

VRACs and other ion channels and transporters in the regulation of cell volume and beyond

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Abstract | Cells need to regulate their volume to counteract osmotic swelling or shrinkage, as well as during cell division, growth, migration and cell death. Mammalian cells adjust their volume by transporting potassium, sodium, chloride and small organic osmolytes using plasma membrane channels and transporters. This generates osmotic gradients, which drive water in and out of cells. Key players in this process are volume-regulated anion channels (VRACs), the composition of which has recently been identified and shown to encompass LRRC8 heteromers. VRACs also transport metabolites and drugs and function in extracellular signal transduction, apoptosis and anticancer drug resistance.

Osmolytes

Dissolved substances that contribute to osmolarity; in biology, the term mainly refers to substances playing a significant part in osmoregulation. Osmolytes can be electrically charged or neutral, as only the concentration of particles matters.

Hypo- or hypertonic

Condition in which the concentration of osmolytes that are not freely membrane permeable is lower or higher, respectively, on the reference side of a membrane, compared with the other side. This concentration difference creates an osmotic pressure.

Cell size and shape are determined by developmental programmes that involve changes in gene transcription¹. In proliferating cells, cell size regulation depends on growth rates and cell cycle progression and has been most thoroughly studied in unicellular organisms. How cell size feeds back to transcription remains largely unknown, but it may involve, among other factors, the titration of DNA-binding proteins¹. By contrast, vertebrate cell volume regulation^{2–4}, as discussed here, involves the rapid adjustment (within minutes to a few hours) of cellular volume in response to external challenges or during the execution of cellular functions. Cell volume regulation is of fundamental importance for almost every vertebrate cell. Cell swelling, shrinkage and regulatory volume adjustments generally occur without significant exocytic insertion or endocytic retrieval of surface membrane. As cells are not ideal spheres and often have membrane wrinkles, they have enough plasma membrane reserve to undergo substantial swelling before bursting^{5,6}. Acute volume regulation involves the net transport of potassium (K⁺), sodium (Na⁺), chloride (Cl[−]) and organic osmolytes across the plasma membrane (FIG. 1). This transport occurs through various ion channels and transporters (BOX 1) and is followed by osmotically driven water transport, often through specialized water channels (BOX 2). As channels and transporters recruited for cell volume regulation almost always have additional important roles, cell volume regulation is inextricably linked to other cellular functions (BOX 3).

While also addressing sensors of cell volume and subsequent signal transduction cascades, this Review

focuses on effectors of cell volume regulation⁴ — that is, proteins responsible for the transport of osmolytes across the plasma membrane — in mammals and discusses their involvement in short-term cell volume regulation and other cellular processes. Particular attention is paid to volume-regulated anion channels (VRACs), which are key players in vertebrate cell volume regulation that not only transport Cl[−], but also various organic osmolytes. VRACs have been studied biophysically and physiologically for decades^{7,8}, but their molecular composition (of LRRC8 proteins) and the ability of particular LRRC8 heteromers to transport certain metabolites and drugs have only been established recently^{9–11}.

Necessity of volume regulation

Biochemical reactions and equilibria depend on the concentrations of the reaction partners involved. Consequently, cells must avoid short-term changes in volume that will globally affect the concentrations of cellular constituents and thereby interfere with cellular functions. For instance, molecular crowding and ionic imbalance caused by cell shrinkage slows diffusion and affects vesicle traffic¹². Furthermore, changes to intracellular ion concentrations resulting from volume changes can affect cellular excitability and signalling by altering ion gradients across the plasma membrane. Cell swelling is dangerous as it may — in extreme cases — lead to cell bursting.

When vertebrate cells are exposed to a hypo- or hypertonic external medium, they swell or shrink, respectively, because they lack rigid extracellular cell walls (as found, for example, in plants and bacteria), and osmotic

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doi:10.1038/nrm.2016.29
Published online 1 April 2016

Osmolarity

Concentration of solute particles per kg of solution, irrespective of their ability to freely cross a given membrane.

gradients drive water across their outer membrane. Even disregarding differences in volume-regulatory processes, the rate of osmotic cell swelling or shrinkage can vary widely between cell types because of differences in the water permeability of their plasma membranes. Biological lipid bilayers have finite water permeability, which can be markedly increased by dedicated water channels termed aquaporins^{13,14} (BOX 2). Although most mammalian cells are protected from large external osmotic disturbances, because the extracellular space of these tissue-embedded cells is controlled by systemic osmoregulation¹⁵, some cells — such as those in the gastrointestinal tract or distal kidney tubules — may be exposed to substantial changes in extracellular

osmolarity. Moreover, with a pathological decrease in extracellular Na^+ levels (known as hyponatraemia), extracellular osmolarity may be reduced to a degree that causes cerebral oedema. Osmotic challenges may also originate in the cytoplasm, for instance, during the breakdown of macromolecules into a large number of (hence osmotically more active) building blocks or during glucose stimulation of pancreatic β -cells¹⁶.

In almost all cells, osmotic swelling or shrinkage is followed by cell-intrinsic regulatory processes that are called regulatory volume decrease (RVD) or regulatory volume increase (RVI), respectively, which tend to restore the initial cell volume (FIG. 1). RVD and RVI are generally studied (see [Supplementary information S1](#) (box)) using

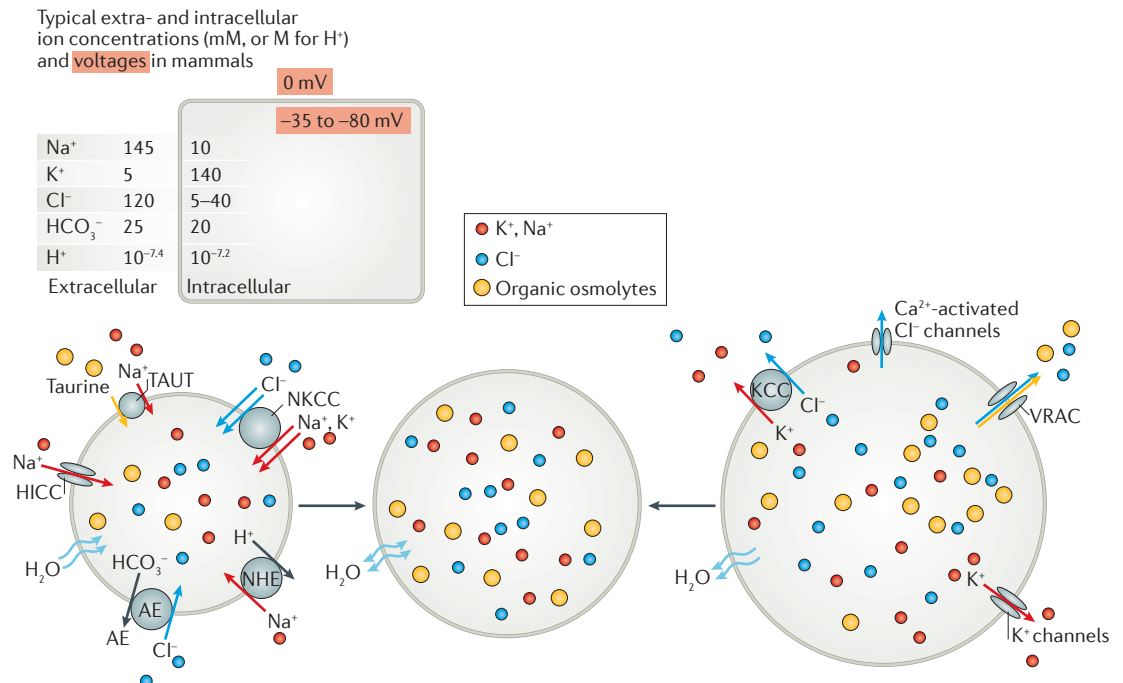
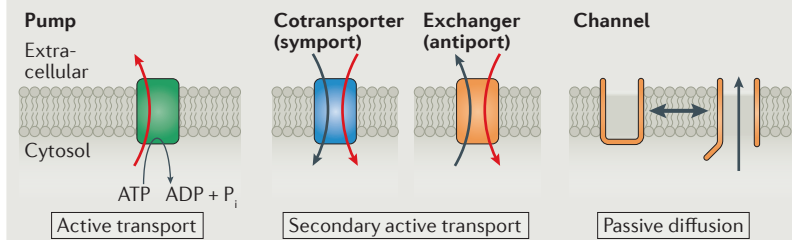


Figure 1 | Plasma membrane channels and transporters involved in cell volume regulation. Cells regulate their volume by adjusting the levels of intracellular osmolytes, including the inorganic ions Cl^- , K^+ and Na^+ , and small organic compounds such as taurine or amino acids. This generates osmotic gradients, which then drive water across the membrane by diffusion through the bilayer or, more efficiently, through aquaporin water channels (BOX 2). Shrunk cells (left) recover their volume by regulatory volume increase (RVI). Depending on the cell type, this mainly involves the parallel activation of Na^+/H^+ exchangers (NHEs; mainly NHE1) and $\text{Cl}^-/\text{HCO}_3^-$ anion exchangers (AEs; mainly AE2), or the stimulation of $\text{Na}^+/\text{K}^+/\text{Cl}^-$ cotransporters (NKCCs; mainly NKCC1). Both processes increase cytoplasmic Na^+ and Cl^- concentrations. In some cells, the activation of hypertonicity-activated, non-selective cation channels (HICCs), for which molecular correlates have not been firmly established, can also be involved and mainly leads to Na^+ influx, which requires a parallel conductance for electroneutrality of overall transport (not shown). In all three cases, the Na^+/K^+ -ATPase (not shown) will gradually replace intracellular Na^+ with K^+ . Na^+ -coupled uptake of taurine by Na^+ - and Cl^- -dependent taurine transporter TAUT (also known as SLC6A6) rather contributes to long-term uptake of this organic osmolyte during RVI. In regulatory volume decrease (RVD; right), cells recover their volume by extruding K^+ , Cl^- and organic osmolytes like taurine. Loss of KCl may occur through K^+/Cl^- cotransporters (KCCs; mainly KCC1, 3 or 4) or through parallel Cl^- and K^+ channels. Key players in RVD are the ubiquitously expressed volume-regulated anion channels (VRACs), which are LRRC8 heteromers. These, depending on subunit composition, conduct not only Cl^- , but also organic osmolytes. In some cells, in which swelling induces a rise in intracellular Ca^{2+} concentration, Cl^- may also exit through various Ca^{2+} -activated Cl^- channels. Channel-mediated Cl^- exit must be electrically balanced by K^+ efflux, which can occur through a range of constitutively open or swelling-activated K^+ channels. Inset: typical extra- and intracellular concentrations of ions involved in mammalian volume regulation. Note that the direction of passive (channel-mediated) ion transport depends not only on the concentration difference, but also on the voltage across the plasma membrane (the electrochemical gradient as given by the Nernst equation; see glossary term ‘Electrochemical gradients’). The intracellular Cl^- concentration shows substantial variation between cell types. In most cells, with the notable exception of most adult neurons, its electrochemical potential will favour Cl^- exit (through VRACs or other Cl^- channels).

Box 1 | Ion transporting proteins

Ion transporting proteins are membrane-embedded proteins or protein complexes that span the lipid bilayer several times to create an ion translocation pathway. Some transporters function as monomers, whereas others are multimers of identical, homologous or non-homologous proteins. They may form a single translocation pathway, but some may have two or more translocation pathways (for instance, one per subunit in the dimeric CLC chloride channels or in tetrameric water channels (BOX 2)).

Functionally, ion transporting proteins can be classified into pumps, transporters and channels. Pumps can transport fixed numbers of ions against their electrochemical gradients through conformational changes driven by direct input of metabolic energy, mostly ATP hydrolysis. This type of transport is called 'active'. Examples include H^+ -ATPases that transport protons, and the Na^+/K^+ -ATPase (which extrudes 3 Na^+ ions and imports 2 K^+ ions in one transport cycle) that is a major determinant of all other ion plasma membrane ion gradients in mammalian cells. Ion transporters are generally defined as also requiring a conformational change per translocation of a fixed number of ions, but they operate without direct input of metabolic energy. The transporter subclasses are cotransporters (symports) that couple the movement of two or more ion species in the same direction (for instance, KCC K^+/Cl^- cotransporters or NKCC $Na^+/K^+/2Cl^-$ cotransporters), or exchangers (antiports) that obligatorily exchange an ion from one side of the membrane for another one on the other side (such as Na^+/H^+ exchangers (NHEs) or Cl^-/HCO_3^- anion exchangers (AEs)). Strict coupling means that the gradient of one ion can drive the other ion against its gradients (termed 'secondary active' transport). Mammalian cells often use the large inwardly directed Na^+ gradient to drive the transport of other ions or organic substrates. The charge of transported ions and the coupling ratio determines whether their transport is 'electroneutral' (meaning that it does not generate electrical currents; examples are KCC and NKCC cotransporters and AEs), or 'electrogenic' (such as CLC $2Cl^-/H^+$ exchangers or NCX $3Na^+/Ca^{2+}$ exchangers). Ion channels, by contrast, have a conduction pathway (that is, a 'pore') for the passive diffusion of ions along their electrochemical gradient. Their transport is intrinsically electrogenic. Channels are generally 'gated' (opened and closed) by a conformational change. Once a channel is open, a non-fixed, large number of ions can pass through. Ion channels generally have much higher transport rates than ion transporters or pumps.



large non-physiological changes in osmolarity. Under most physiological conditions, however, cell volume regulation occurs in the absence of large osmotic gradients or can be initiated without any osmotic imbalance, a process termed near-isotonic volume regulation. Cell volume changes, or 'side-products' of volume regulation such as changes in the voltage across the plasma membrane, may influence other cellular processes (BOX 3). For instance, hepatocyte metabolism can be influenced by cell swelling or shrinkage¹⁷, and glucose-induced swelling of β -cells may stimulate insulin secretion, because activation of VRACs may lead to Cl^- efflux and thereby to depolarization of the cell¹⁸ (BOX 3). In these cases, changes in cell volume may be signals for separate cellular events.

Even in the absence of osmotic challenges, cells must regulate their volume, for instance, during cell division and growth². Volume-regulatory mechanisms may also play a part in cell migration, with local intracellular

osmolarity and volume being increased at the leading edge and decreased at the trailing edge^{19,20}. The passage of salt and water across apical and basolateral membranes must be closely balanced during transepithelial transport, and this may involve volume-sensitive ion transporters. Cells can also change their cell volume in response to external stimuli. For instance, hepatocytes shrink or swell upon stimulation by glucagon and insulin, respectively²¹.

Finally, cell volume changes during cell death, with necrosis being generally associated with cell swelling and apoptosis with cell shrinkage. Cell shrinkage precedes other apoptotic changes^{22–24} in most mammalian cells, with only a few exceptions²⁵. Inhibition of this apoptotic volume decrease (AVD) by extracellular hypertonicity²⁶, various K^+ channel blockers^{22,27}, inhibitors of VRACs^{22,28} or genetic disruption of underlying LRRC8 proteins¹¹ suppressed drug-induced apoptosis. These findings suggest a role for AVD in the progression of apoptosis and in drug resistance in cancer^{11,22–24}.

Cellular osmolytes

Cells regulate their volume by adjusting the intracellular concentrations of dissolved particles (osmolytes) that secondarily drive water flux across their membrane (FIG. 1). Osmolytes can be electrically charged (such as Cl^- , K^+ or glutamate⁻) or neutral (such as myo-inositol or taurine, a 'zwitterion' that carries both a positive and a negative charge at physiological pH). To exert significant osmotic pressure, osmolytes must be poorly membrane-permeable and should be present at sufficiently high concentrations. Among inorganic ions, only K^+ (with intracellular concentration ($[K^+]_i$) ~140 mM), Na^+ ($[Na^+]_i$ ~10 mM) and Cl^- ($[Cl^-]_i$ ~5–40 mM, varying with cell type) are important osmolytes in vertebrate volume regulation (see FIG. 1, inset). Although intracellular cytosolic bicarbonate concentrations ($[HCO_3^-]_i$) are sufficiently high (~20 mM), the equilibrium between $[HCO_3^-]_i$ and $[H^+]_i$ at a given, constant concentration of CO_2 (~5%) implies that large changes in $[HCO_3^-]_i$ during volume regulation would lead to sizeable, possibly harmful, changes in intracellular pH (pH_i). Hence, HCO_3^- is poorly suited as a volume-regulatory osmolyte. An important consideration with charged osmolytes is the requirement for the maintenance of overall electroneutrality of transport. In RVD, for instance, the efflux of Cl^- and K^+ must be almost exactly matched, either by directly coupled, electroneutral transport through K^+/Cl^- cotransporters or by indirect electrical coupling of K^+ and Cl^- channels. Even very small imbalances between the transport of positive and negative charges lead to large changes in the plasma membrane voltage, which must be maintained within a maximal range of a few tens of mVs.

Besides inorganic ions, small organic compounds are key cellular osmolytes. These include taurine, sorbitol, betaine, myo-inositol and glycerophosphocholine. The concentrations of these compounds seem to be sufficient for their function as osmolytes. For instance, tissue concentrations of taurine typically range from 3 to 40 mM²⁹. Particularly high concentrations of organic

Depolarization

A change in the resting voltage of cells (-40 to -80 mV inside versus outside) to more positive values. A change to more negative potentials is called hyperpolarization.

Box 2 | Water flux across membranes: the role of aquaporins

Although water can diffuse across lipid bilayers without the need for water-transporting proteins, dedicated water channels (aquaporins) can, depending on the cell type, drastically enhance (by 5–50-fold) the water permeability of biological membranes^{13,14}. As cell volume regulation involves osmotically driven water transport across the plasma membrane, expression of aquaporins can significantly affect the speed of cell volume regulation (and, of course, the rate at which cells swell or shrink upon changes in extracellular osmolarity; see, for example, REF. 136). There are 13 aquaporin (AQP) genes in humans, of which many encode proteins (including AQP1, 2, 4, 5 and 8) that function as bidirectional water channels¹³. Some aquaporins can also transport glycerol and possibly other small molecules, owing to their wider channel pore. Aquaporin channels function as tetramers of ~30 kDa AQP proteins that have six membrane-spanning domains and two short helical segments that dip into the pore from either side, as revealed by crystal structures. Each of the four subunits has its own pore. Whereas aquaporins have a major, well-established role in osmotically driven water transport, it should be mentioned that some transporters, including Na⁺/K⁺/2Cl⁻ cotransporter 1 (NKCC1) or K⁺/Cl⁻ cotransporter 4 (KCC4) have been suggested to cotransport water together with ions¹³⁷, and that one report suggested that volume-regulated anion channels (VRACs) may also be water-permeable to some degree¹³⁸. Therefore, osmolyte transporters involved in volume regulation might also, to a minor degree, participate in facilitating water transport across membranes, and thereby further contribute to cell volume changes.

osmolytes are found in kidney medulla³⁰, which needs to be hyperosmolar to concentrate urine. After exposure to hypertonicity causing cell shrinkage and an initial, rapid RVI response based on ionic fluxes, these organic osmolytes accumulate in cells, mainly through Na⁺ gradient-driven transporters, which are regulated by the hypertonicity-activated transcription factor TONEBP (also known as NFAT5 or OREBP). The gradual substitution of the accumulated ions by biologically rather inert organic osmolytes allows intracellular ion concentrations to slowly return to near-normal levels, thereby avoiding the harmful effects of high intracellular ionic strength and altered plasma membrane ion gradients. Whereas organic osmolytes play a minor part in short-term RVI because of their low uptake rates, some organic osmolytes like taurine or glutamate play a part in short-term RVI, because they can be rapidly released by VRACs (FIG. 1).

Mechanisms of cell volume regulation

Short-term regulatory volume changes (RVD and RVI) require a fast reversal of the osmotic gradients that led to cell swelling or shrinkage, respectively. This is accomplished by the activation of plasma membrane channels and transporters (BOX 1) that transport Na⁺, K⁺, Cl⁻ or (in RVD) organic osmolytes. The net transport by both channels and transporters is determined by the electrochemical gradients of their substrates and does not involve direct expenditure of metabolic energy. However, ion pumps (mainly the Na⁺/K⁺-ATPase) are ultimately responsible for the generation of cellular ion gradients. Inhibition of the Na⁺/K⁺-ATPase — for example, during anoxia — eventually leads to a dissipation of ion gradients and to cell swelling owing to Donnan effects. None of the channels or transporters that are involved in RVD or RVI is exclusively, and often not even mainly, dedicated to cell volume regulation. Given the molecular diversity of ion channels and transporters, there is a considerable redundancy of volume-regulatory effectors (TABLE 1).

Different cells may use different sets of ion transport proteins for regulating their volume. This section will describe the major mechanisms and principles governing volume regulation by these membrane effectors.

Regulatory volume increase. RVI mainly involves the cellular uptake of Na⁺, K⁺ and Cl⁻. Excess intracellular Na⁺ is secondarily exchanged for K⁺ by the Na⁺/K⁺-ATPase. Although large electrochemical gradients favour Na⁺ entry, which could secondarily drive Cl⁻ influx in response to cellular depolarization, Na⁺- and Cl⁻-selective channels are generally not used in RVI. However, hypertonicity-induced cation channels (HICCs) that conduct both Na⁺ and K⁺ can be involved in RVI in some cells^{31,32}. The molecular identity of these channels has not yet been firmly established.

RVI probably mainly involves Na⁺/K⁺/2Cl⁻ (NKCC) cotransporters or a parallel activation of Na⁺/H⁺ and Cl⁻/HCO₃⁻ exchangers (FIG. 1; TABLE 1). NKCCs couple the movement of one Na⁺, one K⁺ and two Cl⁻ ions in the same direction and hence do not generate currents (that is, they are 'electroneutral'). In this case, the inwardly directed Na⁺ and Cl⁻ gradients of mammalian cells drive K⁺ uptake against its considerable outward gradient. Mammals have two genes encoding NKCC proteins. NKCC1 (also known as SLC12A2) is almost ubiquitously expressed, whereas NKCC2 (SLC12A1) is mainly found in the kidney, where it mediates salt reabsorption. NKCC1 is activated by cell shrinkage in a phosphorylation-dependent manner³³ (see below). Apart from mediating RVI, it has basal transport activity that leads to the accumulation of intracellular Cl⁻. This role of NKCC1 is shared with Cl⁻/HCO₃⁻ exchangers and is important for VRAC-mediated efflux of Cl⁻ in RVD.

Other major players in RVI are Na⁺/H⁺ exchangers of the NHE (SLC9A) family^{34,35} (FIG. 1; TABLE 1). Mammals express nine different NHEs, which reside in the plasma membrane or in intracellular organelles. Consistent with its role in pH_i regulation, the widely expressed NHE1 is activated by acidic pH_i (REF. 36). Cell shrinkage activates NHE1 by shifting its activation curve towards more alkaline pH_i and by relieving inhibition by extracellular Na⁺ (REF. 37). As the vast majority of H⁺ ions that are extruded by NHE1 are derived from intracellular buffers, proton efflux per se is osmotically irrelevant and NHE activation increases cellular osmolarity through Na⁺ influx. In the presence of CO₂, the cytoplasmic alkalization caused by NHE1 activation leads to slow HCO₃⁻ accumulation, which is counteracted by Cl⁻/HCO₃⁻ exchangers, mostly the widely expressed anion exchange 2 (AE2; also known as SLC4A2) isoform. AE2 is activated by intracellular alkalization³⁸ and, with physiological ion gradients, mediates Cl⁻ influx and HCO₃⁻ efflux (FIG. 1; TABLE 1). The indirect coupling of NHEs and anion exchangers by pH_i results in electroneutral uptake of NaCl with only minor effects on pH_i or [HCO₃⁻]_i.

Regulatory volume decrease. RVD involves the exit of K⁺ and Cl⁻ through electroneutral K⁺/Cl⁻ cotransporters or through parallel K⁺ and Cl⁻ channels (prominently including VRACs), as well as an efflux of organic

Electrochemical gradients

The passive, diffusive transport of an ion across a membrane through a channel is driven not only by the concentration difference of that ion, but also by the voltage across that membrane. The driving force is given by the difference in electrochemical potential according to the Nernst equation, for example, for a singly charged anion A⁻ by $E_A = (RT/F) \cdot \ln([A^-]_i / [A^-]_o)$, with R being the gas constant, T the absolute temperature, F Faraday's constant, and [A⁻]_i the intracellular and [A⁻]_o the extracellular concentration of the anion.

Donnan effects

Effects on the voltage and concentration differences of charged particles across a semipermeable membrane, caused by the impermeability of the membrane to one ionic species.

GABA (γ -aminobutyric acid) type A receptor (GABA_A receptor).

A pentameric anion channel that is opened by the binding of GABA. Channels of this type mediate fast synaptic inhibition in the nervous system by allowing a passive influx of Cl⁻ which hyperpolarizes the membrane. This requires a low intracellular Cl⁻ concentration, as found in most adult neurons.

Glycine receptor

A pentameric anion channel that inhibits neuronal excitability by mediating a hyperpolarizing Cl⁻ influx upon extracellular binding of the amino acid and neurotransmitter glycine.

Conductance

Electrical conductance is the inverse of electrical resistance. For instance, the K⁺ conductance of a biological membrane is a quantitative measure of the current mediated by K⁺ channels divided by the driving force (given by potential and concentration differences across the membrane). It depends on the number of open K⁺ channels and how readily they allow the passage of K⁺ ions.

osmolytes through VRACs. K⁺/Cl⁻ cotransporters (KCCs) are encoded by four genes, *KCC1–KCC4* (*SLC12A4–SLC12A7*). By coupling the large outward gradient of K⁺ (~140 mM/5 mM) to Cl⁻ transport, KCCs lower [Cl⁻]_i to less than 5 mM at thermodynamic equilibrium. Low values of [Cl⁻]_i (~5–10 mM) are maintained in adult neurons, in which KCC2 ensures synaptic inhibition by generating the appropriate driving force for Cl⁻ entry through GABA (γ -aminobutyric acid) type A receptor (GABA_A receptor) and glycine receptor channels³⁹. In most other cells, [Cl⁻]_i is about 20–40 mM, and activation of KCCs leads to net KCl efflux, thereby decreasing cell osmolarity and volume. All KCC isoforms can be activated by cell swelling, with KCC3 being more sensitive than KCC1 (REF. 40). KCC2 additionally displays constitutive activity⁴¹. KCC1 is almost ubiquitously expressed, whereas KCC3 and KCC4 have more restricted expression patterns and KCC2 is neuron-specific. A role for K⁺/Cl⁻ cotransport in erythrocyte RVD was recognized at an early stage⁴². Mammalian erythrocytes express KCC1 and KCC3 (REF. 43), with KCC3 mediating the bulk of swelling-induced K⁺/Cl⁻ cotransport in mouse erythrocytes⁴³. Increased erythrocyte volume and higher susceptibility to osmotic lysis suggested that RVD was severely compromised in *Kcc1*^{-/-} *Kcc3*^{-/-} but not in either *Kcc1*^{-/-} or *Kcc3*^{-/-} erythrocytes⁴³,

indicating that KCC1 and KCC3 can partially substitute for each other. Conversely, erythrocyte volume was reduced in mice carrying a *Kcc1* mutation that constitutively activates KCC1 (REF. 44). These mouse models^{43,44} demonstrate that volume-regulatory processes can determine steady-state cell volume *in vivo*. RVD was also reduced in cultured hippocampal neurons from *Kcc3*^{-/-} mice, and in renal proximal tubules, RVD was more severely affected in *Kcc3*^{-/-} than in *Kcc4*^{-/-} mice⁴⁵.

Another, probably more widespread, mechanism in RVD involves the exit of K⁺ and Cl⁻ through independent K⁺ and Cl⁻ channels that are coupled by the plasma membrane voltage² (FIG. 1; TABLE 1). The opening of K⁺ channels hyperpolarizes the membrane and thereby increases the driving force for Cl⁻ exit. Conversely, Cl⁻ channel opening depolarizes the membrane of most cells and hence increases K⁺ efflux. K⁺ conductance exceeds that of Cl⁻ under resting conditions in most cells. Swelling-induced opening of Cl⁻ channels may then be sufficient to elicit RVD.

In many, but by no means all cells, swelling is associated with an increase in [Ca²⁺]_i (for a list of such cells, see REF. 2). In these cells, RVD may involve the opening of Ca²⁺-activated K⁺ and Cl⁻ channels^{46–48}. These channels are molecularly diverse. Ca²⁺-activated K⁺ channels belong to small, intermediate and big conductance classes

Box 3 | Additional processes associated with volume regulation

Cell division and migration, and necrotic and apoptotic cell death provide examples of processes that are directly linked to volume regulation. Obviously, overall cell volume increases during the generation of daughter cells, but so far it remains largely unclear whether and how this relates to the change in expression of various ion channels and transporters during the cell cycle. Cell migration may depend, in addition to cell–matrix interaction, on the cytoskeleton and motor proteins, as well as on cell volume regulatory processes^{19,20}. It is thought that the leading edge of migrating cells recruits transporters involved in regulatory volume increase (RVI) — such as Na⁺/H⁺ exchanger 1 (NHE1) and anion exchange protein 2 (AE2) (for examples, see REFS 139,140), as well as aquaporins^{141,142} — thereby increasing local intracellular osmotic pressure and volume. Conversely, the trailing edge may decrease its volume by regulatory volume decrease (RVD), which may involve various K⁺ channels (for example, EAG2 (REF. 143)) and possibly volume-regulated anion channels (VRACs)¹⁴⁴. Cells may indeed generate sufficient differences in hydrostatic pressure over distances of several micrometres and timescales of several seconds¹⁴⁵, and recent experimental and theoretical work showed that under spatial confinement (as possibly found during cell migration within tissues), such ‘osmotic engines’ can support migration even if actin polymerization and myosin contractility are blocked²⁰. Furthermore, activation of VRACs, in parallel to K⁺ channels, participates in apoptotic volume decrease (AVD) that might foster the progression of apoptosis^{11,22,146} (although probably not in all cell types^{25,146}). For the role of VRACs in apoptosis, see the main text and FIG. 3a. It should be noted that in many processes, including cell migration and AVD, the primary trigger that activates the cell volume-changing channels and transporters is not a change in cell volume. This does not exclude, of course, the possibility that the activation of respective transporters uses part of the same, poorly understood signal transduction machineries that are used for RVI or RVD.

The ion transport associated with cell volume regulation not only changes intracellular osmolarity, but also affects the plasma membrane voltage (in the case of ion channels) or intracellular pH (pH_i; in the case of Na⁺/H⁺ or Cl⁻/HCO₃⁻ exchangers (NHEs or AEs)). In general, these potentially deleterious effects are avoided by neutralizing currents through other channels (for example, by parallel operation of K⁺ and Cl⁻ channels) or by the activation of transporters with opposite effects on pH_i (acid-extruding NHE1 is activated by acidic pH_i, whereas base-extruding AE2 is activated by alkaline pH_i, resulting in a pH-mediated coupling of these transporters). Nonetheless, relatively minor changes in pH_i and plasma membrane voltage may be observed during volume regulation in some cells. In insulin-secreting pancreatic β -cells, for instance, an increase in glucose concentration might lead to cell swelling, activation of VRAC and Cl⁻ efflux. The subsequent depolarization may open voltage-dependent Ca²⁺ channels and raise cytosolic Ca²⁺ levels, and thereby trigger exocytosis of insulin-containing granules^{16,147}. However, this hypothesis, like many others, will require confirmation using the tools available after the identification of VRAC components^{9,10}.

Many of the ion transporters and channels have crucial roles in other processes. For instance, NHE1 and AE2 are major regulators of pH_i. Like NKCC1, they also have important roles in salt and water transport across several epithelia. An imbalance of ion transport across basolateral and epithelial membranes of epithelia may change cell volume, in particular, during acute stimulation of transepithelial transport. This might activate some of these volume-regulatory processes. In line with these considerations, recent work suggests a role for VRAC in β -adrenergic-induced fluid secretion in salivary glands¹⁴⁸.

(SK, IK and BK channels). Both the bestrophin⁴⁹ and TMEM16 (also known as anoctamin)⁵⁰ gene families may encode Ca^{2+} -activated Cl^- channels. Whereas *Drosophila melanogaster* Bestrophin 1 is dually regulated by $[\text{Ca}^{2+}]_i$ and cell swelling^{51,52}, mammalian bestrophins are inhibited upon hypertonic shrinkage⁵³ but, in the absence of increases in $[\text{Ca}^{2+}]_i$, fail to activate upon

hypotonic swelling⁵¹. Similarly, TMEM16 channels do not seem to be swelling-activated without an increase in $[\text{Ca}^{2+}]_i$ (REFS 46,48). These channels must be distinguished from canonical volume-regulated Cl^- channels, which are nearly ubiquitously expressed in vertebrates, display a distinct, well-studied biophysical fingerprint^{7,54} and comprise LRRC8 heteromers⁹ (FIG. 2).

Table 1 | Main ion channels and transporters involved in cell volume regulation

Effector	Protein(s)	Transport	Regulation	Other roles
Regulatory volume increase (RVI)				
Na^+/H^+ exchangers (NHEs)	Mainly NHE1 (possibly also NHE2 and NHE4), widely expressed across tissues	1:1 exchange of external Na^+ for internal H^+ (electroneutral)	<ul style="list-style-type: none"> Activated by acidic intracellular pH (pH_i) Several mechanisms discussed for shrinkage-activation 	<ul style="list-style-type: none"> Regulation of pH_i (counteracts acidification) Transepithelial transport (of acid and Na^+; also prominent for NHE3)
$\text{Cl}^-/\text{HCO}_3^-$ exchangers	Mainly anion exchanger 2 (AE2), widely expressed	1:1 exchange of external Na^+ for internal H^+ (electroneutral)	Activated by alkaline pH_i	<ul style="list-style-type: none"> Regulation of pH_i (counteracts alkalization) Transepithelial transport (of acid and Cl^-) Raising intracellular Cl^- concentration ($[\text{Cl}^-]_i$)
$\text{Na}^+/\text{K}^+/2\text{Cl}^-$ (NKCC) cotransporters	Mainly NKCC1, widely expressed	Coupled uptake of 1Na^+ , 1K^+ and 2Cl^- into cells (electroneutral)	Activation through SPAK and OSR1 kinases that are activated by Cl^- -sensing WNK kinases (inhibited by $[\text{Cl}^-]_i$), leading to inhibition of NKCC1 by $[\text{Cl}^-]_i$	<ul style="list-style-type: none"> Transepithelial transport (of Cl^- or K^+) Raising $[\text{Cl}^-]_i$
Hyperosmolarity-induced non-selective cation (HICC) channels	Unclear (possibly several distinct channels, some candidates that require confirmation)	Passive influx of Na^+ and efflux of K^+ (electrogenic; may need parallel Cl^- influx for electroneutrality)	?	?
Regulatory volume decrease (RVD)				
K^+/Cl^- cotransporters (KCCs)	KCC3, KCC4 and KCC1	Coupled efflux of 1K^+ and 1Cl^- (electroneutral)	Inhibited by SPAK and OSR1 kinases, which are in turn activated by WNK kinases. These are directly inhibited by $[\text{Cl}^-]_i$, leading to activation of KCCs by $[\text{Cl}^-]_i$	<ul style="list-style-type: none"> Lowering of $[\text{Cl}^-]_i$ Transepithelial transport
Volume-regulated anion channels (VRACs)	Various heteromers of LRRC8A with LRRC8B, C, D or E (LRRC8D increases taurine permeation), nearly ubiquitously expressed	<ul style="list-style-type: none"> Passive efflux of Cl^- (in cells with appropriate Cl^- gradient; electrogenic) Passive efflux of organic osmolytes (for example, taurine or glutamate; electrogenic or electroneutral (electrogenic operation needs parallel K^+ channels)) 	Several mechanisms discussed (ionic strength, ROS, RHO signalling, cytoskeleton, tyrosine phosphorylation and others)	<ul style="list-style-type: none"> Effector of apoptotic volume decrease (AVD) Release of signalling molecules (glutamate, aspartate, taurine) Drug uptake (for example, Pt-based anticancer drugs) Transepithelial transport (?), insulin secretion (?)
Calcium-activated Cl^- channels	Bestrophins (BEST1, BEST2?), TMEM16/anoctamin proteins (ANO1, ANO6?), distinct tissue distributions	Passive efflux of Cl^- (in cells with appropriate Cl^- gradient; electrogenic (needs parallel K^+ channels))	<ul style="list-style-type: none"> Activated by rise in cytosolic Ca^{2+} Can mediate RVD only in subset of cells that raise $[\text{Ca}^{2+}]_i$ in response to swelling and express one of these proteins 	Transepithelial Cl^- transport and others
Calcium-activated K^+ channels	Various SK, IK and BK channels, distinct tissue distributions	Passive efflux of K^+ (electrogenic; needs parallel Cl^- channels)	<ul style="list-style-type: none"> Activated by rise in cytosolic Ca^{2+} Can mediate RVD only in a subset of cells that raise $[\text{Ca}^{2+}]_i$ in response to swelling and express one of these channels 	Various
Other K^+ channels	Two-pore domain K2P K^+ channels (for example, TRAAK, TREK and TASK2) and various voltage-dependent K_v channels, distinct tissue distributions	Passive efflux of K^+ (electrogenic; needs parallel Cl^- channels)	Membrane stretch and curvature, lipid mediators (K2P channels)	Various

OSR1, oxidative stress-responsive 1 protein; Pt, platinum; ROS, reactive oxygen species; SK, IK and BK channels, small, intermediate and big conductance K^+ channels; SPAK, SPS1-related proline/alanine-rich kinase; WNK, with no lysine.

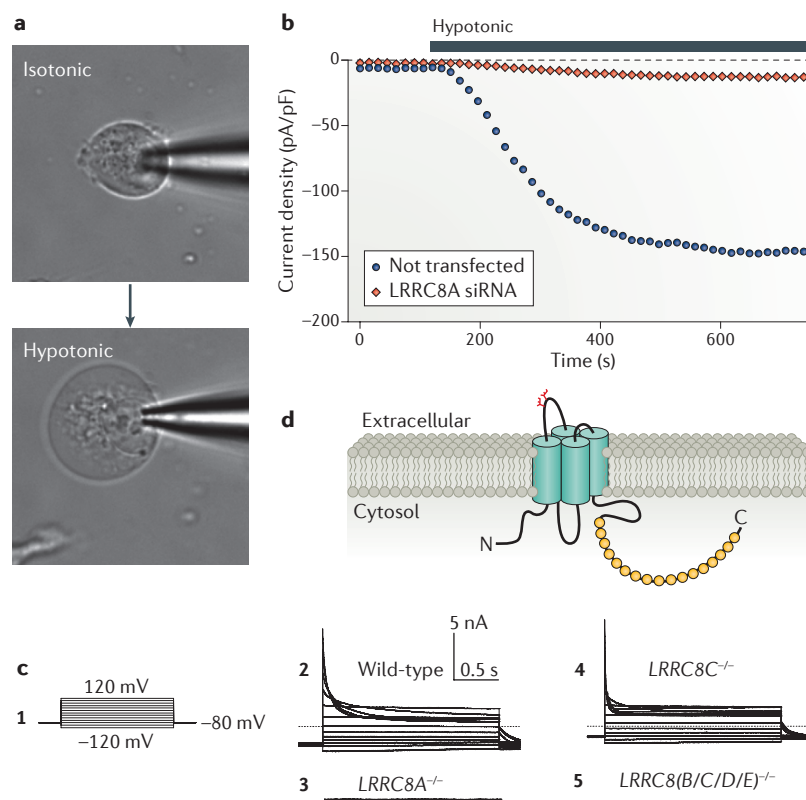


Figure 2 | Properties of volume-regulated anion channels (VRACs). **a** | VRAC currents ($I_{Cl,vol}$) can be measured using the patch-clamp technique. An isolated HEK cell attached to a cover slip is approached by a glass pipette filled with saline (shown on the right). After contact with the plasma membrane is established, negative pressure of the pipette solution (suction) seals the polished glass pipette with the membrane. This ensures mechanical stability and a high electrical resistance ($G\Omega$ range). Further suction ruptures the membrane patch and allows electrical access to the cell interior in the 'whole-cell configuration' of the patch-clamp technique. An electrical amplifier attached to the pipette allows measurements of cell voltage and current injection. The cell is then superfused with hypotonic solution (typically ~25%), leading to slow cell swelling as shown below. **b** | VRAC-mediated $I_{Cl,vol}$ currents develop over minutes after exposure to hypotonicity during the course of cell swelling. Ionic conditions are chosen in such a way as to virtually exclude cation currents. Anion currents elicited by test pulses to negative voltages are shown. Short interfering RNA (siRNA)-mediated knockdown of the essential VRAC subunit LRRC8A nearly abolishes these currents. **c** | To obtain a biophysical fingerprint of currents, the cell interior is clamped by the amplifier through the patch pipette to several distinct voltages. The first panel (1) shows a superposition of these sequentially applied voltage steps. The current that the amplifier needs to inject into the cell to attain the desired voltage is measured. This equals the current flowing through all plasma membrane channels of the cell. Superimposed currents of hypotonically swollen wild-type HCT116 cells are shown in (2). These currents show outward rectification (larger currents at positive than at negative voltage) and inactivation at positive voltages (currents decline over time at constant voltage). Both features are typical of $I_{Cl,vol}$ but are unlikely to be physiologically important. (3) $LRRC8A^{-/-}$ cells, which lack the essential VRAC subunit LRRC8A, lack $I_{Cl,vol}$ currents, but (4) cells lacking LRRC8C (or B, D or E; not shown) display currents with changed inactivation kinetics. (5) Parallel disruption of $LRRC(B-E)$ genes also abolishes $I_{Cl,vol}$. **d** | Topology model of a LRRC8 subunit (not to scale) with 4 transmembrane spans and a cytosolic carboxyl terminus containing 17 leucine-rich repeats that gave rise to its name (leucine-rich repeat-containing 8). VRACs are hypothesized to be hexamers¹⁰⁵ of LRRC8A and at least one of the other LRRC8 proteins (B–E)⁹. Images in part **a** are courtesy of Florian Ullrich, Leibniz-Institut für Molekulare Pharmakologie (FMP) and Max-Delbrück-Centrum für Molekulare Medizin (MDC), Berlin, Germany. Images in parts **b** and **c** adapted with permission from REF. 9, AAAS.

Various Ca^{2+} -independent K^{+} channels can be activated by cell swelling. These include voltage-dependent K_v channels, such as several KCNQ (K_v7) channels⁵⁵ and K2P channels^{56,57}, some of which directly respond to membrane stretch^{58–60} (TABLE 1). Swelling- or stretch-activation of a channel, however, does not automatically imply its involvement in cell volume regulation. Even if the pharmacological inhibition of a channel impairs RVD, this does not prove that its swelling-activation is important for, or triggers, RVD. This stems from the fact that blocking any major constitutive K^{+} conductance may impair RVD by preventing charge compensation for Cl^{-} efflux through volume-regulated Cl^{-} channels.

Two molecularly identified mammalian Cl^{-} channel types, VRACs⁷ and Cl^{-} channel protein 2 (CIC-2)⁶¹, have been shown to be swelling-activated, and residues crucial for swelling-activation have been mapped in CIC-2 (REFS 61,62). Furthermore, a distinct swelling-activated, ATP-conducting 'maxi-anion channel' has been described⁶³, but its molecular identity is unknown and its contribution to volume regulation is ill-defined. CIC-2 and VRACs are widely expressed across tissues but strongly differ in their biophysical properties^{7,63,64}. Although glial cells appeared slightly swollen in *Clcn2*^{-/-} mice⁶⁵, convincing evidence for a role of CIC-2 in volume regulation is lacking⁶⁶. This contrasts with VRACs, which play a major part in RVD in a wide range of cells⁷. This may be related, in part, to the fact that VRACs (unlike CIC-2) also conduct organic osmolytes and are more strongly regulated by cell swelling.

Sensors and transducers in volume regulation. While the list of effectors in cell volume regulation is becoming reasonably complete, our understanding of the mechanisms involved in sensing cell volume and in the complex signal transduction to plasma membrane effectors is frustratingly incomplete. It is clear that cells cannot directly sense their volume, which is an extensive parameter. Mechanical forces at the outer membrane, concentrations of intracellular ions or molecules, or altered interactions of macromolecules following changes in cell shape are candidates for parameters that indirectly reflect volume changes.

In principle, 'volume' sensing might involve mechanosensitive plasma membrane proteins such as signalling receptors or ion channels. Mechanosensitive channels may directly be effectors (that is, significantly transport osmolytes), or they may change intracellular concentrations of ions such as Ca^{2+} that regulate other channels⁶⁷ or transporters. Mechanosensitive channels may either directly sense the lateral tension in the lipid bilayer (known as the 'force from lipid' model) or be gated by mechanical push or pull from extra- or intracellular molecules (called the 'tethered model'⁶⁸). The bacterial MscL channel is a prime example of a directly mechanosensitive channel⁶⁹, and many vertebrate channels may be similarly mechanosensitive⁷⁰. For instance, TRAAK (also known as KCNK4) K2P K^{+} channels respond to changes in both pressure and membrane

curvature⁷¹. Recent biophysical and structural studies provide strong evidence for direct ‘force from lipid’ mechanosensitivity of TRAAK and TREK2 (also known as KCNK10) K⁺ channels^{58,59}. However, the large plasma membrane reserve of most cells (in the form of wrinkles and infoldings)⁶ suggests that direct membrane stretch may require rather extreme swelling. Therefore, changes in membrane curvature or forces exerted by the cytoskeleton or the extracellular matrix are more likely to modulate ion transport during cell volume changes. Indeed, many channels and transporters involved in volume sensing and/or regulation are thought to be gated or influenced by cytoskeletal elements^{72–74}. However, only experiments involving procedures such as mutation⁷³ or outcompeting⁷² of cytoskeletal binding sites on target proteins can prove the specificity of signalling; the mere disruption of cytoskeletal elements is not sufficient. ‘Force from lipid’ activation and ‘tethering’ may, in principle, converge on the same transport protein, as has been suggested for TRAAK channels⁷¹ and NHE1 (REF. 75). Alterations in cell volume can also affect the concentrations of plasma membrane lipids. These include phosphoinositide phosphates such as phosphatidylinositol-4,5-bisphosphate that influence many targets, including various ion channels; however, the mechanisms by which cell volume affects these lipids remain unclear^{76,77}.

In addition to processes at the plasma membrane, cell swelling or shrinkage may be sensed by associated changes in the intracellular concentrations of ions or other molecules. For instance, VRACs could be activated by reducing the intracellular ionic strength^{78,79}, even when purified VRACs were reconstituted into lipid bilayers⁸⁰, suggesting that VRACs are both sensors and effectors of cell volume regulation. Fascinating insights were also obtained for the regulation of cation-Cl[−] cotransporters. Both NKCCs and KCCs are phosphorylated by SPAK (SPS1-related proline/alanine-rich kinase) and OSR1 (oxidative stress-responsive 1 protein), which are serine/threonine kinases that bind to a site in their carboxyl termini^{13,81,82}. Phosphorylation of these transporters activates Cl[−]-accumulating Na⁺-driven transporters, but inhibits Cl[−]-extruding K⁺/Cl[−] cotransporters, resulting in a molecular switch for cytosolic Cl[−] homeostasis and volume regulation. SPAK and OSR1 themselves are activated by phosphorylation through WNK (with no lysine) kinases. WNKs are activated by autophosphorylation in response to a reduction in [Cl[−]]_i or hypertonic shrinkage^{83,84}. Excitingly, the crystal structure of the WNK1 kinase domain revealed a bound Cl[−] ion that inhibits autophosphorylation⁸⁵. Thus, WNK1 — like probably also VRACs^{78–80} — may represent a genuine (indirect) sensor of cell volume changes.

Various signalling pathways have been implicated in cell volume regulation^{2,7}. As an example, VRACs were shown to be affected by cytoplasmic ionic strength^{78–80}, NADPH oxidase and reactive oxygen species^{86,87}, RHO-family GTPases and the cytoskeleton^{74,88}, tyrosine phosphorylation⁸⁹, leukotrienes⁹⁰, plasma membrane cholesterol⁹¹, and integrins and epidermal growth factor

(EGF) receptor signalling⁹², among others. None of these regulatory processes seems to be prominent in all cell types. Generally, the promiscuity of the proposed signalling pathways and their crosstalk hampers conclusions on their relevance in physiological settings. This problem is further complicated by the mostly drastic and often unspecific experimental interventions that have been used to investigate cell volume regulation and the underlying signalling pathways, and — until recently — by our ignorance concerning the molecular identity of VRACs.

Solving the puzzle of VRAC composition

Swelling-activated Cl[−] currents have been known since the 1980s^{93–95}, and the biophysical properties, regulation and roles of the underlying VRACs have been studied extensively (reviewed in REFS 5, 7, 8, 96). More than eight molecular VRAC ‘candidates’ (including the multidrug resistance P-glycoprotein pICln and ClC-3) have been proposed, but all were disproved^{5,8}. Therefore, until the recent discovery of LRRC8 proteins as crucial VRAC components^{9,10}, physiological roles ascribed to VRACs mostly hinged on experiments with nonspecific drugs, and the field stagnated.

VRACs are also known as VSORs (volume expansion-sensing outwardly rectifying anion channels⁵) or VSOACs (volume-stimulated organic osmolyte and anion channels⁹⁷), although the latter name refers specifically to channels also transporting organic osmolytes, which some authors thought to be distinct from VRACs^{48,98,99}. We now know that the observed variability in properties of VRACs, including their ability to transport organic substances, is due to differences in their LRRC8 subunit composition^{9,11}.

VRAC-generated currents. VRAC currents (named I_{Cl,vol} or I_{Cl,swell} when activated by swelling) show moderate outward rectification, variable channel inactivation at inside-positive voltages (FIG. 2c) and I[−]>Cl[−]>Br[−] selectivity. They also require intracellular ATP⁹⁷ and background levels of [Ca²⁺]_i, and can be inhibited by various nonspecific drugs⁷. Depending on their subunit composition¹¹, VRACs also conduct organic substances such as taurine^{9–11,100}. I_{Cl,vol} activates over minutes after reducing extracellular osmolarity by ~25% to induce cell swelling (FIG. 2a,b). However, VRACs can also be activated to a lesser degree without cell swelling by pro-apoptotic drugs^{11,87} and can be opened or potentiated by the stimulation of certain plasma membrane receptors^{86,101–103}.

Typical I_{Cl,vol} currents have been observed in many vertebrate cell types^{7,104}, suggesting an almost ubiquitous expression. However, these currents have not been found in non-chordates such as insects⁵¹. This is consistent with the fact that the expression of protein components of VRACs (discussed below) is observed in vertebrates and some other chordates, but not in non-chordates (with the surprising exception of sea anemones)¹⁰⁵. In many studies^{2,7}, pharmacological inhibition of VRACs severely impaired or blocked RVD. This pivotal role of VRACs in volume regulation may partially be owing to its ability to also mediate efflux of organic osmolytes.

Outward rectification

A channel is called outwardly rectifying if its currents are larger at inside-positive than at inside-negative voltages, which corresponds to an outward transport of positive charge. For anion channels this corresponds to an inward transport of anions.

Channel inactivation

The spontaneous closure of a channel at constant voltage.

LRRC8 proteins as core components of VRACs. Using a functional assay in genome-wide short interfering RNA (siRNA) screens, two groups independently identified LRRC8A as an essential VRAC component^{9,10}. CRISPR–Cas9-mediated gene ablation⁹ and partial knockdown with siRNAs¹⁰ revealed that LRRC8A is required for $I_{Cl,vol}$. However, LRRC8A needs at least one other LRRC8 isoform (LRRC8B, C, D or E) to form functional channels, as demonstrated by parallel disruption of multiple LRRC8 genes⁹ (FIG. 2c) or by re-expressing LRRC8 isoforms in cells engineered to lack all five LRRC8 proteins⁹. LRRC8A physically interacts with the other LRRC8 isoforms^{9,106} and is required for the transport of these isoforms (in a heteromeric complex with LRRC8A) to the plasma membrane⁹. Hence, LRRC8 heteromers are crucial VRAC components⁹. However, heterologous co-expression of LRRC8 isoforms has so far failed to significantly increase $I_{Cl,vol}$ above native levels⁹. It is therefore possible that yet another subunit is necessary to form functional VRACs in cells, although purified LRRC8 heteromers were sufficient to yield currents in artificial bilayers⁸⁰. Alternatively, heterologous expression may have yielded few correctly assembled heteromers, or strict feedback regulation might exist to limit VRAC currents despite an increased abundance of channels.

RVD was reduced in HeLa cells upon LRRC8A knockdown¹⁰ and was virtually abolished in *LRRC8A*^{−/−} or *LRRC8(B/C/D/E)*^{−/−} HEK cells^{9,11}, demonstrating that VRACs are major players in cell volume regulation. Notably, swelling-induced taurine efflux was also reduced by LRRC8A knockdown in HeLa cells¹⁰, and by genomic disruption of either LRRC8A or all other LRRC8 subunits (B to E) in HCT116 and HEK cells^{9,11}. Hence, LRRC8 heteromers are also essential for swelling-induced taurine release, and VRACs and ‘VSOACs’ are either identical or at least share subunits.

LRRC8 proteins have four predicted transmembrane domains and up to 17 carboxy-terminal leucine-rich repeats (hence their name leucine-rich repeat containing 8) (FIG. 2d). They were originally speculated to be receptors with an extracellular leucine-rich repeat-containing interaction domain¹⁰⁷, but it is now established that their amino and carboxyl termini are cytoplasmic^{9,10,105,106}. The transmembrane part of LRRC8 proteins has weak but significant sequence homology to pannexins¹⁰⁸, which are probably hexameric plasma membrane channels that have structural but not sequence similarity to gap junction-forming connexins¹⁰⁵. Similar to VRACs, pannexins conduct ions and organic compounds and are blocked by carbenoxolone¹⁰⁸. On the basis of their similarity to pannexins¹⁰⁵, it was suggested that VRACs function as LRRC8 heterohexamers⁹, but the exact number of LRRC8 proteins constituting VRACs remains unknown. As there are five different LRRC8 proteins, an assumed heterohexameric assembly suggests that many different VRACs, with different subunit composition and properties, may exist. These may coexist in the same cell, as most LRRC8 genes are widely expressed across tissues and cells.

LRRC8 composition and VRAC function. First insights into the roles of different VRAC subunits have been obtained. For instance, the combination of LRRC8 subunits determines the inactivation of $I_{Cl,vol}$ at positive voltages (FIG. 2c), and inactivation characteristics of native $I_{Cl,vol}$ correlate with the expression of LRRC8 isoforms⁹. Importantly, the subunit composition of a VRAC determines its substrate selectivity¹¹ and single-channel conductance⁸⁰. Small changes in halide ion selectivity with LRRC8 composition were observed in one study⁸⁰ but not in another⁹. Much larger effects of subunit composition were observed when comparing the transport of Cl^- with that of organic substrates¹¹. LRRC8D was crucial, although not absolutely required, for the transport of taurine and the anticancer drug cisplatin¹¹, and of the antibiotic blasticidin¹⁰⁶. The ratio of cisplatin or taurine to Cl^- transport was much larger with LRRC8A/LRRC8D than with LRRC8A/LRRC8C heteromers¹¹. These changes in selectivity, together with more recent reconstitution experiments⁸⁰, strongly bolster previous suggestions^{9,10} that LRRC8 proteins themselves form the channel pore. The reported differences between Cl^- -conducting ‘VRACs’ and organic osmolyte-conducting ‘VSOACs’, which were based on divergent regulation and drug sensitivities of $I_{Cl,vol}$ and osmolyte fluxes^{46,98,99,109}, probably reflect the distinct properties of differently composed LRRC8 heteromers¹¹.

VRACs beyond volume regulation

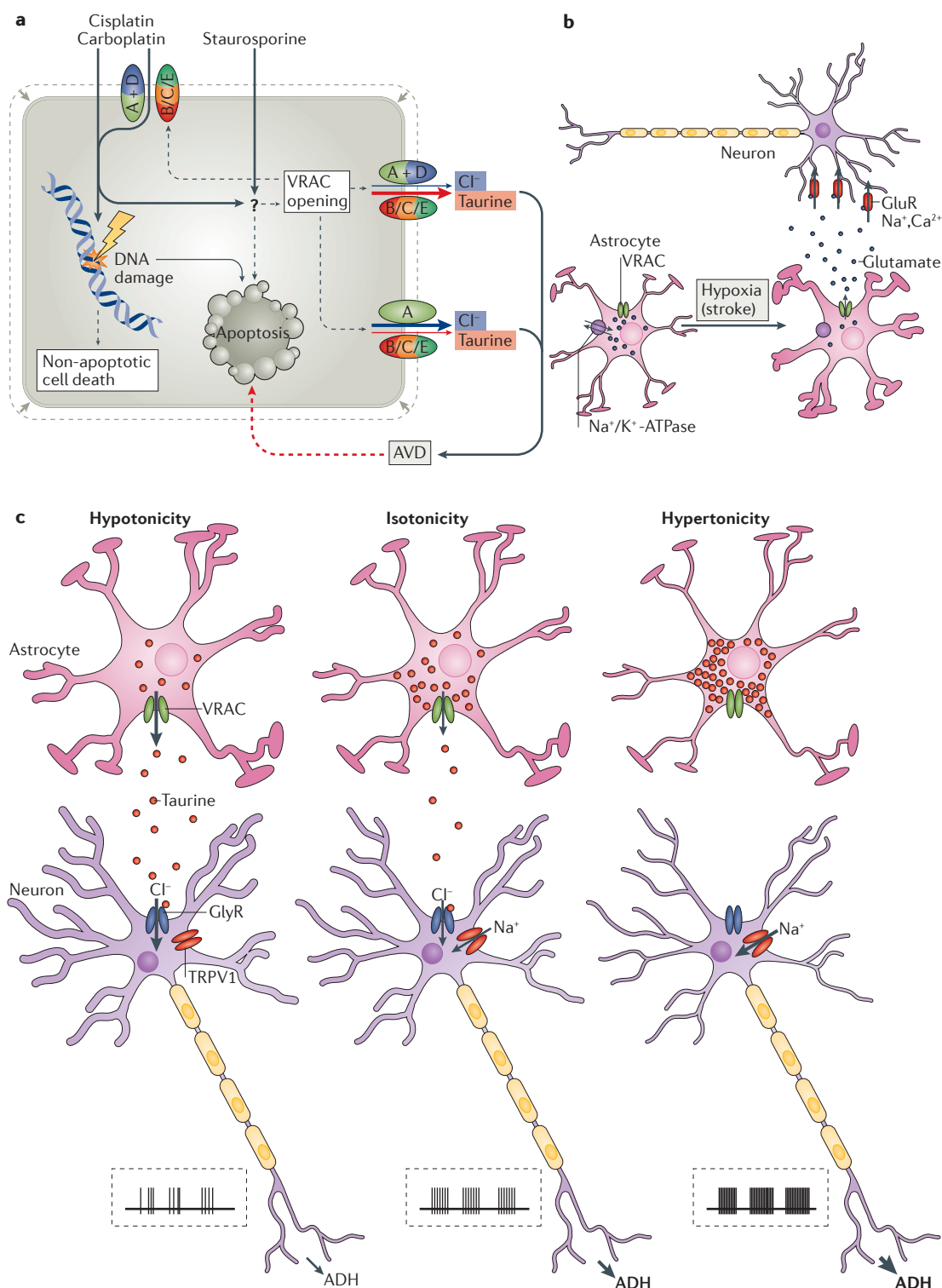
As discussed above, volume-regulatory transport proteins have roles beyond volume regulation. These additional roles are particularly evident in the case of VRACs, as these channels transport not only Cl^- , but also organic osmolytes, metabolites that are involved in extracellular signalling and even clinically important drugs.

VRACs in apoptosis and cancer drug resistance. VRAC-like currents can be induced without cell swelling by pro-apoptotic compounds such as staurosporine or by platinum-based drugs (Pt-drugs) such as cisplatin^{87,110}. These currents probably play a major part in AVD, which is a hallmark of programmed cell death that generally precedes other features of apoptosis such as caspase induction²². Inhibition of drug-induced apoptosis by several nonspecific VRAC inhibitors (and various K^+ channel blockers²⁷) suggested that AVD facilitates the progression of apoptosis^{22,23,28,111} by mechanisms that are poorly understood but that might involve a decrease in intracellular cations²⁷. Several drug-resistant cancer cell lines display less $I_{Cl,vol}$ AVD, RVD and swelling-activated taurine efflux compared to their parent cells^{28,112,113}. Of note, a histone deacetylase inhibitor (which can activate transcription of certain genes) restored cisplatin sensitivity and $I_{Cl,vol}$ of resistant cells¹¹³. It was therefore hypothesized that VRAC downregulation causes drug resistance by reducing AVD and, as a consequence, apoptosis.

Following the identification of LRRC8 proteins as VRAC components, this hypothesis was rigorously tested¹¹. Disruption of the essential VRAC subunit LRRC8A indeed suppressed cisplatin- or staurosporine-induced activation of anion and taurine transport and of

apoptosis. However, there was an unforeseen twist, as it was revealed that as well as functioning in AVD, VRACs participate in the uptake of cisplatin (but probably not of staurosporine). Under resting conditions, about 50% of cellular uptake of cisplatin or carboplatin (which are both widely used anticancer drugs) occurs through LRRC8A- and LRRC8D-containing VRACs¹¹. Drug uptake was much higher when VRACs were opened by cell swelling.

Both *LRRC8A* and *LRRC8D* genes were independently identified in a genomic screen for carboplatin resistance¹¹. By mediating cisplatin uptake and facilitating AVD-dependent apoptosis, VRACs may therefore have a dual role in cancer drug sensitivity (FIG. 3a). At the low Pt-drug concentrations used to treat tumours in patients, non-apoptotic cell death is thought to be prevalent, and VRAC-related drug sensitivity depends



Astrocytes

A major class of glial (non-neuronal) cells in the central nervous system, which have important functions, for instance, in buffering extracellular ion concentrations, in removal of neurotransmitters and in neuronal metabolism.

predominantly on VRAC-dependent drug uptake rather than on VRAC-dependent AVD¹¹. The role of VRACs in Pt-drug sensitivity may be clinically relevant, as *LRRC8D* downregulation correlated with poor survival of patients with cancer who were treated with Pt-drugs¹¹. No down-regulation of *LRRC8A* was apparent in the patients' tumours, suggesting that VRAC-dependent cell volume regulation, which is abolished with a loss of *LRRC8A*, but not *LRRC8D*, is important for the growth of tumour cells.

VRAC-mediated transport of metabolites. As VRACs are able to transport anticancer Pt-drugs¹¹ and structurally distinct antibiotics¹⁰⁶, these channels could potentially transport a broad range of small compounds. Such transport is surely not indiscriminate, as compounds such as the larger Pt-drug oxaliplatin apparently do not permeate VRACs¹¹.

Metabolites such as taurine, glutamate, aspartate and inositol (and possibly even ATP¹¹⁴) are believed to pass through VRACs or have been confirmed as *LRRC8* channel substrates^{9–11,115}. As already mentioned, their release may be important for cell volume regulation. Indeed, quantitative considerations¹¹⁶ indicate that loss of inorganic ions can only decrease cell volume by 20–30%, less than is typically observed in AVD, suggesting an important role for organic osmolytes¹¹⁶. A key role for taurine in volume regulation is indicated by diminished, but not abolished, RVD in *LRRC8D*^{−/−} HEK cells that display severely reduced swelling-induced taurine efflux but normal $I_{Cl,vol}$ (REF. 11). A VRAC-mediated release of

organic osmolytes might be particularly relevant for RVD in neurons, which (as discussed above) maintain a low $[Cl^-]_i$ that is set by constitutively active *KCC2* (REF. 39) to enable inhibitory Cl^- influx through *GABA_A* receptors. Indeed, Cl^- entry through VRACs, together with Na^+ influx through glutamate receptors, may contribute to neuronal swelling in excitotoxicity¹¹⁷ (see below). The loss of valuable intracellular metabolites through VRACs probably has no deleterious consequences during AVD, as such cells will typically die. In RVD, the disadvantage of such a loss may be offset by the advantage of avoiding cell lysis with extreme swelling. Moreover, the loss of metabolites is less pronounced and more easily compensated for following small volume changes. Released osmolytes such as glutamate and taurine, however, can have significant effects on neighbouring cells. Two notable examples are discussed here.

Glutamate is an excitatory neurotransmitter that activates cognate receptors on neurons. In excess, glutamate stimulation can lead to excitotoxicity, which prominently involves overstimulation of Ca^{2+} -permeable glutamate receptors, contributing to neuronal cell death in stroke and other pathologies of the central nervous system. This condition is induced upon ischaemia, and several mechanisms for ischaemia-induced glutamate release have been proposed¹¹⁸, including reversal of Na^+ -driven glutamate transporters of astrocytes. Notably, one of the proposed mechanisms involves the release of glutamate from astrocytes through VRACs^{119–122} (FIG. 3b). Astrocytes are more susceptible to ischaemic swelling than neurons¹²² and may be the main source of VRAC-released amino acids in the brain. VRAC-dependent glutamate release probably occurs mainly in the area surrounding the hypoxic core (termed the penumbra)¹²¹, because VRACs need permissive intracellular concentrations of ATP and hence sufficient oxygen levels. Although cell swelling may be less pronounced in the penumbra, astrocytic VRAC opening may be potentiated by stroke-related increases of substances like ATP and reactive oxygen species^{102,115,123}. Animal experiments indeed showed that VRAC inhibitors blunted hypoxia-induced release of excitatory amino acids in the brain¹²⁴. In addition to its role in astrocytic glutamate release, Cl^- influx into neurons through VRACs might further contribute to pathology by promoting neuronal swelling initiated by Na^+ entry through glutamate receptors¹¹⁷. Thus, VRAC inhibition might be beneficial in stroke. Several studies have indeed reported protective effects of anion transport inhibitors in stroke models^{125,126}. However, all of the VRAC inhibitors used in those studies also affect other channels and cellular processes. Using *LRRC8* mutant mouse models subject to stroke might provide more definitive results.

Besides glutamate, the VRAC substrate taurine has many cellular functions²⁹. For instance, taurine activates inhibitory neuronal glycine receptor and *GABA_A* receptor channels^{127,128}. Interestingly, VRAC may link cellular to systemic volume regulation by releasing taurine from glia in the osmosensing supraoptical nucleus (SON)^{15,127,129,130} (reviewed in REF. 131) (FIG. 3c). Neurons of the SON secrete antidiuretic hormone (ADH; also

◀ **Figure 3 | Some proposed roles of volume-regulated anion channels (VRACs) beyond volume regulation.** **a** | Apoptosis-inducing compounds such as the anticancer drug cisplatin or the alkaloid staurosporine can enter cells by passive diffusion across the plasma membrane and promote cell death. Additionally, by an unknown mechanism (indicated here by '?') that may involve reactive oxygen species, both drugs open VRACs, leading to efflux of Cl^- and taurine (the latter preferentially through *LRRC8D*-containing VRACs¹¹) and, consequently, to apoptotic volume decrease (AVD), which is believed to stimulate the progression of apoptosis. Opening of VRACs also further potentiates cisplatin uptake in a feedforward mechanism, as cisplatin (but not staurosporine) can also enter cells through *LRRC8D*-containing VRACs¹¹. Therefore, whereas disruption of the indispensable VRAC subunit *LRRC8A* suppresses both cisplatin- and staurosporine-induced apoptosis through inhibition of AVD, *LRRC8D* disruption suppresses cisplatin- but not staurosporine-induced cell death through inhibition of enhanced cisplatin uptake¹¹. A, B, C and D indicate the various *LRRC8* subunits. **b** | A role for VRAC in stroke has been proposed. Occlusion of brain blood vessels leads to hypoxia, thereby decreasing cellular ATP levels and impairing ion pumping by the Na^+/K^+ -ATPase. This entails swelling of astrocytes, opening of VRACs on their plasma membranes and the subsequent release of glutamate that binds to neuronal glutamate-gated cation channels (GluR). The massive opening of these receptors results in an influx of Na^+ and Ca^{2+} , which leads to neuronal damage (termed 'excitotoxicity'), in addition to that directly caused by hypoxia. **c** | VRAC-mediated taurine release may link cellular to systemic osmoregulation^{15,129,130}. Neurons of the supraoptical nucleus (SON) poorly regulate their volume^{132,133}. In hypertonic solution, these neurons shrink and depolarize, owing to the opening of transient receptor potential cation channel subfamily V member 1 (TRPV1) cation channels and Na^+ influx. This results in increased action potential firing of SON neurons (shown schematically in graphs), thereby stimulating the release of antidiuretic hormone (ADH) in the pituitary, which in turn increases renal water retention. Conversely, under hypotonic conditions, astrocytes neighbouring SON neurons undergo swelling and open their VRACs, thereby releasing taurine. Taurine then activates glycine receptors (GlyR) on SON neuronal cell bodies, leading to Cl^- influx and hyperpolarization and inhibition of SON neurons, thereby decreasing ADH release. This collectively results in a push–pull mechanism controlling SON neuronal activity and body fluid homeostasis. Image in panel **c** adapted with permission from REF. 129, Elsevier.

known as vasopressin) and oxytocin, which are hormones involved in body fluid homeostasis. These neurons function as osmosensors and were previously thought to lack RVD and RVI¹³²; however, they do slowly develop $I_{Cl,vol}$ and undergo RVD when swollen for more than 10 minutes¹³³. In a hypertonic environment, SON neurons shrink and depolarize owing to Na^+ influx through transient receptor potential cation channel subfamily V member 1 (TRPV1) cation channels that are opened by a shrinkage-induced pushing force from a channel-attached microtubule scaffold⁷². Depolarization, in turn, increases their firing rate, leading to increased secretion of ADH and resulting in enhanced renal water retention. Whereas extracellular hypertonicity directly activates SON neurons, hypotonicity is sensed by SON astrocytes, which release taurine in response to hypotonic swelling through VRAC-like channels^{127,129,130}. By activating glycine receptor Cl^- channels on neuronal cell bodies, taurine hyperpolarizes and thereby inhibits the activity of SON neurons and secretion of vasopressin. The interplay between direct hypertonicity-induced excitation of SON neurons and inhibition by VRAC-mediated astrocytic taurine release upon hypotonicity allows for efficient systemic osmoregulation in both directions (FIG. 3c).

Conclusions and outlook

Cell volume regulation is crucial for almost every vertebrate cell. It involves the regulated transport of osmolytes such as ions or small organic compounds across the plasma membrane. The resulting osmotic gradients drive water across the plasma membrane and restore cell volume. The transport proteins (effectors) involved in osmolyte transport almost always have additional cellular functions. This is particularly true for VRACs, which transport not only inorganic anions, but also metabolites with functions in extracellular signalling. The recent discovery of LRRC8 proteins as essential components of VRACs^{9,10} has already led to the conclusion that the same type of channel is able to conduct both ions and organic osmolytes, and that it is involved both in RVD and

AVD. This discovery has also revealed that VRACs can mediate the uptake of anticancer drugs, an observation of potential importance for tumour drug resistance¹¹. The biological importance of VRACs is highlighted by a recently reported study in *Lrrc8a*^{-/-} mice¹³⁴. The phenotypes of these mice, which were erroneously interpreted¹³⁴ in terms of VRACs being plasma membrane receptors with extracellular leucine-rich repeats^{107,135}, include high embryonic and postnatal lethality and pathological changes in many organs¹³⁴. It has also been reported that a heterozygous truncation of the LRRC8A protein is associated with a congenital absence of serum γ -globulins, but this observation was based only on a single patient¹³⁵. Although the severe phenotypes of *Lrrc8a*^{-/-} mice underline the importance of VRACs, the survival of a few mice¹³⁴ and the viability of *Lrrc8*^{-/-} cells lacking all LRRC8 isoforms^{9,11} prove that VRACs are not strictly required for processes such as apoptosis, cell death, division, growth and migration. However, this does not exclude the important, but partially redundant, roles of VRACs in these processes. The diverse cellular and systemic roles of VRACs can now be addressed by cell- and tissue-specific disruption of individual subunits. It may emerge that the transport of metabolites and signalling molecules by VRACs is as important as their role in volume regulation. LRRC8 proteins may also have roles independent of their channel function, for instance, by interacting with other proteins.

Identification of sensors and transducers involved in cell volume regulation remains another major challenge. The difficulty of identifying specific pathways is probably due, at least in part, to redundancies at the levels of sensors and effectors. As it is likely that all effectors have additional cellular roles and sometimes only 'moonlight' in cell volume regulation, their regulation is expected to be complex. A good way to study the signalling pathways involved may be to work back from known effectors to identify signals directly impinging on them. Owing to the recent progress in our understanding of the composition and functions of VRACs, such analysis should now also be possible for these channels.

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Acknowledgements

The author thanks Rosa Planells-Cases for help with figures, and members of his laboratory for critical reading of the manuscript. Work on VRACs is supported in the author's laboratory by the European Research Council Advanced Grant (FP/2007-2013) 294435 'Cytovolion' and the Deutsche Forschungsgemeinschaft (Exc 257 'Neurocure' and JE164/12).

Competing interests statement

The author declares no competing interests.

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