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Disruption of Ionic and Cell Volume Homeostasis In Cerebral Ischemia: The Perfect Storm

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Abstract

The mechanisms of brain tissue damage in stroke are strongly linked to the phenomenon of excitotoxicity, which is defined as damage or death of neural cells due to excessive activation of receptors for the excitatory neurotransmitters glutamate and aspartate. Under physiological conditions, ionotropic glutamate receptors mediate the processes of excitatory neurotransmission and synaptic plasticity. In ischemia, sustained pathological release of glutamate from neurons and glial cells causes prolonged activation of these receptors, resulting in massive depolarization and cytoplasmic Ca^{2+} overload. The NMDA subtype of glutamate receptors is particularly important as it represents the main initial route for the Ca^{2+} influx. High cytoplasmic levels of Ca^{2+} activate many degradative processes that, depending on the metabolic status, cause immediate or delayed death of neural cells. This traditional view has been expanded by a number of observations that implicate Cl^- channels and several types of non-channel transporter proteins, such as the $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ cotransporter, Na^+/H^+ exchanger, and $\text{Na}^+/\text{Ca}^{2+}$ exchanger, in the development of glutamate toxicity. Some of these ion transporters increase tissue damage by promoting pathological cell swelling and necrotic cell death, while others contribute to a long term accumulation of cytoplasmic Ca^{2+} . This brief review is aimed at illustrating how the dysregulation of various ion transport processes combine in a 'perfect storm' that disrupts neural ionic homeostasis and culminates in the irreversible damage and death of neural cells. The clinical relevance of individual transporters as targets for therapeutic intervention in stroke is also briefly discussed.

Keywords

excitotoxicity; cellular edema; volume-regulated anion channels; Na^+ ; K^+ ; 2Cl^- cotransport; Na^+/H^+ exchange; $\text{Na}^+/\text{Ca}^{2+}$ exchange

1. Introduction: Overview of Ischemic Brain Damage

Ischemic stroke is a loss of neural function resulting from a transient or permanent reduction in cerebral blood flow. In the majority of strokes, such a reduction is restricted to isolated regions of the brain and is caused by an occlusion of one of the major brain arteries, either by an embolus or thrombosis. In some cases, local perfusion deficits are associated with hemorrhage. Less commonly, cerebral blood supply may be completely interrupted due to cardiac arrest. Because brain metabolism is almost exclusively dependent on oxidation of

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blood-derived glucose, reductions in cerebral blood flow below a threshold of ~50% of their normal levels cause a rapid decline in tissue metabolism, which is followed by severe tissue damage (for review see [1–3]).

The degree of brain tissue damage is determined by the severity and duration of the perfusion deficit [4]. In focal strokes, ischemic tissue is divided into an 'INFARCTION CORE' and 'PENUMBRA'. The core is the area of the brain where blood flow is reduced below ~15–20% of its normal levels. In the core, rapid anoxic depolarization causes immediate loss of membrane potential and integrity, and neural cells die very rapidly. The penumbra is the tissue surrounding the core where the blood flow is partially preserved due to collateral circulation. Reduction of perfusion to 20 to 50% of normal levels places tissue at severe risk, while the higher rates of blood flow allow for survival. Cells in penumbra may retain their viability for hours or perhaps days, although penumbral neurons typically lose their excitability within a few minutes after the onset of ischemia [1,4,5].

Over time, anoxic depolarization in the core starts to spread to the outer regions. Increased concentrations of extracellular K^+ and excitatory neurotransmitters trigger repetitive depolarizations in the penumbral tissue, referred to as 'PERI-INFARCT DEPOLARIZATIONS'. Peri-infarct depolarizations place an additional metabolic burden on the already compromised tissue and promote propagation of the ischemic lesion, which may grow as much as 30 to 100% compared to its initial size [1,3–5].

The major cause of cell death in the ischemic tissue is an uncontrolled elevation of intracellular $[Ca^{2+}]$ [6–8]. In the core, levels of extracellular Ca^{2+} drop from ~2 mM to 0.05–0.08 mM, reflecting major Ca^{2+} movement into the intracellular space. Two main Ca^{2+} permeability pathways, which contribute to this shift, are glutamate-gated N-METHYL-D-ASPARTATE (NMDA) RECEPTOR-CHANNELS (NMDA is the selective agonist for these receptors) and voltage-gated Ca^{2+} channels. Both NMDA and voltage-dependent Ca^{2+} channels require strong membrane depolarization in order to be fully activated. In the ischemic tissue, membrane depolarization is determined by a metabolic inhibition of the Na^+, K^+ -pump, but is greatly accelerated by Na^+ influx via the voltage-gated Na^+ channels and glutamate-gated α -AMINO-3-HYDROXY-5-METHYL-4-ISOXAZOLEPROPIONIC ACID (AMPA) RECEPTOR-CHANNELS (AMPA is the selective agonist for these receptors). The resulting sustained and typically irreversible increases in cytoplasmic $[Ca^{2+}]$ directly or indirectly trigger numerous pathological processes. Because these processes are largely activated by two excitatory amino acid neurotransmitters, glutamate and aspartate, they are collectively termed 'EXCITOTOXICITY' [6,9,10].

Excitotoxic cell damage and death involve activation of numerous damaging cascades, which are set in motion, directly or indirectly, by elevations in cytosolic Ca^{2+} . Some key damaging events include activation of Ca^{2+} -dependent proteases and phospholipases, alterations in the plasma membrane permeability, depolarization of mitochondria and release of mitochondrial pro-apoptotic proteins, production of reactive oxygen and nitrogen species, and activation of the DNA-repairing enzyme polyADP-ribose polymerase (PARP). Depending on their metabolic status, neural cells may die via necrotic or apoptotic mechanisms. The complex biology of the ischemic cell death is discussed in detail in several comprehensive reviews [1, 3,5].

This review focuses on the ischemic dysregulation of several ion transporters, which are not directly involved in transmembrane Ca^{2+} fluxes and therefore receive little attention in the ischemia literature. Failure to maintain proper ionic gradients plays a major role in determining the fate of the neural cells in the ischemic brain and contributes to spatial propagation of the ischemic infarction. The main emphasis here is placed on the *in vivo* data obtained in animal

ischemia models because they are more clinically relevant. However, because many of the molecular mechanisms involved in ischemic cell damage have been deciphered *in vitro*, the important *in vitro* experiments are also briefly discussed. For more extensive coverage of the relevant topics the reader is encouraged to consult several comprehensive reviews [11–14], and particularly [15].

2. Transmembrane Na⁺ and Cl[−] fluxes are underappreciated contributors to the excitotoxic tissue damage: an osmotic connection

Although excitotoxicity is frequently considered synonymous with Ca²⁺-dependent cell death, early studies clearly distinguished two neurotoxic effects of glutamate: rapid toxicity determined mainly by the influx of Na⁺ and Cl[−] and osmotic swelling, and a more delayed Ca²⁺-dependent cell death [6,7,9]. The first form of excitotoxicity is reliant on the presence of extracellular Na⁺ and Cl[−], but not Ca²⁺, and manifests as rapidly forming dendritic varicosities followed by generalized somatic swelling and necrotic cell death [6,16–18]. Replacement of extracellular Na⁺ or Cl[−] with impermeant ion species prevents glutamate-induced cell swelling and strongly reduces cell death. Pharmacological evidence suggests that Na⁺ enters the cell mainly via the glutamate-gated AMPA and NMDA channels, while the identity of the Cl[−] influx pathway is less clear. Several studies proposed that Cl[−] influx may be at least partially mediated by GABA_A RECEPTOR-CHANNELS (GABA stands for gamma-aminobutyric acid, a glutamate-derived inhibitory neurotransmitter), as GABA_A receptor blockers partially prevent excitotoxic cell injury [19,20]. However, a recent study by Inoue and Okada demonstrated that, in cortical slices and cultured neurons, glutamate-induced Cl[−] fluxes are largely mediated by the VOLUME-SENSITIVE OUTWARDLY RECTIFYING (VSOR) CL[−] CHANNEL [21]. VSOR is also known in the literature as VOLUME-SENSITIVE ORGANIC OSMOLYTE-ANION CHANNEL (VSOAC) or VOLUME-REGULATED ANION CHANNEL (VRAC) [22–24]. In this review the acronym VRAC will be used.

Application of VRAC blockers prevents both glutamate agonist-induced dendritic swelling and neuronal death [21,25]. Apparently, VRAC is functionally important for Cl[−] accumulation and pathological cell swelling during exposure to excitotoxins, as well as for cell volume recovery when excitotoxins are removed [21]. *In vivo*, two VRAC blockers, Merck compound L644,711 and tamoxifen, potently protect brain tissue against ischemic damage in animal models of focal transient and permanent ischemia [26–29]. However, as discussed in the next section, the protective actions of VRAC blockers are not restricted to preventing pathological cell swelling, but also involve other mechanisms.

Uncontrolled cell swelling is frequently associated with necrosis and may be harmful to neuronal and glial cells for multiple reasons [13,30]. The simplest mechanism of cell damage is osmotic lysis. Yet, animal cells have large reserves of surface membranes and can increase their volume by 2–3-fold without subjecting their plasma membranes to substantial mechanical stress [31,32]. Furthermore, animal cells possess VOLUME REGULATORY MECHANISMS serving to protect against abrupt swelling. In majority of cell types, cell swelling activates Cl[−] efflux via the above mentioned VRAC in conjunction with K⁺ loss via SWELLING-ACTIVATED K⁺ CHANNELS. Under physiological conditions these two independent permeability pathways mediate electrically coupled loss of K⁺ and Cl[−], which is accompanied by an efflux of osmotically obligated water, and REGULATORY VOLUME DECREASE, or RVD [30,33,34]. However, in pathology, anoxic and/or glutamate-driven depolarization disrupts cell volume regulation due to an inhibition of the Na⁺,K⁺-pump, dissipation of K⁺ and Cl[−] electrochemical gradients, and blockade of volume regulatory channels [13,21,25,35]. Na⁺ begins to accumulate uncontrollably in the cells via leak mechanisms, voltage-gated Na⁺ channels, and other Na⁺-transporting pathways, which are considered in following

sections. In glial cells, ischemia additionally opens the unique ATP-dependent nonselective cation channels that greatly increase Na^+ influx and promote astroglial swelling [36].

The cell swelling can be detrimental due to activation of various ion transporting pathways even when K^+ gradients and intracellular ATP levels are relatively well preserved (as it would be expected on periphery of penumbra). The above mentioned regulatory loss of K^+ and Cl^- protects cells against swelling, but at the same time decreases the transmembrane K^+ gradient and membrane potential, thereby affecting neuronal excitability. Cell swelling potentially enhances the activity of the Na^+, K^+ -pump in both neurons and glia [37–39]. In electrically active neuronal cells, the Na^+, K^+ -pump represents the main ATP-consuming enzyme. In the face of falling ATP content, the Na^+, K^+ -ATPase starts to compete for ATP with the PLASMA MEMBRANE Ca^{2+} PUMP (PMCA). One recent report indicates that such competition may compromise the activity of the PMCA and promote the necrotic Ca^{2+} overload [40].

Another potential link between cell volume and tissue damage is via modulation of the NMDA channels, which, in cortical neurons, are potentially activated by pathological degrees of cell swelling [41]. In contrast, AMPA and GABA_A receptors are insensitive to cell volume changes [41]. Thus, NMDA channel activation may trigger cell swelling, which, in turn, increases the activity of the NMDA channels, forming a pathological feed-forward mechanism. Cell swelling also activates several other membrane Ca^{2+} permeability pathways, such as the TRANSIENT RECEPTOR POTENTIAL CATION CHANNEL TRPV4 [42,43], and triggers intracellular Ca^{2+} release from the IP_3 -sensitive stores [44]. On one hand, all of the above mentioned processes contribute to the Ca^{2+} overload; on the other hand, they accelerate ATP depletion via stimulating the activity of the Na^+, K^+ - and Ca^{2+} -ATPases.

In summary: glutamate toxicity is strongly promoted by Na^+ and Cl^- accumulation and cell swelling. Cell swelling activates a number of ion transport pathways. Normally such activation is beneficial as it mediates regulatory volume restoration via the RVD process. However, in energetically compromised cells, substantial transmembrane ion fluxes cannot be sustained, and therefore cell swelling becomes continuous and diverts cells to necrotic cell death. Importantly, cell swelling activates or strongly potentiates several Ca^{2+} transporting pathways that likely also contribute to delayed forms of excitotoxic cell injury. Although swelling itself cannot be considered a pharmacological target in stroke, inhibition of several ion transport pathways by pharmacological agents reduces pathological swelling and protects against ischemic cell and tissue damage *in vitro* and *in vivo* (see next three sections).

3. Volume-regulated anion channels and the reversed mode of glutamate transporters are two major sources of pathological glutamate release *in vivo*

Pathological swelling found *in vitro*, in neural cells subjected to glutamate, anoxia, or chemical ischemia, has an *in vivo* equivalent named CELLULAR or CYTOTOXIC EDEMA. Cellular edema is an early and prominent feature of cerebral ischemia, and has been found in human stroke and animal ischemia models (reviewed in [13,45,46]). In humans, cellular swelling in the brain is detected indirectly by means of MAGNETIC RESONANCE IMAGING (MRI). Using DIFFUSION-WEIGHTED IMAGING (DWI) clinicians generate maps of APPARENT DIFFUSION COEFFICIENT (ADC) of water in the brain. Upon movement from the extracellular to the intracellular space, the mobility of H_2O molecules is hindered by high concentration of organic macromolecules; a corresponding decrease in the ADC reflects cellular swelling. In human stroke, cellular edema can be registered as early as measurements are practically possible, *i.e.*, within 90 min after onset of ischemia [47]. In animal models, changes in ADC signal correspond very well to cellular swelling measured using electron microscopy (EM) in perfusion-fixed brain sections [48]. The final volume of the brain damage

in stroke strongly correlates with changes in the ADC [49–51]. Therefore cellular edema is closely associated with ischemic brain damage.

Cell swelling in ischemia is mainly seen in one cell type, astrocytes, and is most pronounced in their perivascular processes called ASTROCYTIC ENDFEET. Some dendritic swelling is also found, but neuronal soma are not changed or even shrunk. The precise reasons for such selective susceptibility of astrocytes to ischemic swelling are unknown; several hypothetical mechanisms are discussed elsewhere [13,45]. A key study that proposed a mechanistic link between astrocytic swelling and brain damage has been done by Kimelberg and co-workers, who found that exposure of cultured astrocytes to hypoosmotic media triggers massive release of several cytosolic amino acids, including glutamate and aspartate [52]. The swelling-activated amino acid release permeability pathway is sensitive to a number of anion channel blockers, and has strong similarities to the already mentioned swelling-activated anion channel VRAC [22,24,52–54]. Since electrophysiological studies confirmed that VRAC possesses measurable permeability to glutamate and aspartate [55–57], this channel has long been considered a hypothetical pathway for pathological release of excitotoxins from swollen cells [13,45,46].

In spite of the extensive search in the field, the molecular identity of VRAC remains unknown [24,58]. Therefore any studies designed to explore the physiological and pathological roles for this channel have to rely on pharmacological tools. Several commonly used VRAC blockers are 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB, IC_{50} ~8–25 μ M, depending on a cell type), 4,4'-diisothiocyano-2,2'-stilbene disulfonate (DIDS, IC_{50} ~5 μ M at negative potentials, but up to 150 μ M at positive potentials), and tamoxifen (IC_{50} <1 μ M) [23,59]. The major problem with these compounds is that they do not discriminate well between VRAC and other Cl^- channels. Tamoxifen, which is frequently described as the potent and selective inhibitor of the VRAC, is completely ineffective in neuronal cells and blocks a number of other anion and cation channels [60–62]. Other VRAC blockers include 1,9-dideoxyforskolin, niflumic acid, calaxarenes, and phloretin [23,24]. The most selective VRAC inhibitor identified so far is the ethacrynic-acid derivative DCPIB (IC_{50} =4 μ M) [63]. At the concentrations of 10–20 μ M, DCPIB nearly completely blocks Cl^- currents via VRAC, but does not affect other Cl^- channels (CFTR, Ca^{2+} -activated Cl^- channels, and ClC -1, -2, -4, and -5), nor voltage gated K^+ , Na^+ and Ca^{2+} channels [63]. Importantly, DCPIB potently blocks swelling-activated glutamate release from cultured astroglial cells [64].

In the rat global ischemia model, using a cortical cup technique, Phillis *et al.* found 30–70% inhibition of glutamate and aspartate release with the anion channel blockers 2 mM 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS), 350 μ M NPPB, 200 μ M dipyridamole, or 20 μ M tamoxifen [65,66]. In a microdialysis study, also done in the global ischemia model, Seki *et al.* found that another anion channel inhibitor 4,4'-dinitrostilbene-2,2'-disulfonic acid (DNDS) reduced ischemic release of excitotoxins by 50–70% , when added at 1 or 10 mM in microdialysis perfusate [67]. In the penumbra of the rat focal ischemia model, 50 μ M tamoxifen reduced ischemic glutamate and aspartate release by ~80%, although absolute total levels of the release were lower than those in the global ischemia [68]. These data suggest that in the infarction core 30% or more of the ischemic excitatory amino acid release may be mediated by the VRAC, and such contribution is likely much higher in the penumbra.

Because cell swelling in penumbra is limited, the important question is whether it is sufficient to activate the VRAC. Several recent *in vitro* studies have found that a number of substances, which are produced or released in ischemia, such as reactive oxygen species H_2O_2 , reactive nitrogen species peroxynitrite, thrombin, and ATP, all drastically increase swelling-activated glutamate release from moderately swollen astrocytes [69–72].

A therapeutic relevance of the VRAC pathway has been tested in animal neuroprotection studies. Anion channel blockers L644,711 and tamoxifen reduced mortality rates and strongly decreases the infarction size in several ischemia models [26–29]. Importantly, tamoxifen has an extended therapeutic window of up to 3 hrs after initiation of ischemia, which is comparable with tissue plasminogen activator, the only drug used to treat human stroke [2,11,27]. However, the specificity of the effects of tamoxifen have to be verified with more selective VRAC blockers because it may protect brain tissue via non-VRAC related mechanisms, such as inhibition of neuronal nitric oxide synthase or due to its antioxidant properties [73,74].

In addition to the release via a VRAC-like pathway, elevated levels of excitatory amino acids in ischemia may occur due to reversal of Na^+ -dependent GLUTAMATE TRANSPORTERS. Under physiological conditions such transporters maintain low extracellular glutamate levels via a process of $[\text{Na}^+]_o$ - and $[\text{K}^+]_i$ -dependent uptake. If the Na^+ and K^+ gradients are collapsed, the transporters start to function in a reverse mode, and pump glutamate outside of the cell [75,76]. *In vivo*, 1 mM dihydrokainate (DHK), a relatively selective inhibitor of the glia-specific glutamate transporter GLT-1, blocks ~50% of ischemic glutamate release in the rat global ischemia model [67] and approximately the same effect was found in the core of the focal ischemia model [77]. However, when perfused in the penumbra of the focal ischemia model, DHK increases, rather than decreases pathological glutamate release [68]. These data indicate that a complete collapse of ionic gradients triggers glutamate release via reversal of the glutamate transporters, but if ionic gradients are partially preserved (as in the penumbra) transporters continue to remove glutamate from the extracellular space.

In summary, a disruption of transmembrane ionic gradients causes pathological glutamate and aspartate release via cell swelling-dependent and swelling-independent mechanisms. Swelling-independent release is mediated by the reversal of glutamate transporters. This mechanism is likely of limited therapeutic value, because glutamate transporters are functionally important in non-ischemic tissue. Second, swelling-activated mechanism of release likely involves anion channel VRAC. Since VRAC activity is restricted to swollen cells, it may be a suitable therapeutic target. Pharmacological inhibitors of VRAC are highly protective in several animal models of ischemia. The VRAC blocker tamoxifen is particularly effective because it is permeable through the blood-brain barrier and therefore may be given systemically. However, the specificity of the neuroprotective effects of tamoxifen has to be verified using more selective VRAC blockers, such as DCPIB.

4. $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ cotransport is involved in NaCl overload and pathological cell swelling

Two proteins of the $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ COTRANSPORTER (NKCC) family, NKCC1 (coded by *SLC12A2*) and NKCC2 (*SLC12A1*), mediate the electroneutral co-transport of Na^+ , K^+ , and 2Cl^- . NKCC1 is ubiquitously expressed and can be found in the CNS in both neuronal and non-neuronal cells, while the NKCC2 expression is limited to kidney cells in the loop of Henle [78,79]. The major biological roles for NKCC include maintaining intracellular $[\text{Cl}^-]$ above the levels determined by the Cl^- electrochemical equilibrium, cell volume regulation, and epithelial NaCl reabsorption. In the brain, $[\text{Cl}^-]_i$ regulation by NKCC1 is very important for neuronal function [80]. High expression levels of NKCC1 in immature CNS neurons and in peripheral sensory neurons increase $[\text{Cl}^-]_i$ and make GABA an excitatory neurotransmitter due to outwardly directed Cl^- fluxes via the GABA_A channels. On the contrary, in mature CNS neurons, NKCC1 is downregulated and replaced with the K^+, Cl^- COTRANSPORTER KCC2, which drives Cl^- out of the cell, thereby turning GABA into the inhibitory neurotransmitter [80]. In shrunken cells, NKCC1 activity is strongly upregulated due to increases in its phosphorylation levels. Activated transporter causes a net movement of ions inside the cell that, at least partially, mediates the process of REGULATORY VOLUME INCREASE, or

RVI [78]. Under pathological conditions activation of NKCC may result in cell swelling (see below).

Both NKCC isoforms are potently blocked by the 'loop diuretics' furosemide and bumetanide ($IC_{50} < 1 \mu M$, but the values may vary depending of cell type and $[Cl^-]_o$) [78]. The diuretic actions of these compounds are related to inhibition of Na^+ and Cl^- reabsorption in the thick ascending limb of the loop of Henle, resulting in increased volumes of urine production. When used at the concentration of $10 \mu M$, bumetanide inhibits NKCC, but not other ion transporters. Furosemide has much lower affinity towards NKCC and inhibits several other anion transport pathways. Therefore, furosemide is not used as the NKCC inhibitor in experimental studies.

In a rat model of transient occlusion of the middle cerebral artery (MCAo), continuous delivery of $100 \mu M$ bumetanide in the cortical tissue significantly reduces infarction volume by ~25% and tissue edema by >70% [81]. This shows that pathological activation of the cortical NKCC1 is a substantial contributor to neuronal death and edema formation. Interestingly, systemic delivery of 7.4–30.4 mg/kg bumetanide also reduced infarction volume by >50% and brain edema by 30 to 50% in the rat permanent ischemia model [82]. To exclude renal effects of bumetanide, this latter study has been performed in nephroctomized animals. Since bumetanide has limited permeability across the blood-brain barrier, these results point to protective endothelial effects of the diuretic. The pathological significance of NKCC1 in ischemia has been verified in the studies of NKCC1 knockout animals. NKCC1^{-/-} mice show 30 to 45% reduction in the infarction volume, as well as ~30–50% reduction in water accumulation (brain edema) in the infarcted hemisphere in a transient ischemia model [83]. As previously mentioned, ischemic cell swelling is largely restricted to astroglial cells, where it is mainly seen in the perivascular processes [13,45]. Notably, astrocytic perivascular endfeet express high levels of NKCC1, which is co-localized with the water channel AQP4 [79].

Molecular mechanisms involved in neuroprotection by bumetanide have been explored in several *in vitro* studies. In cultured neurons, $10 \mu M$ bumetanide strongly reduces cell death triggered by oxygen-glucose deprivation or by application of NMDA [84]. Such protective effects of bumetanide are absent in cultured neurons prepared from NKCC1^{-/-} mice [83]. *In situ*, in hippocampal slices, bumetanide prevents long-term decreases in neuronal $[Cl^-]_i$ that are associated with neuronal death after oxygen-glucose deprivation [85]. In cultured astrocytes, bumetanide and the genetic deletion of the NKCC1 gene potently inhibit high $[KCl]$ -induced cell swelling, which is used to mimic pathological swelling in the ischemic brain, and partially suppress swelling-activated release of excitatory amino acids [76,86,87]. In the acute preparation of the optic nerve, oxygen-glucose deprivation triggers degeneration of immature oligodendroglial cells, and such degeneration is prevented by the application of an AMPA receptor blocker or bumetanide [88]. Since in this *in situ* preparation NKCC is expressed in astrocytes only, the authors concluded that ischemia triggers NKCC-dependent swelling and death of astrocytes, which release excitotoxins and cause a downstream damage to oligodendroglia [88,89]. Importantly, in cultured astrocytes and C6 glioma cells, but not in cultured cerebellar granule neurons, swelling strongly stimulates NKCC activity [39,90]. Therefore, glial cell swelling and NKCC activation are mutually dependent processes and may form a 'pathological loop' promoting the release of excitotoxins *in vitro* and *in vivo*.

In summary, NKCC1 contributes to pathological NaCl accumulation, cell death and formation of brain edema in animal ischemia models. The selective NKCC inhibitor bumetanide is protective when given systemically or delivered locally in brain tissue. Neuroprotective properties of bumetanide likely involve several cellular mechanisms, as it has direct effects on neurons, glial cells, and endothelial cells of the blood-brain barrier. *In vivo*, NKCC activation is one of the probable causes for pronounced astrocytic swelling. Because of its potent diuretic actions, usage of bumetanide for human stroke treatment is not practical. However, if

developed, novel pharmacological compounds discriminating between the NKCC1 and NKCC2 may be therapeutically useful.

5. Pathological roles for Na^+/H^+ exchange: impact on $[\text{Na}^+]_i$ and cell volume

The family of Na^+/H^+ antiporters (NHE) includes nine human proteins NHE1–NHE9 (coded by *SLC9A1–SLC9A9*) that catalyze an electroneutral exchange of Na^+ and H^+ [91,92]. Five of these, NHE1–5, are plasmalemmal transporters whose activity is driven by inwardly directed Na^+ gradients, which are established by the Na^+/K^+ -pump. NHE1 is ubiquitously expressed and plays a major role in the regulation of cytoplasmic pH and cell volume, while NHE2–5 participate in additional tissue-specific processes [33,91]. NHE6–9 are intracellular $\text{Na}^+(\text{K}^+)/\text{H}^+$ exchangers in the secretory and endocytic pathways; they mediate pH regulation in various organelles [91,92].

There are several groups of pharmacological compounds that are employed to potently and selectively inhibit membrane NHEs *in vitro* and *in vivo* (reviewed in [91,93]). Amiloride derivatives, such as ethylisopropylamiloride (EIPA) and dimethylamiloride (DMA), benzoylguanidines, such as HOE-642 and HOE-694, and a number of ‘bicyclic’ inhibitors, such as cariporide, zoniporide, eniporide, SM-20220 and SM-20550, all have K_i or IC_{50} in the 1–100 nM range, but their inhibitory potency may be reduced by physiological concentrations of Na^+ [91,93]. Typically, the house-keeping NHE1 isoform exhibits the highest sensitivity to the NHE blockers.

The idea of a pathological role for NHE1 and a therapeutic potential for the NHE inhibitors has been initially proposed and explored in animal models of myocardial ischemia and reperfusion injury [91,93,94]. Several large-scale clinical trials using the NHE blockers eniporide and cariporide produced mixed, but encouraging, results [93,95].

In a rat transient ischemia model, the NHE blocker SM-20220 (1 mg/kg, delivered i.v. immediately after initiation of ischemia) reduced total infarction area by ~50%, and partially prevented formation of brain edema [96]. These effects may be relevant to preservation of blood-brain barrier function and improved reperfusion rates [97], as well as to reduced accumulation of leukocytes in damaged brain tissue [98]. In the same model, another selective NHE blocker, sabiporide (3 mg/kg, i.v. 20 min before ischemia), reduced both infarction volume and brain edema by ~40% and had a therapeutic window of 1 hr [99]. EIPA (5 mg/kg, i.p. 30 min before ischemia) protected hippocampal neurons and reduced neurological deficits in the gerbil global ischemia model [100], and reduced the ischemic release of excitotoxins in the rat global ischemia (25 μM , applied topically in the cortex) [101]. In a mouse transient focal ischemia model, inhibition of NHE1 with selective blocker HOE-642 (1 mg/kg i.v., 5 min prior ischemia), or a decrease in the NHE1 expression in heterozygous $\text{NHE1}^{+/-}$ animals, both significantly reduced brain infarction by ~30% and tended to suppress the development of brain edema [102]. The $\text{NHE1}^{-/-}$ animals have very high mortality rates and therefore could not be used in ischemia studies.

In cultured cortical neurons, oxygen-glucose deprivation followed by the reoxygenation causes steep rises in $[\text{Na}^+]_i$ and $[\text{Ca}^{2+}]_i$ that is potently inhibited by the selective NHE1 blocker 1 μM HOE-642 [102]. In the same paradigm HOE-642 also potently suppressed neuronal death. Cytosolic Na^+ and Ca^{2+} overload and the effects of HOE-642 were blunted in the neurons prepared from $\text{NHE1}^{-/-}$ animals [102]. Cultured astrocytes typically do not die when subjected to hypoxia-glucose deprivation. Nevertheless, similar to neurons, hypoxic treatment increases astrocytic $[\text{Na}^+]_i$ and induces cell swelling [103]. As in cultured neurons, HOE-642 or the genetic deletion of NHE1 both reduce elevation in $[\text{Na}^+]_i$ and cellular swelling [103]. Since astrocytic swelling has been linked to the release of glutamate and aspartate and ischemic tissue

damage, these findings may be of pathological relevance. A comprehensive analysis of the *in vitro* data on the role of NHE in ischemic cell damage can be found in [15]. Overall, there is a similarity between NKCC1 and NHE1 in terms of their contributions to hypoxic changes in cytosolic Na^+ , Ca^{2+} , and cellular volume. Perhaps this is not surprising. Despite differences in their transport stoichiometry, both transporters are involved in RVI and regulate cellular volume via a net accumulation of NaCl . More work is needed to establish the effects of NHE blockers on brain endothelial cells. Some of the above mentioned protective effects seem to be mediated by protection at the blood-brain barrier interface.

The therapeutic potential for the NHE inhibitors in stroke is not clear. On one hand, a number of highly selective and clinically tested compounds are available, and the results of animal studies are encouraging [93,95]. However, already conducted cardioprotective trials limit the enthusiasm. In an EXPEDITION trial involving coronary artery bypass surgery, the cardioprotective effects of the NHE inhibitor cariporide were offset by an unexpected increase in the incidence of stroke. Furthermore, another NHE blocker, eniporide, was ineffective when applied during reperfusion, suggesting a limited therapeutic window for this class of compounds [95].

6. Pathological reversal of the $\text{Na}^+/\text{Ca}^{2+}$ exchange may contribute to the cytoplasmic Ca^{2+} overload

The $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) transporter family includes three mammalian gene products, NCX1-3 (coded by *SLC8A1–SLC8A3*), which in a majority of cell types catalyze the electrogenic extrusion of one cytoplasmic Ca^{2+} in exchange for three Na^+ taken from the extracellular space [12,104]. Four additional related proteins, NKCX1–4, move four Na^+ from the extracellular space in exchange for ($\text{Ca}^{2+} + \text{K}^+$) out. NCX1 is expressed in the majority of tissues, while NCX2 and 3 are found nearly exclusively in muscle and neural cells (*i.e.*, neurons and glia) [12]. The kinetics of NCX are primarily governed by the Na^+ gradient and transmembrane potential, but are also affected by non-transported Ca^{2+} , protons, ATP, and diverse signaling molecules [104]. In the muscle and nerve cells, NCX (and NKCX) proteins serve as an important “high capacity” Ca^{2+} -extruding mechanism, complementing the Ca^{2+} -ATPase in pumping Ca^{2+} against its electrochemical gradient. However, dissipation of the Na^+ gradient and/or depolarization reverses the mode of NCX operation and, under pathological conditions, results in cytoplasmic Ca^{2+} overload [12,104].

Several groups of inhibitors have been designed to block NCX pharmacologically (reviewed in [12]). The most commonly used blockers are the amiloride derivatives 2',4'-dimethylbenzamil (DMB, $\text{IC}_{50} = 10 \mu\text{M}$) and [*N*-(4-chlorobenzyl)]2,4-dimethylbenzamil (CB-DMB, $\text{IC}_{50} = 7.3 \mu\text{M}$), isothiurea derivative KB-R7943 ($\text{IC}_{50} \sim 1 \mu\text{M}$), and the derivative of diarylaminopropylamine, bepridil ($\text{IC}_{50} = 8.1 \mu\text{M}$). The more recently synthesized SEA0400 (2-[4-[(2,5-difluorophenyl)-methoxyl-phenoxy]-5-ethoxyaniline) is the most potent NCX blocker with $\text{IC}_{50} \sim 10\text{--}90 \text{ nM}$, but it is effective predominantly against the NCX1 isoform. Importantly, two of the NCX inhibitors, KB-R7943 and SEA0400, block the reverse (*i.e.*, “pathological”) mode of $\text{Na}^+/\text{Ca}^{2+}$ exchange.

When observed, the effects of NCX blockers should be analyzed with caution. Bepridil [105] and KB-R7943 [106] have been reported to directly block the NMDA channels in hippocampal neurons. KB-R7943 may also exert its actions by blocking L-type Ca^{2+} channels [107] or, *in vivo*, via lowering the body temperature [108].

In vivo testing of NCX inhibitors in animal ischemia models produced mixed results. In the rat transient focal ischemia model, SEA0400 (3 mg/kg bolus followed by 2-hr infusion of 3 mg/kg/hr) reduced infarction volume by ~50% in the cortex and by ~25% in the striatum [109]. In

the rat permanent focal ischemia model, another group found potent protection with KB-R7943 (10 µg/kg delivered by osmotic minipump over 24 hrs) [108]. However, in the same study three other NCX blockers, bepridil, CB-DMB, and the NCX inhibitory peptide Glu-XIP, all significantly increased the infarction volume. The protective effect of KB-R7943 has been explained by its potent hypothermic actions [108]. Importantly, *in vivo* downregulation of NCX1 and NCX3, but not NCX2, using phosphorothioated antisense oligonucleotides, strongly increased infarction volumes and neurological deficits in a rat permanent focal ischemia model [110]. Consistent with the latter data, two NCX blockers, bepridil and the more selective CB-DMB, produced an irreversible loss of electrical activity in striatal spiny neurons in the penumbra of focal ischemia, as compared to untreated ischemic controls [111]. Overall, *in vivo* data suggest a beneficial role for NCX in maintaining ionic homeostasis in the peri-infarct area.

The neuroprotective and damaging properties of the NCX blockers have been explored in more detail *in vitro* and *in situ*. Several groups reported that NCX blockers rescue both neurons and glia from hypoxia/reoxygenation or glutamate-induced injury [112–114]. However, the others found increased cell death upon NCX inhibition [115,116]. Of special interest is the involvement of NCX in white matter injury (reviewed in [14]). Rat optic nerve and spinal cord white matter tracks are functionally protected against anoxic injury by removal of extracellular Ca^{2+} or by NCX inhibitors benzamil, bepridil, DCB, and KN-R7943 [117–119]. These data are of particular importance, as ischemic white matter injury is less understood and may contribute substantially to neurological deficits in human stroke [120]. However, some of the previously reported effects of NCX inhibitors have to be re-evaluated because bepridil and KB-R7943 also inhibit NMDA receptors [105,106] and L-type Ca^{2+} channels [107], two pathways that may also contribute to the anoxic injury of various white matter components [14,121–124].

In summary, reversal of NCX may contribute to Ca^{2+} overload in some, but not all, forms of ischemic cell death. This transporter may be of particular importance for axonal damage in white matter. However, NCX is beneficial for the survival and functional recovery of neurons in the ischemic penumbra. This likely explains why a number of NCX blockers increase, rather than decrease, brain damage in animal ischemia models. Therefore, the inhibition of NCX does not currently seem to be a viable strategy for pharmacological intervention in stroke.

7. Conclusions and perspectives

The failure of ionic homeostasis is a hallmark of ischemic brain damage. Current research efforts in the field are heavily focused on transmembrane Ca^{2+} fluxes and severe disturbances in cytoplasmic and mitochondrial Ca^{2+} homeostasis. In contrast, the pathological dysregulation of Na^+ and Cl^- transport receives relatively little attention. The present analysis of experimental data suggests that a pathological accumulation of Na^+ and Cl^- plays a very important role in development of ischemic cell and tissue injury. The damaging effects of high cytosolic $[\text{NaCl}]$ are realized via pathological cell swelling, the impact of Na^+ overload on Ca^{2+} homeostasis, and through an increased metabolic burden that is placed on the cell due to enhanced work of the Na^+, K^+ - and Ca^{2+} pumps.

The neuroprotective actions of the inhibitors blocking Na^+ and Cl^- transport pathways may stem from their actions in at least three cellular sites: (1) tested compounds may preserve the function of the blood-barrier and prevent formation of vasogenic brain edema by acting on endothelial cells; (2) they may directly reduce necrotic and apoptotic death of neuronal cells by preserving their ion homeostasis; (3) the inhibitors may also prevent the pathological release of excitotoxins from astroglial cells by inhibiting astrocytic swelling and the dissipation of ion gradients. Additionally, ion transport blockers may also protect white matter components

(i.e., axonal fibers and their myelinating oligodendroglial cells). While interpreting and comparing the *in vitro* and *in vivo* data, it is important to take into consideration the blood-brain permeability of individual compounds and their effects at each of the above mentioned sites.

A comprehensive understanding of the complex pathobiology of ischemic tissue damage will greatly benefit the development of future therapeutic treatments in stroke. The failure of so many neuroprotectants in clinical trials represents a significant challenge and calls for novel treatment approaches, perhaps involving multiple therapeutic targets (for recent discussion see [3,125,126]).

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