point of injection to the registering electrode gives information on the tortuosity of the extracellular space. Fluctuations of TMA<sup>+</sup>/TEA<sup>+</sup> concentrations under steady-state conditions reflects changes in the volume of the interstitial liquid. Using this type of assay, several groups found that electrical stimulation of neurons causes significant reductions in the extracellular space (cell swelling) in brain preparations from lower vertebrates and mammals [see for example (Svoboda & Sykova, 1991; Jing, Aitken, & Somjen, 1994; Larsen et al., 2014; Larsen & MacAulay, 2017) and comprehensive review by (Sykova & Nicholson, 2008)].

The oldest method for monitoring changes in the volume of interstitial liquid is the electrical impedance assay, which was developed by A. Van Harreveld and co-workers (Van Harreveld, Dafny, & Khattab, 1971; Van Harreveld, 1972). This approach employs measuring changes in the extracellular resistance (impedance) upon the delivery of electric currents with their parameters selected to not allow charge transfer across cellular membranes. Using this technique or its modifications, several groups demonstrated dynamic activity-dependent changes in ionic concentrations and volume of the extracellular space (Dietzel, Heinemann, Hofmeier, & Lux, 1982; Dietzel, Heinemann, & Lux, 1989; Amzica & Neckelmann, 1999). F. Amzica and D. Neckelmann were able to deduce which cell type is swollen, by adding an additional approach and measuring the activity-dependent changes in capacitance (proportional to cell volume) in both neurons and astrocytes in the cortex of anesthetized cats. The capacitance changes of patched astrocytes closely followed neuronal activity and were most pronounced during seizure-like events, suggesting that swelling of astroglia modifies ECF volume. In contrast, apparent changes in neuronal cell volume were smaller and did not correlate with degree of excitation (Amzica & Neckelmann, 1999).

Overall, each of the abovementioned methodologies reveal measurable changes in the volume of the intracellular and extracellular compartments in response to neuronal activation. These are mediated by the activity-dependent fluxes of ions and movement of osmotically obligated water. Changes in the volume and geometry of the extracellular compartment are sufficient to impact neuronal excitability in a normal brain, but are particularly profound in neuropathologies [see review by (Sykova & Nicholson, 2008) and section 12.6]. Depending on the study, cellular swelling has been ascribed to either neurons or astrocytes, but most likely multiple cell types are affected. Thus, N.N. Haj-Yasein *et al.* analyzed ECF shrinkage (inversely related to cell swelling) in response to high-frequency stimulation in hippocampal slices prepared from either wild type animals or animals carrying deletion of the astrocytic water channel aquaporin-4 (AQP4). In the regions

enriched with astrocytic processes, nerve stimulation triggered shrinkage of the extracellular space that was strongly influenced by the deletion of AQP4, suggesting that it occurred in astrocytes. In contrast, in the areas devoid of astrocytic processes, changes in the extracellular space were more profound but independent of AQP4 expression, pointing to their neuronal origin (Haj-Yasein et al., 2012).

Another important example of physiologically relevant cell volume changes can be found in hypothalamic neuroendocrine structures, which sense minor osmolarity changes in systemic circulation. The supraoptic (SON) and the paraventricular (PVN) nuclei of the hypothalamus contain magnocellular neurons, whose main function is regulation of the body's water-electrolyte homeostasis (Bourque & Oliet, 1997; Hussy, Deleuze, Desarmenien, & Moos, 2000; Bourque, 2008). These neurons project to the pituitary gland and secrete the antidiuretic hormones, vasopressin and oxytocin. Once released into systemic circulation, vasopressin in humans and vasopressin and oxytocin in rodents regulate water reabsorption in the kidney. Neuronal activity in the SON and PVN and release of antidiuretics are activated by hyperosmolarity and inhibited by hypoosmolarity via several distinct mechanisms, which are comprehensively discussed in several specialized reviews (Hussy et al., 2000; Bourque, 2008; Noda & Hiyama, 2015). Briefly: (i) Magnocellular neurons in the SON and PVN are regulated via the excitatory inputs from the upstream osmosensitive neurons located in the organum vasculosum lamina terminalis (OVLT) and the subfornical organ (SFO). (ii) Both the OVLT and SFO cells and the SON and PVN neurons are intrinsically osmosensitive, via the mechanism deciphered in the laboratory of C.W. Bourque. This group discovered that hyperosmolarity directly stimulates osmosensitive neurons via opening the volume-sensitive splice variant of the TRPV1 channel, which lacks a portion of its amino terminus (N-TRPV1) (Bourque, Oliet, & Richard, 1994; Voisin, Chakfe, & Bourque, 1999; Ciura & Bourque, 2006; Ciura, Liedtke, & Bourque, 2011). (iii) An alternative, non-cell-autonomous mechanism responsible for sensing hypoosmolarity was identified by the group of N. Hussy, who found that even moderate reductions in medium osmolarity stimulate specialized populations of SON and PVN astrocytes, which contain extremely high cytosolic levels of taurine. Once swollen, astrocytes release taurine via a VRAC-like channel (Deleuze, Duvold, & Hussy, 1998; Bres et al., 2000). Taurine inhibits the activity of SON and PVN magnocellular neurons by acting as the endogenous ligand for their inhibitory glycine receptors (Hussy, Deleuze, Pantaloni, Desarmenien, & Moos, 1997). A similar, astrocyte-specific mechanism was found in OVLT, where swelling triggers  $\text{Ca}^{2+}$  influx via TRPV4 and amplifies astrocytic taurine release via VRAC (Ciura et al., 2018). Overall, the direct activation of neurons by shrinkage and their indirect inhibition by taurine release from swollen astrocytes mediate the CNS osmolarity sensing.

## 12.5 A SPECIAL CASE FOR ASTROCYTIC SWELLING AND CELL VOLUME CONTROL

A large variety of brain pathologies – including hyponatremia, stroke, traumatic brain and spinal cord injuries, epilepsy, hepatic failure, and hypoglycemia – are associated with the prominent pathological swelling of astrocytes [see reviews (Norenberg, 1994; Kimelberg, 1995; Mongin & Kimelberg, 2005; Stokum, Gerzanich, & Simard, 2016) and section 12.6

for further discussion and references]. The phenomenon of pathological swelling in the CNS has been historically termed cytotoxic brain edema (Klatzo, 1967; Kimelberg, 1995; Stokum et al., 2016). This unusual name originates from the early studies on the effects of certain toxins, such as dinitrophenol, triethyltin, and hexachlorophene, on brain tissue. The latter toxic substances trigger the shift of electrolytes and water from the extracellular to the intracellular space, without the net accumulation of water in the brain or swelling of brain tissue. To avoid confusion, H.K. Kimelberg introduced the more accurate term of cellular edema (Kimelberg, 1995). Cellular/cytotoxic edema is usually explained by oncotic (Donnan) swelling of metabolically compromised cells. This is in contrast to vasogenic edema, which is caused by disruption of the integrity of the BBB at the blood-brain interface. Vasogenic edema causes the accumulation of plasma proteins and net water influx into the brain tissue; and is associated with massive and potentially life-threatening brain swelling. Cellular edema typically precedes vasogenic edema, but these two processes are connected. Despite much work in the field, the molecular mechanisms underlying cellular edema are incompletely understood. The major unanswered question is why cell swelling is preferentially seen in one cell type – astrocytes. In this section text and Fig. 12.3, we present several prevalent hypotheses.

### 12.5.1 Water permeability and aquaporin channels

As shown in Fig. 12.3A, one popular explanation for the preferential swelling of astrocytes is the high water permeability of their membranes, which is facilitated by the presence of water channels, aquaporins (AQP). Astrocytes abundantly express the AQP4 protein, particularly in their processes contacting brain capillaries and larger vessels (Amiry-Moghaddam & Ottersen, 2003). Deletion of AQP4 in astrocytes reduces their water permeability by ~sevenfold at 12°C but only twofold at 37°C (Solenov, Watanabe, Manley, & Verkman, 2004). It is well known that water can move in and out of the cell through many alternative routes, passively or via assorted channels and transporters conducting major osmolytes [see (MacAulay, Hamann, & Zeuthen, 2004) and section 12.5.2]. Nonetheless, despite the relatively modest decrease in water permeability at physiological temperatures, AQP4-null animals show high resistance to systemic water intoxication, (hyponatremia) as compared to their wild type counterparts (Manley et al., 2000). Specific enrichment of AQP4 in astrocytic endfeet is dependent on the adaptor proteins,  $\alpha$ -syntrophin and dystrophin (Amiry-Moghaddam et al., 2003). Deletion of  $\alpha$ -syntrophin impedes water movement from the blood to the brain and reduces tissue edema in an animal model of stroke (Amiry-Moghaddam et al., 2003). Because brain and cell swelling in neuropathologies develops on a slow scale, it is not clear if the twofold changes in water permeability in AQP-null astrocytes or disruption of AQP4 localization in the astrocytic endfeet seen in  $\alpha$ -syntrophin knockouts can explain the observed resistance to cellular and/or tissue edema. Perhaps, AQP4 plays more complex roles in regulating water movement and osmotic gradients, via modulating activities of ion channels and transporters. E.g., AQP4 can form functional complexes with the non-selective cation channel TRPV4 (Benfenati et al., 2011), the SUR1-TRPM4 channel complex (Stokum et al., 2018), and also regulates the activity of VRAC (Benfenati et al., 2007).



### 12.5.2 Neurotransmitter uptake

A well-supported mechanism for astrocytic swelling is related to the critical role for these cells in the re-uptake of the major amino acid neurotransmitters: glutamate, aspartate, and GABA (Danbolt, 2001; Zhou & Danbolt, 2013). Excitatory glutamate and aspartate molecules are taken inside the cell by excitatory amino transporters (EAATs) with 3 Na<sup>+</sup> ions in exchange for 1 K<sup>+</sup> out per each transport cycle, along with the uncoupled conduction of Cl<sup>-</sup> (Machtens et al., 2015; Fahlke, Kortzak, & Machtens, 2016) (Fig. 12.3B). Uptake of the inhibitory amino acid GABA occurs through GAT transporters along with ~2–3 Na<sup>+</sup> and 1 Cl<sup>-</sup> (Willford, Anderson, Spencer, & Eskandari, 2015). There is an extensive literature on glutamate-induced astrocyte swelling in cultures and *in situ*, indicating that such swelling involves the accumulation of Na<sup>+</sup> and Cl<sup>-</sup>, and is Ca<sup>2+</sup>-dependent (Koyama, Sugimoto, Shigenaga, Baba, & Iwata, 1991; Schneider, Baethmann, & Kempfski, 1992; Bender, Schousboe, Reichelt, & Norenberg, 1998; Izumi, Kirby, Benz, Olney, & Zorumski, 1999; Koyama et al., 2000)). Under pathologic conditions such as epilepsy or ischemic stroke, high extracellular glutamate levels can significantly contribute to astrocytic swelling via transporter or metabotropic receptor-mediated mechanisms (Hansson, Johansson, Westergren, & Ronnback, 1994; Bender et al., 1998). Importantly, in addition to increasing the osmotic load of the cell, the neurotransmitter transporters also readily move water (Zeuthen & MacAulay, 2002; MacAulay et al., 2004). E.g., the astrocytic glutamate transporter EAAT1/GLAST has the water permeability of roughly one tenth of that of the water channel AQP1 (MacAulay, Gether, Klaerke, & Zeuthen, 2001).

### 12.5.3 K<sup>+</sup> buffering and Donnan swelling

Another well-recognized function of astroglial cells is the buffering of extracellular K<sup>+</sup>, which is accomplished via the activity of K<sup>+</sup> channels, the Na<sup>+</sup>,K<sup>+</sup>-pump and the NKCC1 cotransporter, and further facilitated by redistributing the intracellular K<sup>+</sup> throughout the astrocytic syncytium (Kofuji & Newman, 2004; MacAulay & Zeuthen, 2012). Their high K<sup>+</sup> uptake capacity and negative membrane potential make astrocytes prone to K<sup>+</sup>-induced swelling (Fig. 12.3C). Moderate elevations in [K<sup>+</sup>]<sub>o</sub> during physiological excitation are thought to be the key reason for the dynamic changes in astrocyte volume in healthy brain tissue [section 12.4, but see alternative view in (Larsen & MacAulay, 2017)]. Work in astrocyte cultures suggests that supraphysiologic increases in [K<sup>+</sup>]<sub>o</sub> (up to 10–15 mM) trigger cell swelling via stimulation of the bumetanide-sensitive NKCC1 (Larsen et al., 2014). The same mechanism has been identified for the activity-dependent astrocyte swelling in optic nerve (MacVicar, Feighan, Brown, & Ransom, 2002). However, in hippocampal slices, bumetanide does not prevent the activity-dependent shrinkage of the interstitial space (and by extension, astrocytic swelling), suggesting that other mechanisms may dominate this process *in situ* and *in vivo* (Larsen et al., 2014).

In pathologies such as cerebral hypoxia and ischemia, extracellular K<sup>+</sup> levels can rise as high as 80 mM (Hansen, 1985). This leads to robust astrocytic swelling via the Donnan mechanism: membrane depolarization creates an inward driving force for Cl<sup>-</sup>, which in turn sustains the electromotive force for the additional influx of Na<sup>+</sup> and K<sup>+</sup> and continued cell swelling (Rutledge & Kimelberg, 1996; Mongin, Cai, & Kimelberg, 1999). In astrocyte cultures, high K<sup>+</sup>-induced cell swelling depends on the extracellular [Cl<sup>-</sup>], and is completely

blocked by  $\text{Cl}^-$  channel blockers and strongly reduced by  $[\text{Ca}^{2+}]_i$  buffering, perhaps because  $[\text{Ca}^{2+}]_i$  facilitates  $\text{K}^+$  accumulation via  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  channels or other  $\text{Ca}^{2+}$ -dependent pathways (Rutledge, Aschner, & Kimelberg, 1998; Mongin et al., 1999). There is experimental evidence that the NKCC1-dependent mechanism appears as equally important as the role of Donnan swelling in promoting astrocyte swelling *in situ* and *in vivo* (see section 12.5.6).

#### 12.5.4 Ammonia uptake and metabolism

Acute and chronic liver diseases frequently lead to increases in the systemic levels of ammonia, which at physiological pH exists in a balance between the charged and uncharged forms ( $\text{NH}_4^+ \leftrightarrow \text{NH}_3$ ).  $\text{NH}_3$  freely crosses the blood-brain barrier, and  $\text{NH}_4^+/\text{NH}_3$  buildup in the brain tissue results in the development of hepatic encephalopathy (see section 12.6.3). Astrocytic swelling is a major feature of this disease and its degree highly correlates with blood ammonia levels (Norenberg, 1977; Swain, Butterworth, & Blei, 1992; Norenberg, 1998). Uncharged  $\text{NH}_3$  enters the cell via passive diffusion through the cell membrane, in a manner dependent on the intracellular pH (Antonenko, Pohl, & Denisov, 1997). In contrast, the charged  $\text{NH}_4^+$  enters the cell through NKCC1 (taken up at the  $\text{K}^+$ -binding site) and via  $\text{K}^+$  channels (Kinne, Kinne-Saffran, Schutz, & Scholermann, 1986; Nagaraja & Brookes, 1998; Allert, Koller, & Siebler, 1998; Marcaggi & Coles, 2001) (see Fig. 12.3D). Ammonia influx through  $\text{K}^+$  channels causes astrocytic membrane depolarization and leads to Donnan swelling (Allert et al., 1998). Extensive *in vitro* and *in vivo* studies, the majority of which have been done in the laboratory of M.D. Norenberg, have shown that elevated levels of ammonia cause persistent astrocytic swelling (Norenberg et al., 1991; Norenberg, 1998). The mechanisms of such swelling are multifaceted and complex. However, it is known that they largely depend on ammonia assimilation by the astrocytic enzyme glutamine synthetase. For further information on ammonia transport, metabolism, and their relationship to cell swelling, see (Marcaggi & Coles, 2001; Norenberg, 1998) and section 12.6.3.

#### 12.5.5 SUR1/TRPM4 non-selective cation channels

A series of studies performed by J.M. Simard and colleagues identified an additional mechanism for astrocytic swelling, which may be selectively relevant to pathological states. Biophysical studies in reactive astrocytes revealed a nonselective cation permeability ( $\text{NC}_{\text{Ca-ATP}}$ ), which was activated in metabolically inhibited cells and caused their swelling and blebbing (Chen & Simard, 2001). The  $\text{NC}_{\text{Ca-ATP}}$  channel contains the sulfonylurea receptor SUR1 that is typically coupled with Kir6.x proteins to form  $\text{K}_{\text{ATP}}$  channels (Chen, Dong, & Simard, 2003). However, in the case of  $\text{NC}_{\text{Ca-ATP}}$  the non-selective cation permeability is mediated by the transient receptor potential melastatin 4 (TRPM4) channel (Woo, Kwon, Ivanov, Gerzanich, & Simard, 2013). In an animal stroke model, SUR1 expression steadily increased up to 6 hours after the initiation of ischemia, and treatment with the sulfonylurea inhibitor glibenclamide reduced brain edema and tissue damage (Simard et al., 2006). Their most recent work demonstrated that SUR1-TRPM4 can form a tripartite complex with AQP4, and such association mediates the pathological, high-capacity influx of water that is ~7.6-fold higher than that stimulated by TRPM4 alone, and ~3.2-fold higher than that induced by the SUR1-TRPM4 assembly (Stokum et al., 2018) (Fig. 12.3E). TRPM4 knockout mice displayed reduced astrocytic cell swelling following cold-injury

(Stokum et al., 2018), but additional studies are needed to determine if this heteromeric complex is critical for cell swelling in other pathologies.

### 12.5.6 NKCC1 activity

As mentioned in section 12.1.2, NKCC1 is one of the primary shrinkage-activated transporters, which contribute to RVI in a variety of cell types (Russell, 2000). In astrocyte cultures, pharmacological inhibition or genetic deletion of NKCC1 prevents the high  $[K^+]_o$ -induced astrocytic swelling and swelling-activated glutamate release (Su, Kintner, & Sun, 2002; Su, Kintner, Flagella, Shull, & Sun, 2002). This is paradoxical because NKCC1 is inhibited by cell swelling. This transporter is regulated by a protein kinase cascade, which incorporates the With No Lysine (K) (WNK) protein kinases WNK1–4 and the two homologous downstream protein kinases, Sterile20-related Proline-Alanine-rich Kinase (SPAK) and Oxidative Stress Responsive kinase 1 (OSR1) (Kahle, Ring, & Lifton, 2008; Alessi et al., 2014). Typically, the activity of the WNK/SPAK/OSR is augmented by cell shrinkage and inhibited by cell swelling with matching effects on NKCC1 (Russell, 2000; Hoffmann et al., 2009). However, astrocytes represent an interesting exception: in these cells, swelling activates NKCC1 nearly as potently as cell shrinkage (Mongin et al., 1994; Mongin et al., 1996). One potential mechanism is the activation of WNK kinases by low intracellular  $Cl^-$ . Astrocytes are high- $Cl^-$  cells, in which swelling and subsequent RVD likely reduce  $[Cl^-]_i$  and relieve the known inhibition of WNK kinases by this anion (Kahle et al., 2008; Pacheco-Alvarez & Gamba, 2011; Piala et al., 2014; Terker et al., 2016). The end result of this unusual regulation would be an amplification of cell swelling due to additional osmolyte intake via NKCC1 (Fig. 12.3F).

### 12.5.7 $Na^+$ -bicarbonate cotransporters and other emerging mechanisms

Recent studies from the N. MacAulay laboratory (Larsen et al., 2014; Larsen & MacAulay, 2017), proposed alternative mechanisms of astrocytic swelling in hippocampal slices. They found that electrical stimulation of neuronal networks causes increases in intracellular  $[K^+]_i$  and shrinkage of the interstitial space, reflecting cellular (astrocyte?) swelling. In brain slices, cell swelling was largely insensitive to the inhibitors of glutamate transporters (TBOA),  $K^+$  channels ( $Ba^{2+}$ ), and NKCC1 (bumetanide). Instead, it was lessened by the non-selective inhibitor of the  $Na^+$ -bicarbonate cotransporter NBCe1, DIDS, and poorly selective monocarboxylate transporter blocker,  $\alpha$ -cyano-4-hydroxycinnamate (Larsen & MacAulay, 2017), suggesting the contribution of these two latter transport mechanisms. Although this work remains to be validated using more selective molecular biology and genetics tools, the underlying ideas are supported by the literature. Thus, S.J. Mulligan and co-workers found that the  $K^+$ -induced astrocytic swelling in hippocampal slices is largely dependent on the presence of bicarbonate in the media, and is strongly reduced by blockers of NBCe1 and GABA<sub>A</sub> receptors (Florence, Baillie, & Mulligan, 2012). To what extent these additional mechanisms can contribute to pathological swelling of astroglia remains to be tested.

## 12.6. FAILURE OF CELL VOLUME CONTROL IN BRAIN PATHOLOGIES

In this section, we discuss several examples of neuropathologies which are associated with defective cell volume control (persistent cell swelling), as well as the potential relevance of cell volume changes to disease outcomes.

### 12.6.1 Hyponatremia

Hyponatremia is the most prevalent electrolyte disorder, which manifests as a drop in serum  $\text{Na}^+$  levels below the threshold of  $\sim 135$  mM (Adrogue & Madias, 2000). On average, this condition affects 1.72% of the U.S. population, but hospitalized patients, elderly people, and individuals with neurological disorders are disproportionately affected (Upadhyay, Jaber, & Madias, 2006; Mohan, Gu, Parikh, & Radhakrishnan, 2013). Most often, hyponatremia develops as a result of impaired capacity of the kidney to excrete water. It could be caused by underlying kidney pathologies, severe hormonal imbalance, or due to the syndrome of inappropriate antidiuretic hormone secretion (SIADH). SIADH is very common and in hospital settings; it can be triggered by anesthesia and several commonly prescribed medications or may develop in an idiogenic fashion. In neurological patients and endurance athletes, hyponatremia can also be caused by excessive water intake (Fraser & Arieff, 1997; Adrogue & Madias, 2000).

Although all tissues are affected, the brain represents the target organ of this disorder and is responsible for all major symptoms. Acute hyponatremia is typically associated with headaches, nausea, fatigue, confusion and hallucinations. In its most severe form, acute hyponatremia progresses to seizures and coma and puts patients at significant risk for brain damage and death. The latter outcomes are driven by expansion of the brain tissue and compression of blood vessels, particularly in the brainstem, leading to the life-threatening dysregulation of blood pressure, heart rate, thermal regulation and respiratory control (Fraser & Arieff, 1997; Adrogue & Madias, 2000; Podesta et al., 2015). Cell swelling in hyponatremia is the closest analogue of the model osmotic cell swelling utilized in cell cultures and other model studies. As already mentioned, in the hyponatremic brain swelling is mainly seen in astrocytes, particularly in the astrocytic processes surrounding blood vessels [e.g., (Wasterlain & Torack, 1968; Manley et al., 2000; Risher, Andrew, & Kirov, 2009)]. There is conflicting information on neuronal swelling, with some studies showing no swelling of neuronal somata or processes (Andrew et al., 2007; Steffensen et al., 2015), while others report typical hypoosmotic increases in neuronal volume in brain slice models (Murphy et al., 2017).

Irrespective of which cell types are swollen (or not), rapid adaptation of the brain to hyponatremia is mediated by the loss of inorganic ions,  $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Cl}^-$ , which move from the cytosol to the interstitial space to the circulatory system (Melton et al., 1987; Gullans & Verbalis, 1993). The loss of  $\text{K}^+$  is the main driving mechanism of RVD but, when it occurs in the brain within the constraints of a small extracellular space, it leads to rapid dissipation of  $\text{K}^+$  gradients, membrane depolarization, and neuronal excitation that can precipitate seizure development (Andrew, Fagan, Ballyk, & Rosen, 1989; Andrew, 1991; Somjen, 2004). Swollen cells also release a variety of small organic molecules – including glutamate, aspartate, *myo*-inositol, and the amino sulfonic acid taurine – and these can be measured in

the extracellular space using a microdialysis approach (Wade, Olson, Samson, Nelson, & Pazdernik, 1988; Lehmann, 1989; Verbalis & Gullans, 1991; Haskew-Layton et al., 2008). On a short time-scale, organic osmolytes are released predominantly via VRAC, but the long-term adaptation to hyponatremia likely involves additional processes (Estevez, O'Regan, Song, & Phillis, 1999; Haskew-Layton et al., 2008; Hyzinski-Garcia et al., 2011). It has been estimated that the loss of organic osmolytes is responsible for >50% of the regulatory loss of osmotically obligated water during compensatory changes in hyponatremia (Sterns et al., 1993). Yet, because aspartate and glutamate are the excitatory neurotransmitters, their release is not benign as it causes activation of neuronal glutamate receptors, with the NMDA subtype likely being the main target (Lauderdale et al., 2015; Mongin, 2016). Furthermore, activation of VRAC in astrocytes severely impairs extracellular glutamate scavenging because it interrupts its removal and metabolic conversion into the non-toxic glutamine (Haskew-Layton et al., 2008; Hyzinski-Garcia et al., 2011).

One interesting aspect of brain adaptation to hyponatremia is the preferential loss of one organic osmolyte, taurine. This phenomenon has been reported *in vitro* and *in vivo* and interpreted in the context of the privileged role of taurine in brain cell volume control and with the implied existence of taurine-selective volume regulatory pathways (Wade et al., 1988; Pasantes Morales & Schousboe, 1988; Lehmann, 1989; Verbalis & Gullans, 1991; Moran, Maar, & Pasantes-Morales, 1994; Pasantes-Morales, Lezama, Ramos-Mandujano, & Tuz, 2006). We have recently tested this hypothesis and found that the differences between the osmotic behavior of taurine and excitatory amino acids are determined by the rates of their reuptake and synthesis. Once glutamate uptake and *de novo* synthesis via transamination are pharmacologically blocked, astrocytes release cytosolic taurine and aspartate at very similar rates (Schober & Mongin, 2015). This suggests that the preservation of cytosolic glutamate and aspartate during RVD are homeostatic mechanisms related to their numerous roles in metabolism and brain signaling.

In contrast to the acute disease, chronic hyponatremia, which develops and/or persists for more than 48 hours, is typically “asymptomatic”. This happens because the brain tissue slowly adapts to new osmotic conditions via the already mentioned loss of inorganic and particularly organic osmoles. Despite an apparent lack of symptoms, chronic hyponatremia still requires correction because it is strongly associated with a host of mild neurological deficits and increases morbidity and mortality of co-existing clinical conditions (Adrogué & Madias, 2000; Podesta et al., 2015). However, its treatment often comes with a separate set of dangers. Rapid correction of systemic and  $\text{Na}^+$  levels leads to osmotic demyelination syndrome, a demyelinating pathology that most commonly affects the central basis of pontis (pontine myelinosis) but also the nearby regions of thalamus and subcortical areas (Adrogué & Madias, 2000; Sterns & Silver, 2006; Podesta et al., 2015). To avoid demyelination, correction of chronic hyponatremia is performed by introducing additional electrolytes at a very slow rate. The etiology of demyelination is likely related to lowered levels of organic osmolytes, particularly *myo*-inositol, which play incompletely understood protective roles in the preservation of myelin integrity. In one experimental study, the intravenous supplementation of *myo*-inositol dramatically decreased mortality rates and preserved

myelin structures in rats subjected to rapid correction of chronic hyponatremia (Silver, Schroeder, Sterns, & Rojiani, 2006; Sterns & Silver, 2006).

### 12.6.2 Epilepsy

Epilepsy is the fourth most common chronic neurologic disorder, which affects more than 2 million individuals in the U.S. and 65 million people worldwide (Hirtz et al., 2007; Thurman et al., 2011). The term epilepsy encompasses a constellation of diseases with somewhat different etiologies, all of which manifest as recurrent, unprovoked seizures which are usually initiated in the cortical areas or the hippocampus and then spread throughout the brain (McNamara, 1994; McNamara, Huang, & Leonard, 2006). While the main focus of epilepsy research is on changes in neuronal cells, emerging studies also implicate the complex interactions between neurons and various classes of glia, with the potentially strong involvement of changes in glial gene expression, glutamate metabolism, cellular morphology and cell volume (McNamara et al., 2006; David et al., 2009; Eid, Williamson, Lee, Petroff, & de Lanerolle, 2008; Murphy, Binder, & Fiacco, 2017). As mentioned in the prior section, a hypoosmotic challenge on its own can cause brain hyperexcitability and seizures, strongly suggesting that cell volume is a highly relevant factor.

The experimental evidence for cellular swelling in epilepsy is derived from three types of findings: (i) electron microscopy studies in fixed brain tissue after chemically-induced status epilepticus, (ii) intravital magnetic resonance measurements of water mobility, and (iii) intravital or *ex vivo* monitoring of volume dynamics in the extracellular space. In the study by (Fabene et al., 2006), the first two parameters were compared side-by-side. As reproduced in Fig. 12.4, rats with status epilepticus develop profound swelling of astrocytic cell bodies and processes, and additionally show swelling of dendritic boutons. Interestingly, neuronal cell bodies appear to maintain their volume, or even moderately shrink with an apparent condensation of cytoplasm (Fabene et al., 2006). The same study correlated the phenomenon of cellular swelling with MRI-registered changes in water mobility. Increased signal of diffusion-weighted imaging (DWI) and the inversely related apparent diffusion coefficient (ADC) of water show a consistent and long lasting (for at least 24 hours) decrease in water mobility in the epileptogenic brain areas (Fabene et al., 2006). Similar in nature and amplitude DWI and ADC changes are reliably associated with the movement of water from the extracellular to the intracellular space (Sotak, 2004). Other publications demonstrated that the development of seizure activity is preceded by shrinkage of the extracellular space, signifying increases in cellular volume [e.g., (Binder, Papadopoulos, Haggie, & Verkman, 2004; Slais et al., 2008)]. In brain slice studies, application of hyperosmotic media ameliorates the K<sup>+</sup>-induced electrographic seizures; and, conversely, introduction of mild hypoosmolar media dramatically increases epileptogenic activity, again suggesting that cell volume and/or volume of extracellular space are highly relevant (Traynelis & Dingledine, 1989). Likely causes for hyperexcitability and cellular swelling are discussed below.

The core reason for neuronal dysfunction in epilepsy is the modification of cellular excitability, which is complex in its nature. Still, the initial triggers of disease are ionic in nature. Excessive neuronal activity leads to the accumulation of K<sup>+</sup> and neurotransmitter

molecules in the extracellular space. High  $[K^+]_o$  causes partial membrane depolarization, increases neuronal excitation, and removes the  $Mg^{2+}$  block of the glutamate NMDA receptors, which are all strongly associated with epileptogenic activities (McNamara, 1994). Additionally,  $K^+$ -induced depolarization alters the electrochemical driving force for  $Cl^-$  in neurons and dampens the activity of the inhibitory  $GABA_A$  and glycine receptors (McNamara, 1994). The importance of changes in  $Cl^-$  gradients is strongly supported by studies of the functional roles for electroneutral KCC transporters. In neuronal cells, these transporters set low intracellular  $Cl^-$  levels, and in such a way allow for the inhibitory actions of GABA and glycine (Kahle et al., 2008; Kaila, Price, Payne, Puskarjov, & Voipio, 2014). Deletion or downregulation of the activities of KCC2 and KCC3 result in neuronal hyperexcitability in humans and model organisms (Woo et al., 2002; Huberfeld et al., 2007; Kahle et al., 2014; Kaila et al., 2014). KCC2-null mice develop spontaneous seizures and have high postnatal mortality (Woo et al., 2002), whereas KCC3-null phenotypes are more diverse. In humans, KCC3 mutations cause mental retardation, severe peripheral neuropathy, and loss of the corpus callosum (Howard et al., 2002). In mice, the loss of KCC3 has been alternatively associated with defects in peripheral myelination and neurological deficits, or alternatively, hyperexcitability, reduced seizure threshold, and deafness (Howard et al., 2002; Boettger et al., 2003). The KCC3 phenotypes have been interpreted in the context of defective ability of neurons to regulate their volume (Kahle et al., 2015), but this remains to be proven. Many of the mechanisms listed in section 12.5 can contribute to astrocytic swelling in epilepsy. These may include neurotransmitter uptake, the  $[K^+]_o$ -induced Donnan swelling, and anomalous NKCC1 activity [e.g., (Bender et al., 1998; Palma et al., 2006; Hubel & Ullah, 2016)].

An additional interesting topic is the link between the astrocytic water channel AQP4 and seizure susceptibility. Several studies have identified changes in the volume of the extracellular compartment and its dynamics during hyperexcitability, and found modified seizure thresholds in AQP4 knockout animals (Binder, Oshio, Ma, Verkman, & Manley, 2004; Binder et al., 2006; Lee et al., 2012). These data have been interpreted in the context of reduced water fluxes and/or defective cell volume homeostasis in AQP4-null mice (Verkman, Binder, Bloch, Auguste, & Papadopoulos, 2006; Binder, Nagelhus, & Ottersen, 2012; Murphy et al., 2017). However, even without AQP4, astrocytes retain fairly high water permeability (Solenov et al., 2004). Besides direct effect on water fluxes, the observed changes in AQP4-null animals can be explained by other factors, such as adaptive changes in the expression of other genes or the indirect effects of AQP4 on activities of ion transporters and channels. This protein has been shown to physically associate with or functionally regulate activities of the  $K^+$  channel Kir4.1, volume-sensitive VRAC, and nonselective cation channels TRPV4 and TRPM4/SUR1 [(Nagelhus, Mathiisen, & Ottersen, 2004; Benfenati et al., 2007; Benfenati et al., 2011; Stokum et al., 2018), but see an alternative opinion for Kir4.1 (Zhang & Verkman, 2008)]. Therefore, effects of AQP4 deletion may develop independent of or be upstream of cell volume changes [see (Assentoft, Larsen, & MacAulay, 2015) for detailed discussion].

### 12.6.3 Hepatic encephalopathy

Another syndrome that disproportionately affects the CNS and is strongly linked to astrocytic swelling is hyperammonemia – systemic increases in ammonia levels above the approximate threshold of ~100  $\mu\text{M}$ , which lead to neurologic deficits described as hyperammonemia or hepatic encephalopathy (Blei & Larsen, 1999; Norenberg, Rao, & Jayakumar, 2005; Haussinger & Schliess, 2008; Brusilow, Koehler, Traystman, & Cooper, 2010). In hyperammonemias, arterial ammonia levels frequently reach 1 mM, and can be as high as 5 mM, leading to severe brain edema and high mortality rates due to herniation of the brainstem (Blei & Larsen, 1999; Norenberg et al., 2005; Brusilow et al., 2010). Most frequently, hyponatremia develops due to acute liver failure or chronic liver disease. Depending on the clinical population, 20 to 80% of patients with liver cirrhosis suffer from moderate to severe hepatic encephalopathy. However, other conditions – such as inherited deficiencies of the urea cycle, certain bacterial infections of the gut, and Reye’s syndrome – may also lead to the same type of neuropathology (Felipo & Butterworth, 2002; Brusilow et al., 2010).

The main mechanism of the hepatic encephalopathy is thought to be osmolyte accumulation in the brain due to fixation of ammonia by the astrocytic enzyme glutamine synthetase (Norenberg et al., 2005; Brusilow et al., 2010). Glutamine synthetase produces glutamine from ammonia and glutamate in an ATP-dependent fashion, as a part of its normal physiological activity in the brain, the recycling of excitatory neurotransmitters (McKenna, 2007; Sofroniew & Vinters, 2010; Verkhratsky & Nedergaard, 2018). Because ammonia circulates systemically and freely diffuses into the brain, in hyperammonemia this process serves as an unlimited osmotic sink as long as the brain has energy reserves. The mechanisms of ammonia transport across cell membranes are summarized in section 12.5 and Fig. 12.3D. The osmotic burden due to the activity of glutamine synthetase is further accentuated by the accumulation of brain alanine produced via the work of the alternative detoxification mechanism incorporating glutamate dehydrogenase and alanine aminotransferase (Tofteng et al., 2006; Dadsetan et al., 2013). In experimental hyperammonemias, treatments with the glutamine synthetase inhibitor methionine sulfoximine reduced the amount of brain edema and cell swelling, supporting the causal link of glutamine synthetase to disease development (Blei, Olafsson, Therrien, & Butterworth, 1994; Tanigami et al., 2005).

Because glutamine and alanine synthesis occur in astrocytes, it is not surprising that the main cellular pathology in hepatic encephalopathy is astrocytic. Human pathological studies show the appearance of swollen astrocytes in acute disease or so-called Alzheimer type II astrocytes in chronic pathology, with the latter characterized by enlarged cell somata and nuclei with significant nucleolus. The frequency of observed pathological cells correlates with the severity of encephalopathy (Norenberg, 1998). The mechanisms of astrocytic swelling have been thoroughly explored in primary astrocyte cultures with main contributions coming from the laboratory of M.D. Norenberg. Surprisingly, these cell culture studies indicate that the osmotic effect of glutamine accumulation is not the leading reason for cell swelling. Instead, they propose a variety of alternative mechanisms, including membrane depolarization via ammonia uptake through  $\text{K}^+$  channels followed by Donnan



swelling (Allert et al., 1998), osmotic load due to NKCC1 activity (Jayakumar, Valdes, & Norenberg, 2011), and secondary swelling due to opening of mitochondrial permeability transition pore (Bai et al., 2001) followed by production of reactive oxygen and nitrogen species (Murthy, Rama Rao, Bai, & Norenberg, 2001). To reconcile the conflicting results on the role of astrocytic glutamine in cellular swelling, J. Albrecht and M.D. Norenberg proposed the “Trojan horse” hypothesis, in which the glutamine synthetase-derived glutamine causes astrocytic swelling via its secondary metabolic effects within the cells, particularly in mitochondria (Albrecht & Norenberg, 2006; Norenberg, Jayakumar, Rama Rao, & Panickar, 2007)).

#### 12.6.4 Stroke

Stroke is the fifth most prevalent source of mortality in the U.S. and the second most common cause of death worldwide, with 6.5 million deaths reported globally in 2013 (Feigin, Norrving, & Mensah, 2017). A stroke occurs due to the interruption of blood flow to an area of the brain. Because brain metabolism is almost exclusively dependent on oxidation of blood-derived glucose, reductions in cerebral blood flow below a threshold of ~50% of their normal levels cause a rapid decline in tissue metabolism, and anything below 20% of normal blood flow levels is followed by severe tissue damage (Dirnagl, Iadecola, & Moskowitz, 1999; Brott & Bogousslavsky, 2000; Lo, Dalkara, & Moskowitz, 2003). The majority of strokes (>85%) are ischemic and involve embolic or thrombotic occlusion of a larger blood vessel. A smaller (<15%), but deadlier fraction of strokes are hemorrhagic and result from bleeding through a rupture in the parenchyma or subarachnoid space. The mechanisms of tissue damage are not identical between the two types of stroke because of the nature of the initial insult, but they all share characteristics of ionic dysfunction and cell swelling. In this section we focus on ischemic stroke.

There is a significant body of work demonstrating astrocytic cell swelling in ischemic stroke, through (i) electron microscopy in animal stroke models, (ii) measurements of brain interstitial volume in ischemia, (iii) two-photon microscopy imaging of cell swelling through a cranial window, and (iv) diffusion and perfusion weighted MRI imaging, which are widely utilized in clinical settings. The most commonly used animal model of stroke is focal ischemia induced by transient or permanent occlusion of the middle cerebral artery occlusion (MCAo) (Longa, Weinstein, Carlson, & Cummins, 1989). EM analysis reveals astrocytic swelling in fixed MCAo tissue after only 30 min of ischemia and such swelling persists up to 24 hours after reperfusion (Garcia et al., 1993). Under physiological conditions, the extracellular space makes up ~20% of the brain volume (Thrane et al., 2014). Early methods of measuring interstitial space in the brain used radiotracer diffusion studies or the ion-selective microelectrodes detecting the extracellular marker tetramethylammonium ( $\text{TMA}^+$ ), and found that during severe ischemia the volume fraction drops to as low as 5% [reviewed in (Sykova & Nicholson, 2008)]. More recently, studies using 2-photon microscopy allowed for the direct observation of cell swelling in both animal slices and in vivo. The laboratory of S.A. Kirov found that the cross-sectional area of astrocytes in hippocampal slices increased by ~40% upon 3-min depolarization with high extracellular  $[\text{K}^+]$ , and by ~30% when slices were exposed to oxygen/glucose deprivation (Risher et al., 2009). The same study used a cranial window and 2-photon microscopy to

demonstrate that global ischemia, which was induced by cardiac arrest, prompted very rapid swelling of astrocytic soma by ~30% (Risher et al., 2009). Another publication, which used similar methodology for measuring astrocytic swelling in the ischemic penumbra of the photothrombotic stroke model, found the degree of astrocytic swelling to be in the range of 20–35% (Risher, Croom, & Kirov, 2012).

In clinical settings, tissue edema is visualized using the MRI-derived DWI or ADC signals. The early hyperintensity in MRI signal or matching hypointensity of the ADC signal serve as a reliable representation of the ischemic area and are fairly predictive of the final infarction volume (Neumann-Haefelin et al., 1999; Thijs, Adami, Neumann-Haefelin, Moseley, & Albers, 2002; Kucinski et al., 2002). Acute ischemia-induced changes in DWI and ADC are shown in Fig. 12.5. The human MRI data have been extensively validated by crosschecking DWI imaging with histological outcomes in animal stroke models [e.g., (Neumann-Haefelin et al., 2000)]. The time-dependent development of DWI signal in human stroke correlates well with the evolution of infarction, suggesting that cellular edema contributes to progression of tissue damage (Battey et al., 2014).

The reasons for ischemic swelling of astroglial cells are likely plural and may vary significantly depending on the proximity to the ischemic core. In the ischemic core, cessation of the blood flow and resulting lack of oxygen and glucose lead to the rapid (within 5 min) and complete depletion of energy reserves, inhibition of the  $\text{Na}^+, \text{K}^+$ -pump, and collapse of ionic gradients (Dirnagl et al., 1999). A dramatic rise in the intracellular  $[\text{Na}^+]$  and  $[\text{Ca}^{2+}]$  and extracellular  $[\text{K}^+]$  lead to Donnan cell swelling, which for poorly understood reasons is largely restricted to astrocytes. Astrocytes may retain their membrane potentials, and consequently the electrochemical driving force for ion accumulation, for a longer time than neurons. Furthermore, they are linked into syncytium by gap junctions, and at least in the beginning of the ischemic episode, work as one unified ion sink. Neurons also swell, but their swelling is largely limited to beading in the dendritic arbors, where a high density of ionotropic receptor-channels and water-permeable electroneutral transporters (NKCC1, KCC2, MCT, etc.) facilitate rapid osmotic loading (Steffensen et al., 2015). In contrast, neuronal cell bodies in the ischemic core typically retain their initial volume or even appear to shrink, perhaps due to the rapid redistribution of osmolytes into astrocytic compartments (Garcia et al., 1993).

In the ischemic penumbra, tissue ATP levels are partially preserved, or even elevated in the areas distal to occlusion. Nevertheless, the penumbral tissue is electrically silent as a result of presynaptic failure (Bolay & Dalkara, 1998; Neumann-Haefelin & Witte, 2000; Bolay et al., 2002). This is explained by the difficulties in sustaining metabolism in remote axonal processes and oxidative/nitrosative modification in presynaptic processes [reviewed in (Mongin, Dohare, & Jourdain, 2012)]. Astrocytic swelling in the penumbra likely involves numerous mechanisms, including but not limited to osmolyte fluxes via the  $\text{Na}^+$ -dependent transporters (GLT-1, GLAST, and others). Moreover, astrocytic swelling is promoted by NKCC1 activity. Treatment with the NKCC1 inhibitor bumetanide given prior to the induction of animal ischemia reduces the infarction volume and tissue edema in a MCAo model of stroke (Yan, Dempsey, & Sun, 2001). In astrocyte cultures, bumetanide and genetic deletion of NKCC1 make astrocytes resilient to  $\text{K}^+$ -induced cell swelling and

obliterates the  $K^+$ -swelling-induced release of excitatory amino acids (Su et al., 2002; Su et al., 2002). The major physiological signal for NKCC1 activation is phosphorylation by the WNK signaling cascade [see section 12.5.6 and reviews (Kahle et al., 2008; McCormick & Ellison, 2011)]. This cascade is typically inhibited by cell swelling, rendering NKCC1 silent. Yet, paradoxically, astrocytes and glioma C6 cells respond to hypoosmotic cell swelling with an increase in NKCC1 activity (Mongin et al., 1994; Mongin et al., 1996). As already mentioned, one possible explanation is that cell swelling and RVD reduce the intracellular  $Cl^-$  levels and relieve  $Cl^-$  inhibition of protein kinases belonging to the WNK family (Kahle et al., 2008; Pacheco-Alvarez & Gamba, 2011; Piala et al., 2014; Terker et al., 2016). Genetic deletion of the brain-enriched WNK3 reduces cerebral edema and tissue injury following MCAo stroke (Begum et al., 2015).

Another important cause for ischemic cell swelling is the pathological opening of ATP-sensitive nonselective cation channels. One type, which is assembled from the TRP channels belonging to the melastatin family (TRPM4) and the sulfonylurea receptor-1 (SUR1) appears to dominate brain edema and stroke outcomes (Chen & Simard, 2001; Simard et al., 2006; Simard et al., 2010). The SUR1 subunit expression is upregulated during ischemic hypoxia by HIF1- $\alpha$  activation, and in an animal model of stroke can be detected in neurons, astrocytes, and endothelial cells (Simard et al., 2006). Treatment with the sulfonylurea glibenclamide inhibits these channels, and has been shown to reduce cerebral edema and stroke volume by as much as 50% and increase viability in the malignant MCAo model of stroke (Simard et al., 2006; Simard et al., 2010). Recently, it has been shown that astrocytic SUR1-TRPM4 forms a tripartite complex with AQP4, and in such a way significantly increases the rate of water influx into cells exposed to hypoosmotic media (Stokum et al., 2018) (see section 12.5.5). Importantly, sulfonylureas, the SUR1 antagonists, protect not only animals but also humans and are now in clinical trials for treatment of ischemic stroke (Kunte et al., 2007; Sheth et al., 2016).

## 12.7. VRAC AS A VILLAIN IN NEUROPATHOLOGIES

Persistent cell swelling in neuropathologies disrupts normal neural functions via numerous mechanisms, including volume-dependent changes in ionic gradients and cell excitability. There are strong reasons to believe that among many relevant processes, opening of VRAC is the key to the pathological tissue damage. The uncontrolled release of glutamate and aspartate activates highly abundant receptors on neurons and all classes of glial cells, including the  $Na^+/K^+$  permeable ionotropic AMPA and kainate receptors, the  $Na^+/K^+/Ca^{2+}$ -permeable NMDA receptors, and a variety of metabotropic mGluRs (Meldrum, 2000). Their combined actions cause sustained membrane depolarization and increases in the intracellular  $[Ca^{2+}]$ , which set in motion multiple neurodegenerative cascades causing immediate or delayed neuronal damage and demise. This process is termed excitotoxicity (Choi, 1988; Choi, 1992; Arundine & Tymianski, 2004). In principle, activation of VRAC can impact extracellular excitotoxin levels via at least three interconnected mechanisms: (i) VRAC serves as a conduit for excitatory amino acid release from swollen astrocytes and other cell types; (ii) opening of VRAC shunts glutamate to the extracellular space and renders astrocytic glutamate uptake ineffective; (iii) shunting of glutamate to the extracellular space interrupts glutamate processing via the glutamate-glutamine cycle, also a major mechanism

regulating extracellular glutamate levels (Haskew-Layton et al., 2008; Hyzinski-Garcia et al., 2011). Pathological contributions of VRAC have been most extensively studied in stroke.

*In vivo* studies in animal stroke models consistently demonstrated dramatic increases in extracellular glutamate levels both in the ischemic core and the penumbra (Benveniste, Drejer, Schousboe, & Diemer, 1984; Phillis & O'Regan, 1996; Phillis, Song, & O'Regan, 1997; Seki, Feustel, Keller, Jr., Tranmer, & Kimelberg, 1999; Feustel, Jin, & Kimelberg, 2004; Dohare et al., 2014). This pathological glutamate can be contributed to by several mechanisms, which are not limited to VRAC. Besides VRAC, the best candidates for release routes include reversal of glutamate transporters, opening of connexin hemichannels, and stimulation of P2X<sub>7</sub>-receptor associated pannexin hemichannels; (Szatkowski, Barbour, & Attwell, 1990; Rossi, Oshima, & Attwell, 2000; Ye, Wyeth, Baltan-Tekkok, & Ransom, 2003; Duan et al., 2003; Orellana, Avendano, & Montero, 2014). It should be noted that not all of these mechanisms have been extensively tested *in vivo*. The relative contribution of VRAC was probed with diverse anion channel blockers, including the most selective among the available VRAC inhibitors, DCPIB. These compounds strongly reduced the intras ischemic glutamate levels clearly pointing to VRAC activity in the pathological brain (Phillis et al., 1997; Phillis, Song, & O'Regan, 1998; Seki et al., 1999; Feustel et al., 2004; Zhang, Zhang, Feustel, & Kimelberg, 2008). However, because the specificity of VRAC blockers, including DCPIB, has been questioned (Ye, Oberheim, Kettenmann, & Ransom, 2009; Benfenati et al., 2009; Bowers, Dohare, Kuo, & Mongin, 2013), further work in transgenic animals and/or with more specific molecular biology tools, is needed to unequivocally link VRAC to glutamate release in stroke.

It is important to note the fact that VRAC contributions to pathological glutamate levels are likely context-specific. The inhibitory effects of VRAC blockers and the inferred mechanisms of the intras ischemic glutamate release vary depending on the study. The major determinant appears to be the site of detection, e.g., position of the microdialysis probe within the ischemic brain. In the ischemic core, the main source of glutamate release is reversal of the dihydrokainate-sensitive glutamate transporter GLT1 (Seki et al., 1999). In contrast, in the penumbra, glutamate release is dominated by the tamoxifen- and DCPIB-sensitive VRAC (Feustel et al., 2004; Zhang et al., 2008). There are several potential explanations for paradoxical "silence" of VRAC in the ischemic core, where cell swelling is the most pronounced. VRAC opening requires the non-hydrolytic binding of ATP, and therefore its activity and associated glutamate release are abolished in metabolically-inhibited cells (Jackson et al., 1994; Oike, Droogmans, & Nilius, 1994; Rutledge, Mongin, & Kimelberg, 1999). VRAC is also potently inhibited by arachidonic acid and other polyunsaturated fatty acids, which are abundantly produced in the ischemic tissue (Kubo & Okada, 1992; Nilius, Sehrer, & Droogmans, 1994). In contrast, on the periphery of the ischemic tissue, collateral blood flow partially sustains brain cells metabolism, allowing for sustained VRAC activity. It is in the ischemic penumbra, tamoxifen and DCPIB suppress glutamate release by up to 70% (Feustel et al., 2004; Zhang et al., 2008).

Even if we accept the link between VRAC and pathological glutamate release, the potential impact of this process on tissue viability is not certain due to the very complex nature of ischemic brain injury. Due to the role of VRAC in cell volume control, its activity in stroke

tissue may be beneficial, harmful, or net-neutral. The question about the therapeutic value of this channel has been addressed in animal stroke models by H.K. Kimelberg and co-workers. The systemic administration of the blood-brain barrier-permeable tamoxifen potently protected against brain injury in both the transient and the permanent cerebral ischemia, with maximal protection reaching >80% and an extended therapeutic window up to 3 h after occlusion of the MCA (Kimelberg et al., 2000; Kimelberg, Jin, Charniga, & Feustel, 2003). Due to limited tamoxifen selectivity, its protective effects have been further verified with the more specific (but not blood-brain barrier-permeable) VRAC blocker DCPIB (Zhang et al., 2008). In Fig. 12.6A, we present representative data from the latter study illustrating DCPIB-mediated protection in the ischemic brain. Because DCPIB is not BBB permeable, it required intracerebral injection to achieve neuroprotection. As shown in Fig. 12.6B, systemic administration of the same compound was ineffective, further suggesting that it acts in the brain parenchyma but not on blood vessels. The protective effects of VRAC blockers (tamoxifen) have been next validated in larger animal species, in a canine model of stroke (Boulos et al., 2011).

Altogether, animal data support the major pathological significance for VRAC in stroke and warrant giving it the title of the “villain” in the ischemic brain. The idea of the therapeutic utility of VRAC in stroke and other neuropathologies has been extensively covered in several relatively recent reviews (Kimelberg, 2005; Mongin, 2007; Mongin, 2016).

## 12.8. THE EVOLVING UNDERSTANDING OF VRAC IDENTITY: PROGRESS AND PERSPECTIVES

Despite much progress in the field, many aspects of cell volume regulation in the brain require further mechanistic exploration. One avenue which carries the most promise is the recent and ongoing work on establishing the molecular nature of VRAC and the mechanisms contributing to its activation in swollen cells.

For nearly thirty years, the molecular identity of VRAC remained unknown. In 1988, two electrophysiological studies resulted in the first recordings of swelling-activated  $\text{Cl}^-$  currents in human lymphocytes and intestinal epithelial 407 cells (Cahalan & Lewis, 1988; Hazama & Okada, 1988). Since these two pioneer publications, the biophysical properties of VRAC have been a subject of intense interest and were characterized in numerous cell and tissue types [reviewed in (Strange et al., 1996; Okada, 1997; Nilius et al., 1997; Pedersen et al., 2016; Mongin, 2016)]. Nevertheless, despite numerous attempts to clone VRAC, its molecular identity was established only in 2014. The laboratories of A. Patapoutian and T.J. Jentsch performed independent genome-wide siRNA screens and identified VRAC as heteromeric protein complexes homologous to mammalian pannexins and assembled from the gene products belonging to the leucine-rich repeat containing family 8 (LRRC8) (Qiu et al., 2014; Voss et al., 2014). Both labs discovered that VRAC activity is critically dependent on the expression of the LRRC8A isoform, while the four other members of the same family, LRRC8B through E, play dispensable roles (Qiu et al., 2014; Voss et al., 2014). However, T.J. Jentsch and colleagues made an additional conceptual step and established that LRRC8A has to heteromerize with at least one alternative type of the LRRC8 subunits

in order to form a fully active VRAC (Voss et al., 2014). Moreover, they found that LRRB8C-E partners modify the biophysical properties of VRAC (Voss et al., 2014). These latter finding addressed the long-standing question of why VRACs have similar but not identical profiles in different cell types (Okada, 1997; Nilius & Droogmans, 2003; Okada, Sato, & Numata, 2009). Also importantly, deletion of LRRC8A and disruption of VRAC function blocked the RVD process in model cell lines (Qiu et al., 2014; Voss et al., 2014). The amazing progress in our knowledge of VRAC is covered in the two invited reviews in this book (Okada, Okada, Islam & Sabirov, 2018; Osei-Owusu, Yang, Vitery & Qiu, 2018). The most recent development in this area is the report on the 3D structure of VRAC resolved with X-ray crystallography and cryo-electron microscopy (Deneka, Sawicka, Lam, Paulino, & Dutzler, 2018).

In this chapter, we narrowly focus on the recent discovery of the heterogeneous VRAC complexes with distinct permeability profiles for organic molecules. As seen in the previous sections, our interest in this topic is motivated by the high relevance of organic osmolyte release to normal brain signaling and pathological states. The two original papers which linked VRAC to LRRC8 proteins, based their work on measurements of currents and fluxes of  $\text{Cl}^-$  and other halides. However, they also additionally found that the LRRC8A-containing channels are responsible for swelling-activated release of the zwitterionic taurine (Qiu et al., 2014; Voss et al., 2014). Our laboratory was the first to affirm the critical role of LRRC8A for VRAC activity in brain cells (astrocytes), but also made the side-by-side comparisons of the LRRC8A-dependent release of taurine and the negatively charged amino acids, glutamate and aspartate (Hydzinski-Garcia et al., 2014). The principal finding in our study was that presence of LRRC8A is equally critical for release of both taurine and glutamate/aspartate (Hydzinski-Garcia et al., 2014). On one hand, this was highly encouraging because the VRAC involvement in the release of organic osmolytes has been questioned in the past (Kirk, 1997; Franco, 2003; Shennan, 2008). On the other hand, the finding of a uniform dependence on LRRC8A expression contradicted the enduring idea about the existence of plural swelling-activated release pathways for inorganic anions and organic osmolytes. Over the years, many groups accrued strong, albeit indirect evidence that  $\text{Cl}^-$  and certain organic molecules, such as taurine, may be transported via distinct volume-sensitive pathways [e.g., (Lambert & Hoffmann, 1994; Shennan, McNeillie, & Curran, 1994; Stutzin et al., 1999; Mongin, Reddi, Charniga, & Kimelberg, 1999)]. The hypothesis that taurine has its own independent release route was particularly influential, but not rooted in any molecular knowledge. This has changed with the discovery of the LRRC8 proteins.

The first molecular evidence for multiple VRAC channels responsible for the release of inorganic and organic osmolytes came from the laboratory of T.J. Jentsch. They discovered that LRRC8A/LRRC8D-containing VRAC plays a unique role in transport of the chemotherapy drugs, cisplatin and carboplatin (Planells-Cases *et al.*, 2015). The same study established that expression of the LRRC8D subunit is essential for the release of taurine, but dispensable for swelling-activated  $\text{Cl}^-$  currents (Planells-Cases *et al.*, 2015). These findings led to the conclusion that LRRC8A/LRRC8D-containing heteromers are the long-sought after swelling-activated taurine channel. Several subsequent publications explored the requirements of LRRC8 composition for the release of taurine and other structurally diverse organic osmolytes, including glutamate, GABA glycine, lactate, and *myo*-inositol (Gaitan-



It is fitting that this admittedly non-exhaustive review is concluded with the discussion of structure, functions, and (patho)physiological roles of VRAC. In our opinion, the discovery of the LRRC8 proteins has been and continues to be a powerful catalyst for further progress in this field. Although VRAC studies can be dated back to the late 1980s, only now do we have the molecular biology and genetics tools to tackle some of the perennial questions about this channel and cell volume control in the brain. More work is needed to establish if VRAC and other volume-sensitive channels and transporters, such as KCC, NKCC, and SUR1/TRPM4, are friends or foes in the pathological brain. The emerging clinical studies certainly provide a lot of hope that the relevant information will soon be translated to new treatment modalities.

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## ABBREVIATIONS:

<b>ADC</b>	apparent diffusion coefficient [of water]
<b>BBB</b>	blood-brain barrier
<b>CNS</b>	central nervous system
<b>CSF</b>	cerebrospinal fluid
<b>DRG</b>	dorsal root ganglion
<b>DWI</b>	diffusion-weighted imaging
<b>EGFP</b>	enhanced green fluorescent protein
<b>EM</b>	electron microscopy
<b>ENaC</b>	epithelial Na <sup>+</sup> channels
<b>GPCR</b>	G protein-coupled receptor
<b>HICCs</b>	hypertonicity-induced cation channels
<b>ICF</b>	intracellular fluid
<b>ISF</b>	interstitial fluid
<b>MRI</b>	magnetic resonance imaging
<b>PK</b>	protein kinase
<b>PKC</b>	protein kinase C
<b>RVD</b>	regulatory volume decrease
<b>RVI</b>	regulatory volume increase
<b>TRP</b>	transient receptor potential [channels]



**VRAC** volume-regulated anion channels

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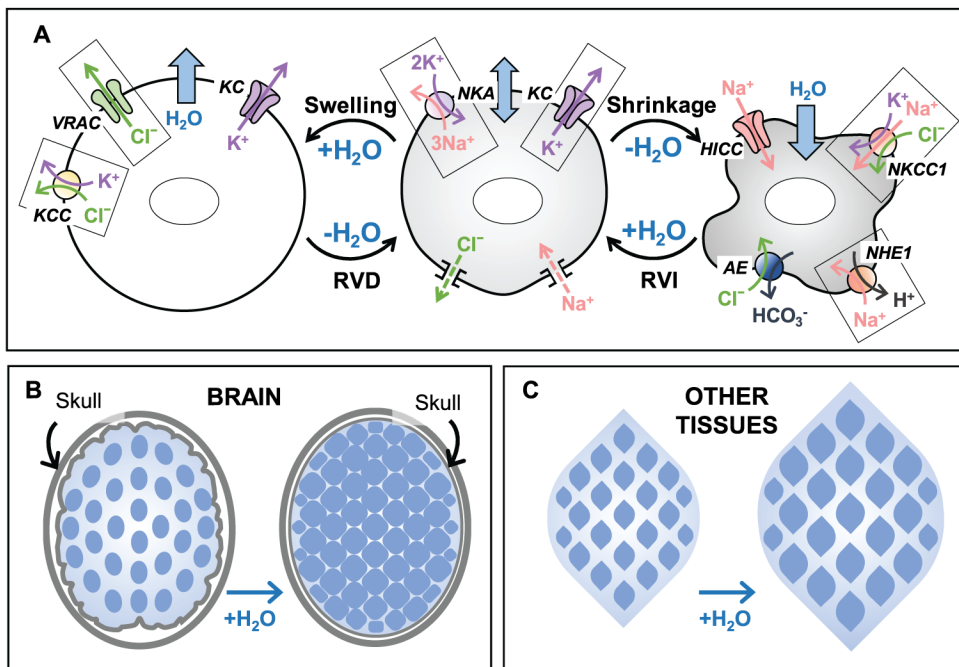
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**Figure 12.1.**

Basic principles of cell volume regulation in mammalian cells and major differences in the osmotic homeostasis within the brain and in other tissues. **(A)** Ion transport mechanisms responsible for cell volume regulation. *Center panel:* Under isosmotic conditions, the work of the Na<sup>+</sup>,K<sup>+</sup>-ATPase (NKA) and the dominant activity of K<sup>+</sup> channels (KC), set the transmembrane ionic gradients and negative membrane potential, as well as compensate for the persistent Na<sup>+</sup> uptake via a variety of mechanisms (Na<sup>+</sup> leak). Importantly, the negative membrane potential drives out intracellular Cl<sup>-</sup>, offsetting the presence of the negatively charged impermeant macromolecules and metabolites in the cytosol. *Left panel:* Hypoosmotic cell swelling triggers activation of the volume-regulated Cl<sup>-</sup>/anion channel (VRAC) and several K<sup>+</sup>,Cl<sup>-</sup> cotransporters (KCC). Cooperative activity of VRAC, KC, and KCC mediates the loss of cytosolic KCl and powers regulatory volume decrease (RVD). *Right panel:* Cell shrinkage upon exposure to hyperosmotic media stimulates the ubiquitous Na<sup>+</sup>,K<sup>+</sup>,Cl<sup>-</sup> cotransporter 1 (NKCC1) and/or the Na<sup>+</sup>/H<sup>+</sup> exchanger 1 (NHE1). NHE works in cooperation with the volume-insensitive Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> anion exchangers (AE). In some cell types, cell shrinkage also opens hypertonicity induced non-selective cation channels (HICC). The combined activity of these transporters and channels leads to cytosolic accumulation of NaCl and KCl and mediates regulatory volume increase (RVI). **(B and C)** The major differences in cell volume control between the brain and other tissues are due to the fixed volume of (extracellular + intracellular) space in the CNS. **(B)** In the brain, due to spatial limitations imposed by the rigid skull, cell swelling under hypoosmotic conditions or in pathologies occurs at the expense of the interstitial volume and may also compress blood vessels and cause ischemia. Also, due to the restricted ion transport across the blood-brain barrier, there is a “fixed” total pool of extracellular and intracellular ions. Therefore, during cell volume regulation, the electrochemical driving forces for ionic fluxes dissipate very quickly. **(C)** In contrast to the brain, the majority of peripheral tissues are not restricted in

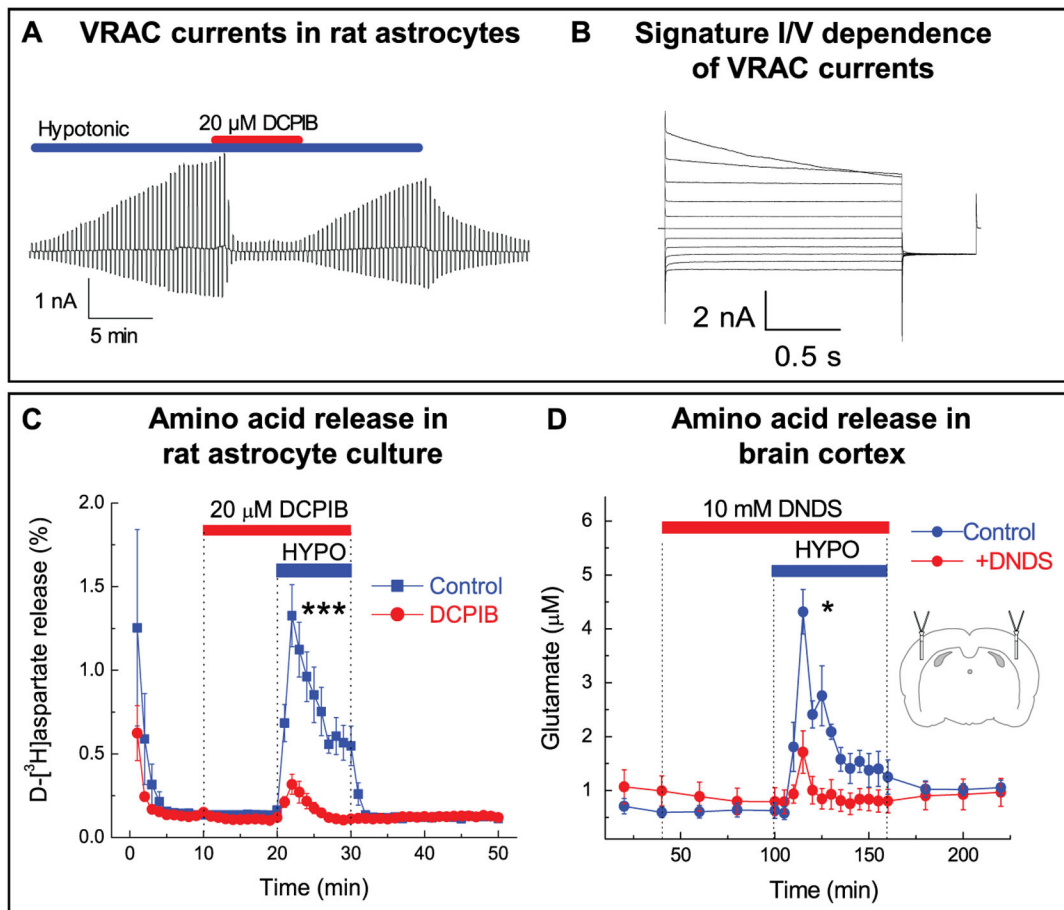
terms of their osmotic expansion or shrinkage, and allow for the relatively rapid exchange of electrolytes between the interstitial space and the blood.

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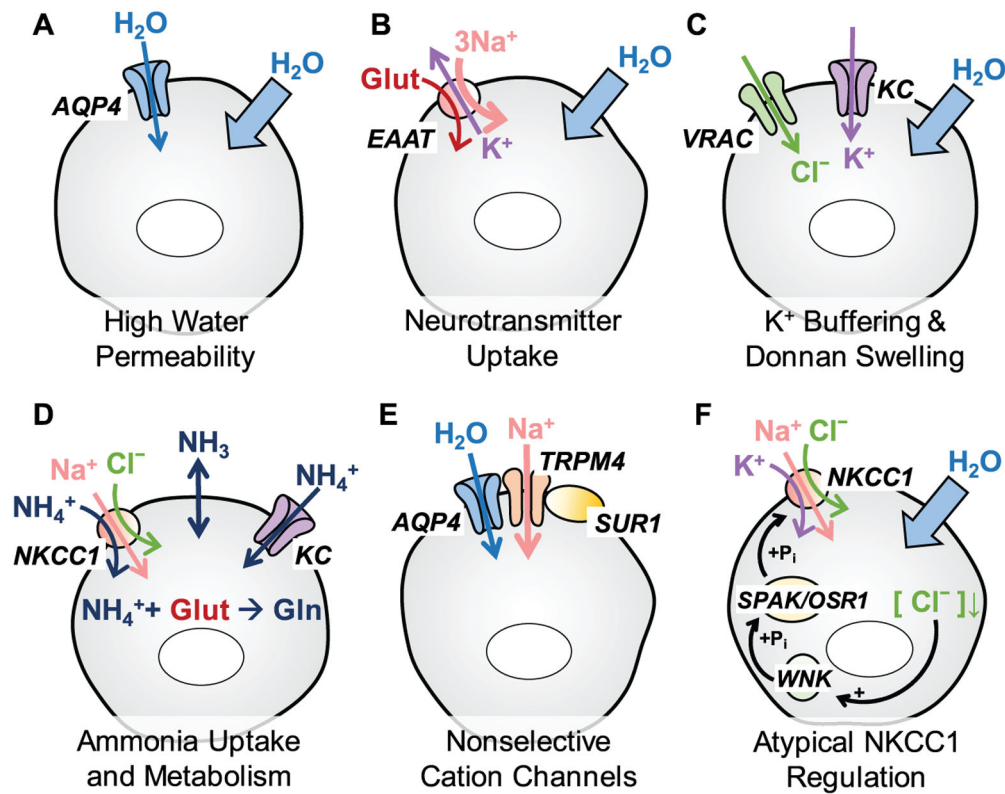
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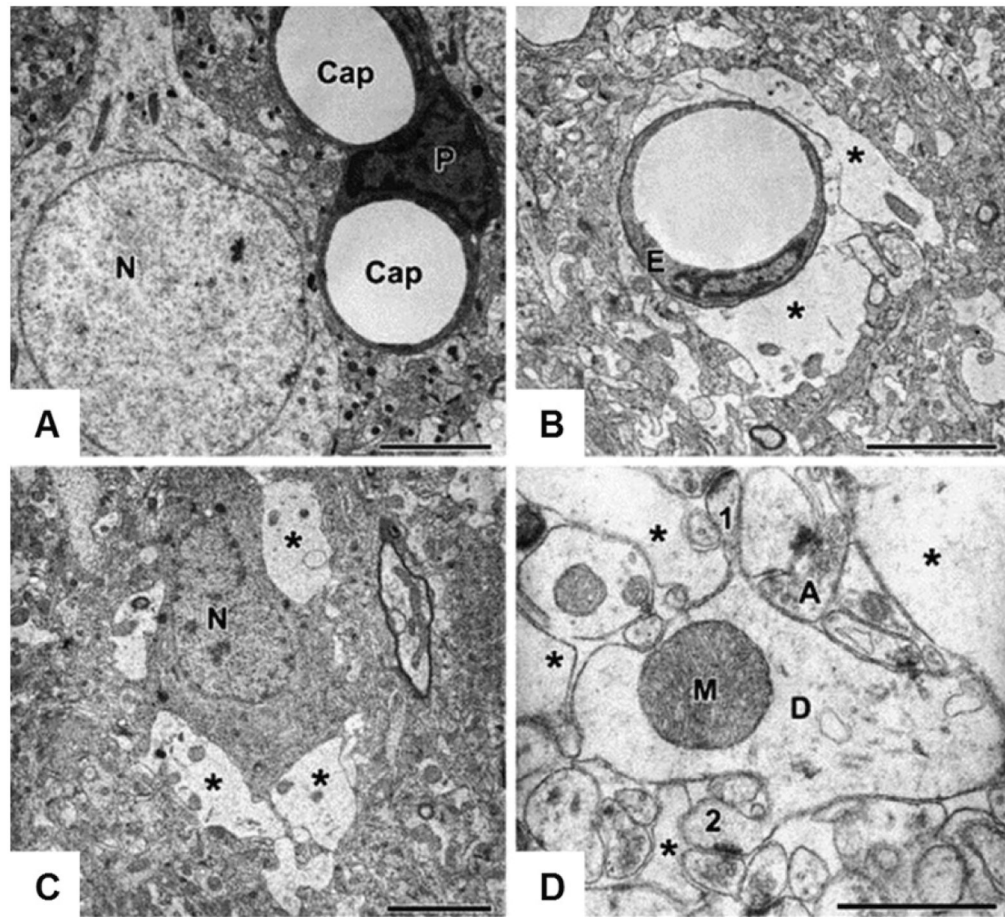
**Figure 12.2.**

Swelling-activated  $\text{Cl}^-$  currents and amino acid release through VRAC *in vitro* and *in vivo*. (A) Representative whole-cell recordings of  $\text{Cl}^-$  currents in primary rat astrocytes exposed to hypotonic medium ( $-60$  mOsm). Activity of  $\text{Cl}^-$  channels was measured by holding cells at  $0$  mV and alternately administering  $\pm 40$  mV voltage pulses. Currents were inhibited by treatment with the VRAC blocker DCPIB ( $20$   $\mu\text{M}$ ). (B) Swelling-activated  $\text{Cl}^-$  currents in astrocytes in response to  $20$  mV step pulses from  $-100$  to  $+100$  mV, displaying the characteristic outward rectification and time-dependent inactivation at positive potentials. (C) Effect of DCPIB on swelling-activated glutamate release in primary astrocytes, traced with the non-metabolizable glutamate analog D- $[\text{}^3\text{H}]$ aspartate.  $***p < 0.001$ , effect of DCPIB. (D) Effect of the non-specific VRAC blocker DNDS on swelling-activated glutamate release in the rat cortex after stimulation with hypotonic artificial cerebrospinal fluid, measured by microdialysis approach and analyzed with HPLC.  $*p < 0.05$ , effect of DNDS. (A-C) adapted from I.F. Abdullaev *et al.* (2006), with permission. (D) Reproduced from R.E. Haskew-Layton *et al.*, 2008, under the Creative Commons Attribution (CC BY) license.



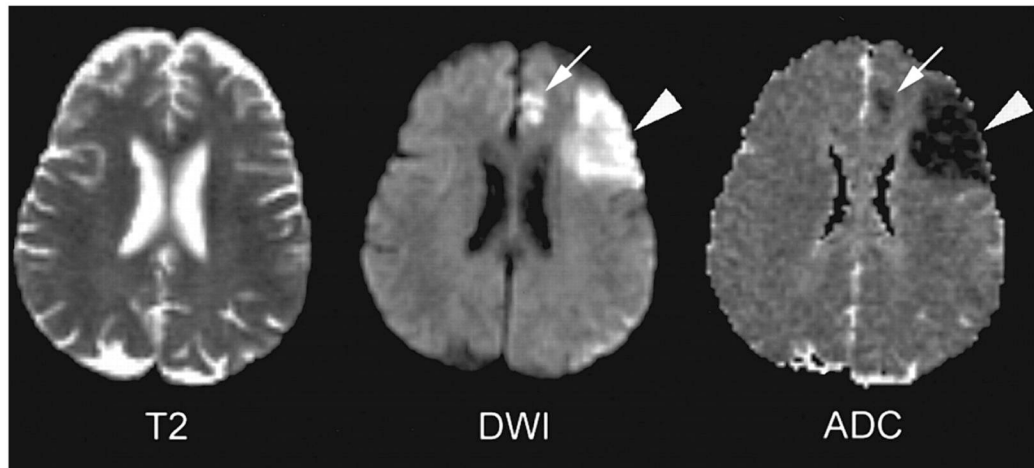
**Figure 12.3.**

Astrocytic properties that may be responsible for preferential propensity of this cell type to cell swelling in neuropathologies. (A) Astrocyte membranes are highly permeable to water due to expression of the water channel aquaporin-4 (AQP4); (B) Astrocytes take up neurotransmitters from the extracellular space in order to maintain normal neuronal activity. Excitatory amino acid transporters (EAAT) take one glutamate in together with 3  $Na^+$  and in exchange of 1  $K^+$ , and promote the accumulation of osmotically obligated water; (C) Buffering of  $[K^+]_o$  through potassium channels (KC) can be associated with the concomitant influx of  $Cl^-$  through VRAC and promote Donnan cell swelling; (D) In normal brain and neuropathologies, astrocytes accumulate extracellular ammonia ( $NH_3$ ) via passive transmembrane diffusion and ammonium ions ( $NH_4^+$ ) through potassium channels (KCs) and NKCC1. Inside the cell,  $NH_3/NH_4^+$  is then assimilated to produce glutamine from glutamate. (E) Upregulation and activation of the nonselective SUR1-TRPM4 channels and their assembly with AQP4 promotes  $Na^+$  uptake and water accumulation; (F) Astrocytic NKCC1 cotransporter contributes to electrolyte and water accumulation in response to high  $[K^+]_o$ . Cell swelling may paradoxically activate NKCC1 via the low  $[Cl^-]_i$ -sensing WNK/SPAK/OSR1 cascade and in such a way further amplify the persistent astrocytic swelling.



**Figure 12.4.**

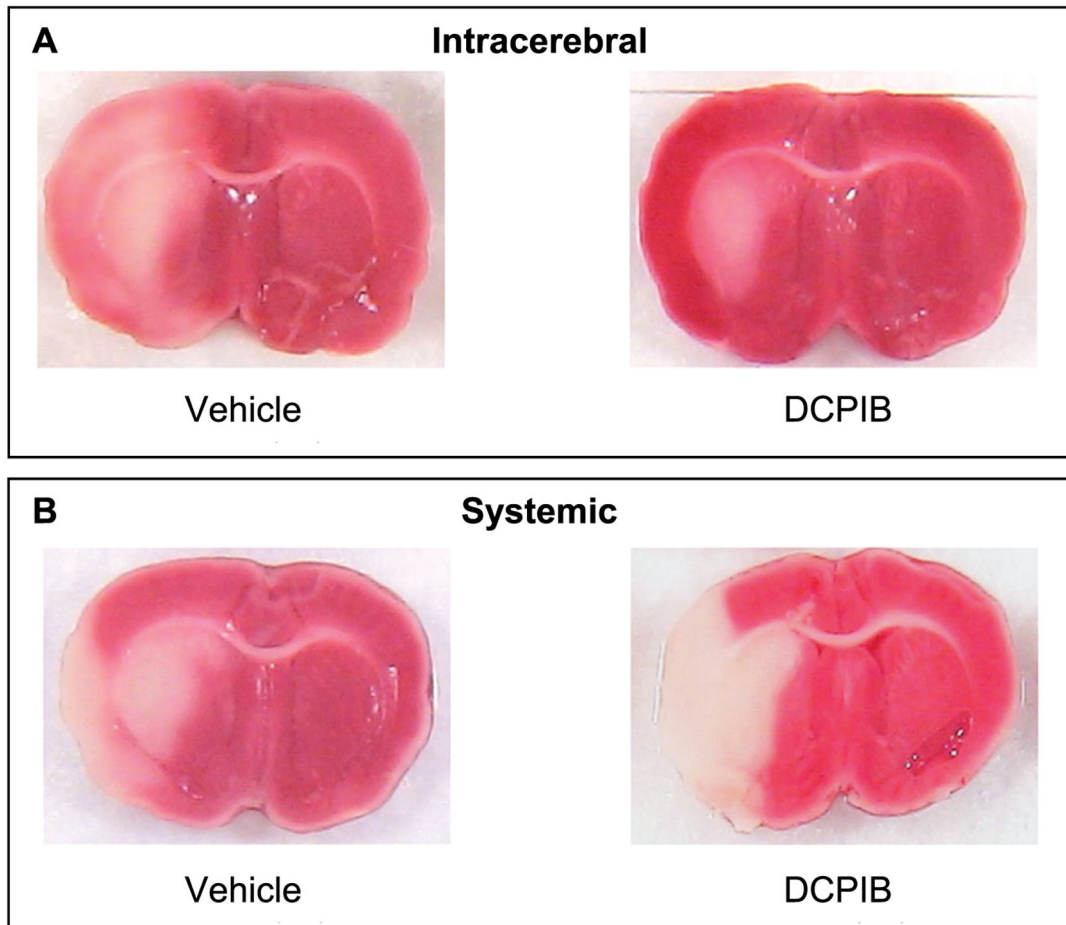
Cell swelling in status epilepticus revealed by electron microscopy (EM). (A) Structural analysis of the control rat neocortex reveals neuronal cell bodies [N], capillaries [Cap], and pericytes [P]. (B and C) Two hours following injection of 4-aminopyridine, EM shows swollen perivascular astrocytic endfeet [asterisks in (B)], which surround an endothelial cell [E]. Swollen astrocytic processes [asterisks in (C)] also border an apparently shrunken pyramidal neuronal body [N]. (D) A higher magnification image in the CA3 layer of the hippocampus displays a swollen dendrite [D] with apparently normal mitochondrion [M], and adjacent electron-transparent swollen astrocytic processes [asterisks]. Additionally labeled are: an axon terminal [A] and dendritic spines [1,2]. The scale bars in the fields (A-C) = 5  $\mu\text{m}$ , and in (D) = 1  $\mu\text{m}$ . Reproduced from P.F. Fabene et al., 2006, with permission.



**Figure 12.5.**

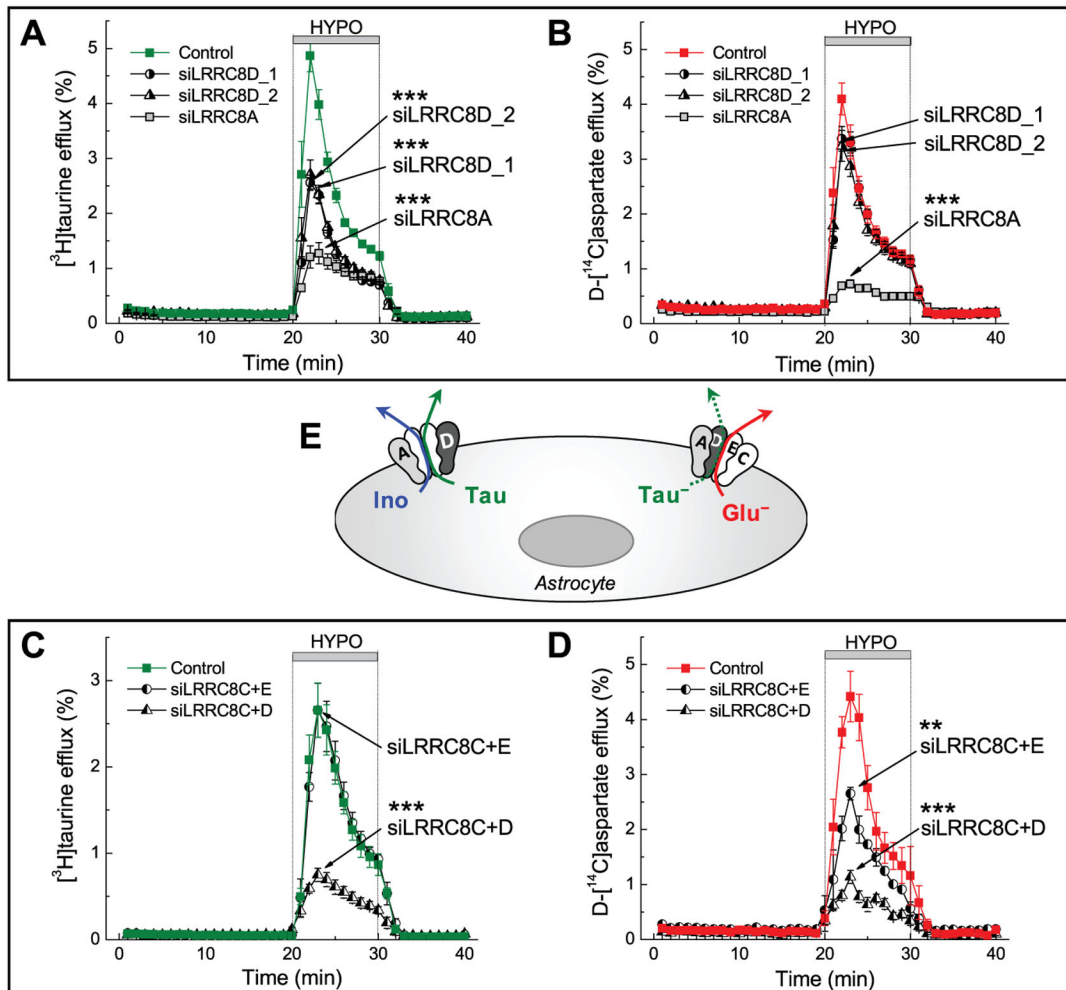
MRI imaging modes which are used in clinical settings to visualize changes in water mobility (cell swelling) following ischemic stroke. *Left panel:* At early times after the onset of focal ischemic stroke, fast spin-echo T2-weighted MRI imaging has yet to show brain damage. *Center panel:* In contrast to the T2 imaging, diffusion weighted imaging (DWI) shows a large hyperintense area in the territories of the prefrontal artery (arrowhead) and a smaller signal in the territory of the anterior cerebral artery (arrow). Hyperintensity reflects a decrease in water mobility upon its shift from the extracellular to the intracellular space. *Right panel:* Apparent diffusion coefficient (ADC) of water identifies the regions of low water mobility as hypointense signals. In 2–3 days, the DWI/ADC positive areas will likely develop full infarction that will be apparent in T2 imaging (not shown). Reproduced from M.G. Lansberg et al., 1999, with permission.





**Figure 12.6.**

Intracerebral treatment with the VRAC blocker DCPIB potently protects against ischemic brain damage in a rat model of stroke induced by 2-hour occlusion of the middle cerebral artery (MCAo). **(A)** Representative images of brain sections prepared from MCAo animals after intracerebral injection of vehicle (DMSO) or 20 µg/kg DCPIB. **(B)** Brain sections from MCAo animal which were treated systemically with intravenous injection of vehicle or 10 mg/kg DCPIB. Animals were euthanized 3 days after ischemia and the extent of brain infarction was revealed by staining brain sections with 2,3,5-triphenyltetrazolium chloride (viable tissue is red). Modified from Y. Zhang *et al.*, 2008, with permission.

**Figure 12.7.**

RNAi analysis reveals that astrocytes express two types of heteromeric LRRC8-containing VRAC channels with differential permeability to charged and uncharged organic osmolytes. (A–D) Primary rat astrocytes were transfected with negative control siRNA (Control), or the gene-specific siRNA targeting the expression of one or more members of the proteins belonging to the family of leucine-rich repeat-containing 8 (LRRC8) proteins. After a 4-day incubation with the gene specific siRNA constructs, astrocytes were simultaneously loaded with  $[^3\text{H}]$ taurine and the glutamate analog D- $[^{14}\text{C}]$ aspartate, and superfused with isosmotic or hypoosmotic (HYPO) media to activate VRAC and quantify the amino acid release. Taurine and glutamate fluxes have been measured simultaneously but, for clarity, are presented in separate panels (A–B) and (C–D). Knockdown of LRRC8A reduced release of all amino acids. Knockdown of LRRC8D reduced  $[^3\text{H}]$ taurine release by ~50% (A), but had no effect on the release of D- $[^{14}\text{C}]$ aspartate (B). Conversely, the combined knockdown of LRRC8C + LRRC8E inhibited the efflux of D- $[^{14}\text{C}]$ aspartate (D), but not  $[^3\text{H}]$ taurine (C). Finally, and surprisingly, the combined knockdown of LRRC8C + LRRC8D inhibited the release of all amino acids to the same extent as the knockdown of LRRC8A. (E) The proposed composition of the two populations of LRRC8-containing VRAC heteromers

which explains the data in (A-D). The LRRC8A+D combination is responsible for the movement of uncharged molecules such as taurine and *myo*-inositol (Ino) (data not shown). Another channel composed of LRRC8A+C+D+E is permeable to negatively charged molecules, including glutamate. Because zwitterionic taurine is partially negatively charged, it shares both pathways. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , amino acid release rates vs. cells treated with the negative control siRNA. Adapted from A.L. Schober *et al.* (2017), with permission.

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