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Ionic Regulation of Cell Volume Changes and Cell Death after Ischemic Stroke

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Abstract

Stroke is a leading cause of human death and disability in the US and around the world. Shortly after the cerebral ischemia, cell swelling is the earliest morphological change in injured neuronal, glial and endothelial cells. Cytotoxic swelling directly results from increased Na^+ (with H_2O) and Ca^{2+} influx into cells via ionic mechanisms evoked by membrane depolarization and a number of harmful factors such as glutamate accumulation and the production of oxygen reactive species (ROS). During the sub-acute and chronic phases after ischemia, injured cells may show a phenotype of cell shrinkage due to complex processes involving membrane receptors/channels and programmed cell death signals. This review will introduce some progress in the understanding of the regulation of pathological cell volume changes and the involved receptors and channels, including NMDA and AMPA receptors, acid-sensing ion channels (ASIC), hemichannels, transient receptor potential (TRP) channels and KCNQ channels. Moreover, accumulating evidence supports a key role of energy deficiency and dysfunction of Na^+/K^+ -ATPase in ischemia-induced cell volume changes and cell death. Specifically, the Na^+ pump failure is a prerequisite for disruption of ionic homeostasis including a pro-apoptotic disruption of the K^+ homeostasis. Finally, we will introduce the concept of hybrid cell death as a result of the Na^+ pump failure in cultured cells and the ischemic brain. The goal of this review is to outline recent understanding of the ionic mechanism of ischemic cytotoxicity and suggest innovative ideas for future translational research.

Introduction

According to the American Heart Association (AHA) 2013 updated statistics^[1], about 795,000 people experience a new or recurrent stroke each year in the United States. This makes stroke one of the leading causes of human death and long-term disability in the US. Similar stroke occurrence and threats to human life and health are seen in other countries. The economic and social burden of stroke is severe in the US and around the world.

Brain ischemia initially causes oxygen and glucose deprivation and relatively acute (from hours to the first 1–3 days) cell death in the region where cerebral blood flow is arrested or severely reduced (80% decrease from the baseline level), eventually leading to an

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This is a review article that does not contain any studies with human or animal subjects. The authors assume that all original studies cited in this review followed institutional and national guidelines for the care and use of laboratory animals and guidelines for clinical trials on patients. However, the authors are not responsible for any violation of the guidelines in the original investigations.

infarction core during this time [2]. The remaining tissue within the peri-infarct region (also known as the penumbra) is partially and chronically (days to weeks) injured. Because of the slow time course of cell death and partial tissue damage in this region, the penumbra has been regarded as savable brain tissue and a preferred therapeutic target in neuroprotective treatments. Although this neuroprotective approach has been effective in protecting the gray matter in animal stroke models, none of neuroprotective treatments has been successfully translated into clinical therapy for stroke patients [3]. The underlying reasons for clinical failures may include, but are not limited to, the narrow therapeutic window of the neuroprotective reagents, anatomic and pathophysiological differences between animal and human strokes (such as the lack of focus on white matter damage in rodent models), the narrow focus on only an individual target (a single signaling pathway/receptor/channel or a single gene), and inappropriate selection/control of stroke subtypes in clinical trials [4–7]. Additionally, it is possible that we still have incomplete understanding of ischemic brain damage. For example, the majority of investigations on cell death mechanisms were carried out in cultured cells, while cells in the ischemic brain are injured or die in a more complicated environment and their fate is affected by interplays of multiple regulatory pathways. It is now clear that in addition to neurons, glial cells, endothelial cells and axonal fibers are susceptible to hypoxia and ischemia, and are of equal importance in ischemic brain damage, treatments and functional recovery [8–10]. For example, investigations from Sun's group and others have implicated the $\text{Na}^+\text{-K}^+\text{-Cl}^-$ cotransporter isoform 1 (NKCC1), an electroneutral cotransporter expressed in astrocytes and the blood brain barrier (BBB), in cerebral edema in rodent models of stroke [4–6, 11]. Pharmacological inhibition or genetic deficiency of NKCC1 decreases ischemia-induced cell swelling, BBB breakdown, cerebral edema, and neurotoxicity. A combination of pharmacological strategies that targets receptors/channels as well as membrane transporters might thus prove beneficial for the treatment of cerebral ischemia. More information on the role of NKCC1 in brain edema and damage mechanisms can be found in previous reviews [6, 11].

The progression of cell death is irreversible in the ischemic core without a timely blood reperfusion. A slower cell death occurs in the peri-infarct region due to the presence of collateral blood perfusion. The active cell death mechanisms crossing the ischemic core and penumbra are characterized by a spectrum of time-dependent pathological events that have been described as ion imbalance, excitotoxicity, acidosis, production of reactive oxygen species (ROS), apoptotic cascade activation, and inflammatory activities. A summary of this ischemic cascade along with stroke risk factors and plasticity for recovery can be learned from previous reviews and other reviews in this special issue [12–14]. Here, we concentrate on ionic mechanisms underlying the cellular toxicity during the acute, sub-acute and chronic stages (hours to days and weeks) after a cerebral ischemic insult. A better understanding of these basic cellular and molecular changes is expected to help in translational stroke research.

Cytotoxic cell swelling

Gray and white matters in the brain are active in electrophysiological activities and chemical signal transmission, thus requiring a high consumption of glucose and oxygen to maintain ionic gradients across the cell membrane. A few minutes after occlusion of cerebral blood flow, ischemic brain tissues become deprived of oxygen and glucose, resulting in mitochondrial dysfunction and reduction of ATP synthesis. In this acute phase after ischemia, a rapid cell swelling (cytotoxic edema) occurs due to excessive Na^+ and Ca^{2+} influx via cation channels/receptors and water flood into intracellular space. Facing the challenge of excessive Na^+ and Ca^{2+} influx as well as K^+ efflux, $\text{Na}^+/\text{K}^+\text{-ATPase}$, which is the major active transporter for Na^+ and K^+ homeostasis and the main cellular machinery of ATP consumption, is over-activated to keep the ionic gradients in affected cells. Sooner or

later in the pathological process, the activity of Na^+/K^+ -ATPase decreased due to ATP depletion and accumulation of injurious factors. In electrophysiological examinations, we were able to directly record the Na^+ pump associated membrane current in cortical neurons and demonstrated that the lack of ATP in combination with the production of ROS severely impaired the activity of Na^+/K^+ -ATPase [15].

Intracellular Na^+ accumulation may reverse the operational direction of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) to cause a Na^+ -dependent Ca^{2+} uptake [16]. NCX are trans-membrane transporters that exchange 3 Na^+ for 1 Ca^{2+} in forward mode (Ca^{2+} extrusion) or reverse mode (Ca^{2+} uptake) depending on the ion gradients and membrane potentials. The ischemia-induced reversed operation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger is mostly reported in glial cells and other non-neuronal cells, which seems consistent with the observations that early swelling is more prominent in astrocytes and oligodendrocytes than in neurons. In the ischemic cortex of a rat model, astrocytic swelling and fragmentation can be seen 30 min after MCAO while neuronal cells are less injured at this early stage [17]. In the sub-cortical region, swollen oligodendrocytes and astrocytes can be identified in the ischemic core by electron microscopy 30 min after MCAO. Three hours after ischemia, axonal swelling appears, and oligodendrocytes undergo pyknosis; 12–24 hours later, pyknotic oligodendrocytes show necrotic deterioration [17]. Demyelination of axonal fibers occurs 30 min after ischemia and is still seen in white matter injury 12–24 hours later [17]. Evidence from a sciatic nerve ischemic model also shows that swelling is the earliest morphological change in white matter injury [18]. Numerous studies have shown that increased Na^+ and Ca^{2+} influx into cells is responsible for cytotoxic cell swelling and the ensuing acute injury.

It is believed that the reverse mode of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in glial cells contribute to Ca^{2+} uptake under ischemic conditions. In neuronal cells, however, the situation may be different. Using whole-cell recordings, we directly measured the $\text{Na}^+/\text{Ca}^{2+}$ exchanger current under normal and ischemic conditions. In cortical neurons we showed that exchangers in these cells might concurrently operate in either the forward or the reverse direction, perhaps in different membrane locations. More importantly, cytotoxic glutamate exposure enhanced the exchanger forward current while the reverse activity was inhibited [19]. Consistent to this observation, knockdown of the exchanger NCKX2 resulted in greater $[\text{Ca}^{2+}]_i$ increases in cortical neurons. Oxygen-glucose deprivation (OGD)-induced cell death in vitro and ischemic infarct formation in a rat stroke model both dramatically increased in the absence of NCKX2 [20]. In NCX3 knockout mice subjected to transient middle cerebral artery occlusion (MCAO), an enlarged area of brain damage was seen compared to wild type mice [21]. Thus it appears that block of the NCX damages neurons while increasing the NCX activity is neuroprotective [22, 23]. These investigations provide specific evidence that after ischemia the neuronal exchangers remains or even increases its forward operation to remove Ca^{2+} from the intracellular space and therefore it has a neuroprotective role in the ischemic gray matter.

Apoptotic cell shrinkage

Cell volume change is a dynamic process. When cells swell under physiological conditions, a counteracting cellular process of regulatory volume decrease (RVD) is activated to offset the cell volume increase [24]. In sharp contrast to cell volume increase commonly seen in necrotic cell death, apoptosis is characterized by cell volume decrease [24–27]. The cell shrinking process or apoptotic volume decrease (AVD) is a ubiquitous aspect of apoptosis regardless of the cell types involved. Evidence from recent years supports the idea that AVD is an integrated episode of a cell death program that is regulated by specific ion channels and molecules in apoptosis [24, 26, 27]. For example, our early investigations provided the original evidence that apoptotic insults evoked marked upregulation of the delayed rectifier K^+

current and excessive K^+ efflux [28]. This ionic mechanism is not only responsible for AVD but also constitutes an active early event in the death program ahead of the activation of caspases and nucleases [24, 26, 29, 30]. To some extent, a selective up-regulation of certain K^+ channels and excessive K^+ efflux in AVD can be regarded as a red flag for cells ready to commit suicide. Thus, an appreciation of ion movement, particularly changes in K^+ efflux and intracellular K^+ in the dying cell, may hold the key to understanding the ionic mechanism of apoptosis.

Although RVD and AVD are distinct cellular events, they share some common mechanisms; for example both involve K^+ efflux and are mediated by activation of certain K^+ and/or Cl^- flux via Cl^- channels [27]. A well-known phenomenon supporting ischemic stimulation of a massive K^+ leak from the intracellular space is the elevated extracellular K^+ concentration (from ~5 mM raises to over 60 mM) in the ischemic brain [31]. This pathological event acts as the ionic mechanism of “spreading depression” that is widely recognized in the post-ischemic brain and is detrimental to brain tissues [32, 33]. Accompanied with increased extracellular K^+ , compelling evidence now supports that a marked intracellular K^+ loss is a common and critical step leading to cell body shrinkage and apoptotic cell death [28, 34]. In cultured cells, stimulation of K^+ efflux and intracellular K^+ depletion induces caspase activation, cytochrome c release and DNA degradation. Therefore, under certain circumstances, especially during sub-acute and chronic phases of an ischemic insult, intracellular K^+ loss is an ultimate consequence that can lead to activation of apoptotic cascades [29, 35]. This K^+ -mediated mechanism underlying apoptosis has been demonstrated in neurons and non-neuronal cells after ischemia, hypoxia and many other pathological insults [27, 29, 30, 36, 37] (please see the review from Aizenman’s group in this special issue).

The following paragraphs will introduce membrane molecules (receptors and channels) that can regulate and/or participate in cell volume changes after a hypoxic/ischemic insult (Fig. 1). Many receptors and cation channels have been identified in excitotoxicity for cell swelling. Some may have dual roles in both cell swelling and cell shrinkage, depending on the insult and pathological conditions. For example, although glutamate receptors, including N-methyl-D-aspartate (NMDA) receptors and some α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/kainate receptors, are well known for their Ca^{2+} permeability and responsibility for excitotoxic Ca^{2+} influx, these non-selective cation receptor channels are also capable of carrying out massive K^+ efflux. We have shown that activation of NMDA receptors and even AMPA/kainate receptors in a low Ca^{2+} condition can lead to significant intracellular K^+ reduction (~50% depletion), cell shrinkage and apoptosis [34, 38].

It is worth pointing out that although an excessive K^+ efflux occurs as a pro-apoptotic event, the disruption of K^+ and Na^+ homeostasis can only occur when the Na^+/K^+ -ATPase counterpart mechanism is impaired. We will discuss this issue and a unique hybrid cell death mechanism associated with Na^+/K^+ -ATPase impairment later in this review. In the next few sections, we inspect the most prominent and also some recently identified receptors/channels that mediate excessive Na^+ and Ca^{2+} influx under hypoxic/ischemic conditions (Fig. 1).

Ionotropic glutamate receptors

Glutamate is the primary excitatory neurotransmitter in the brain, playing key roles in synaptic transmission, synaptic plasticity and cell excitotoxicity via activation of glutamate receptors including NMDA, AMPA and kainate receptors. In the ischemic core, sustained cell membrane depolarization causes increased glutamate release and removal of the voltage-dependent Mg^{2+} block of NMDA receptors. The process of glutamate re-uptake is simultaneously suppressed by ischemia. Accumulation of glutamate in the synaptic cleft and

extracellular spaces over-activates ionotropic glutamate receptors including NMDA, AMPA and kainate receptors. It is believed, with some exceptions, that activation of these receptors especially the extrasynaptic NMDA receptors is a major route for excessive Ca^{2+} influx to trigger cell death [39–42]. The increased intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) activates Ca^{2+} -dependent protein kinases and neutral proteases (calpain) that degrade essential proteins in maintenance of cellular and subcellular integrity. This cell death mechanism, known as excitotoxicity, is responsible for acute neuronal death and has been used to guide stroke interventions.

Glutamate-mediated excitotoxicity is also involved in ischemic axonal injury. In axonal damage, activation of AMPA/kainate receptors mediates the excitotoxic effect [43–45]. The AMPA receptor blocker NBQX preserves axonal structure and functional activity in OGD-treated brain slices and is likely due to a secondary effect resulting from protection of oligodendrocytes by NBQX [46]. Blockade of AMPA/Kainate receptors also suppresses OGD-induced Ca^{2+} entry [47–49]. Aside from *in vitro* evidence, *in situ* and *in vivo* experiments also show that blockade of AMPA/kainate receptors reduces Ca^{2+} -dependent oligodendrocyte death in hypoxic-ischemic acute brain slices and hypoxic-ischemic injury in the developing white matter [46, 50]. Most recent studies show that rat dorsal column axons express glutamate receptor subunit 4 (GluR4) in AMPA receptors and GluR5 and GluR6 in kainate receptors. Application of AMPA/kainate receptor agonists induces progressive elevation of intra-axonal Ca^{2+} and impairs functional compound action potentials (CAP) in dorsal axons [51, 52].

It has long been thought that the NMDA receptor was not involved in excitotoxic oligodendrocyte death because these cells lack functional expression of NMDA receptors [53–55]. This concept is now challenged by several reports showing the existence of NMDA receptor subunits and their functional expression in mature and immature oligodendrocytes of the cerebellum and corpus callosum. It was shown that activation of NMDA receptors contributes to an ischemia-induced intracellular Ca^{2+} increase in oligodendrocyte damage [56–58]. Hence, it is likely that NMDA receptors also participate in hypoxic-ischemic injury of oligodendrocytes. The importance of this contribution in ischemic stroke requires further examination in animal experiments and the human brain.

In a retrospective review, glutamate-mediated excitotoxicity was initially investigated in simplified *in vitro* models and then acute artery ligation animal models. In stroke patients, complex ischemic cascades are activated after years of development of pathophysiological processes prior to the sudden arrest of local blood supply. Although glutamate excitotoxicity is a dominant player in the acute phase of ischemic injury, it may not be responsible for all of the dynamic changes and pathological progression of stroke. Beyond NMDA and AMPA receptors, many other selective and nonselective cation channels have been indicated in the ionic mechanisms responsible for ischemic brain damage [59].

Acid-sensing ion channels (ASICs)

Acid-sensing ion channels (ASICs) are members of the degenerin/epithelial Na^+ channel (DEG/ENaC) family of cation channels [60, 61]. In the central and peripheral nervous systems, ASICs are gated by extracellular H^+ and conduct the acid-evoked inward Na^+ currents with a selective sensitivity to amiloride blockade [62–64]. This is particularly important considering that acidosis, e.g. reduction in pH, develops in the ischemic brain region. In ischemic region, deprivation of energy and oxygen leads to an enhanced anaerobic glucose metabolism and accumulation of lactic acid, along with enhanced H^+ release from ATP hydrolysis. The pH value in the ischemic core may drop to 6.5 or lower [65]. This acidosis occurs in many pathological conditions such as brain trauma, inflammation, epileptic seizure, multiple sclerosis, and ischemic stroke [66–68]. Electrophysiological

characterization reveals a pH_{50} of 6.18 for acidosis to activate ASIC1a in rodent brain neurons, while the pH_{50} in human cortical neurons is 6.60 [69].

Molecular cloning has identified six ASIC subunits that arise from four genes: ASIC1a and ASIC1b; ASIC2a and ASIC2b; ASIC3 and ASIC4 [66]. Each ASIC protein contains two trans-membrane domains (TM1 and TM2), intracellular C and N terminals, and a large cysteine-rich extracellular loop for sensing protons and other unrecognized ligands [70]. These channels have different pH values of half-maximal activation (pH_{50}); pH_{50} 5.8–6.8 for ASIC1a, ASIC1b and ASIC3 and pH_{50} 4.5–4.9 for ASIC2a. ASIC2b or ASIC4 on their own do not form pH-sensitive homomeric channels [66]. Once activated by extracellular acid, ASICs mediate Na^+ influx leading to increased cell excitability and water content. Homomeric ASIC1a channels are also Ca^{2+} permeable and have been implicated in acidosis-mediated neuronal injury under ischemic conditions [71–73].

Ischemic condition mimicked by OGD increases ASIC-mediated inward currents and decreases desensitization of ASIC activity in cultured neurons [71]. Evidence from Ca^{2+} imaging experiment shows that acidosis can induce Ca^{2+} entry via ASIC1a and cytosolic Ca^{2+} elevation in a glutamate receptor-independent manner. In cultured cortical neurons, ASIC1a activation and its mediated Ca^{2+} entry are critically involved in acidosis induced, glutamate-independent neuronal damage. Application of ASIC blockers and low Ca^{2+} significantly prevent this type of injury [74]. In an animal model of ischemic stroke, blockage of ASIC with amiloride or knockout of the ASIC1a gene shows a protective effect by reducing the infarct volume, even where glutamate-mediated excitotoxicity is already suppressed [71]. The therapeutic time frame for ASICs in the stroke brain is much longer than for glutamate receptor antagonists. It can be up to 5 hours and may persist for at least 7 days [71, 75]. Thus, ASICs are identified as a new therapeutic target for ischemic stroke. Some studies have taken the first step to test leading drugs targeting ASICs for stroke treatment [76–79].

Pannexin and connexin hemichannels

There are two major families of proteins, pannexins and connexins contributing to the formation of hemichannels. A Gap junction forms when two opposing connexons or pannexin hemichannels join together between two adjacent cells. Hemichannels in nonjunctional membranes conduct ions and signaling molecules that are smaller than 1 kDa, such as K^+ , Na^+ , Ca^{2+} , ATP, glutamate, glucose, NAD^+ , adenosine and PGE_2 [80–82]. Three pannexin members Px1, Px2 and Px3 have been identified in animals and humans. Px1 and Px2 are abundantly expressed in the brain region of hippocampus, neocortex, cerebellum, thalamus and hypothalamus, whereas Px3 is only found in skin and osteoblasts [83]. At the cellular level, Px1 is localized in neurons, astrocytes, microglia and oligodendrocytes, and Px2 appears only in a subset of GAPDH-positive pyramidal neurons [84–86].

In the central nervous system (CNS), gating of hemichannels is regulated by pH, cations, trans-membrane potential, cytokines/growth factors, neurotransmitters and many other factors [87]. Thus, it is obvious that hemichannels may be involved in trans-membrane signal transduction/transmission and cell-cell communication under physiological and pathological conditions. In glial and neuronal cells, hemichannels have been implicated in cell swelling and disruption of intracellular ionic homeostasis after exposure to hypoxia/ischemia. For example, chemical ischemia increases cell permeability of cortical astrocytes due to opening of Cx43 hemichannels. Na^+ , Ca^{2+} and H_2O pass through Cx43 hemichannels and causes astrocytic swelling and cell death [88]. Down-regulation of Cx43 function reduced cell swelling and astrocytic death in a rat model of traumatic brain injury [89]. An in vitro experiment performed on acutely isolated hippocampal neurons showed that OGD induced a

large inward current at the resting membrane potential [90]. Pharmacological testing and biophysical characterization of this large conductance current indicated that it is mediated by Px1 hemichannels, but not voltage-dependent Na⁺ channels, glutamate receptor channels, ASICs and TRP channels. It was proposed that Px1 hemichannels are activated under ischemic conditions and mediate up-regulated Ca²⁺ entry, glucose and ATP efflux, thus contributing to ionic imbalance and cytotoxicity. The gap junction/hemichannel blocker carbenoxolone (Cbx) was shown to prevent ischemic insult in stroke models [91, 92]. A double-knockout of Px1 and Px2 in mice leads to smaller infarct size in the ischemic brain [93]. Pannexin or connexin hemichannels are emerging as a new molecular target that has therapeutic potential for protecting brain from ischemic attack.

TRP channels

TRP channels are nonselective cation channels containing six families: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), and TRPA (ankyrin) [94, 95]. The first type of TRP channel was identified in the *Drosophila* eye. Now TRP channels are found widely distributed in many organs and tissues of mammals, including the nervous system [96, 97]. The TRP channels function as tetramers and conduct different cations (Na⁺, K⁺, and Ca²⁺) when responding to local changes in their environment such as temperature, mechanical pressure, ion changes, pH changes and many other physical and chemical changes. Opening of TRP channels depolarizes the cell membrane and thereby activates voltage-dependent Na⁺ and Ca²⁺ channels, leading to intracellular Na⁺ and Ca²⁺ accumulation [97]. Therefore, TRP channels are destined to regulate membrane potential and manipulate intracellular Na⁺, K⁺ and Ca²⁺ contents in both excitable and non-excitable cells. Several TRP channel members have been investigated in ischemic/anoxic conditions and implicated in the cytotoxicity of secondary injury.

In order to detect novel ischemic cell death mechanisms beyond glutamate-mediated excitotoxicity, Arts and his colleagues reported that OGD of 1.5 hours or longer evoked an intracellular Ca²⁺ increase in cultured cortical neurons. This Ca²⁺ increase cannot be eliminated by the mixture of blockers containing nimodipine, MK801 and CNQX and thus is not mediated by L-type Ca²⁺ channels, NMDA or AMPA/kainate glutamate receptors [98]. Protection of this anti-excitotoxic combination is confined to 1-hour OGD exposure and allowed intracellular Ca²⁺ to return close to basal level. Their experimental data suggested that prolonged Ca²⁺ entry and cytotoxicity mainly resulted from activation of TRPM7 channels. Inhibition of TRPM7 channels by its blockers or siRNA-mediated knockdown significantly prevents [Ca²⁺]_i accumulation during prolonged OGD and protected anoxic neuronal death [98]. It was also showed that TRPM7 channels are activated by ROS from nitric oxide (NO) signaling. Evidence from an *in vivo* study demonstrated that down-regulation of TRPM7 in rat hippocampus protected neurons against brain ischemia and also preserved behavioral deficits [99]. TRPM7 channels seem to be a potential target for treating cerebral ischemia although the effect of TRPM7 blockers on ischemic infarct formation remains to be verified [99].

TRPM4 channels are also recommended as a mediator for ischemic injury. TRPM4 channels are activated by intracellular Ca²⁺, closed by ATP, permeable to Na⁺, K⁺, Cs⁺ and Li⁺, but impermeable to Ca²⁺ [99]. Study of TRPV4 in rat hippocampal astrocytes shows that cerebral hypoxia/ischemia increases TRPV4 expression and its Ca²⁺ permeability along with the development of astrocytosis [100]. TRPV4 blockade suppresses intracellular Ca²⁺ elevation and attenuates astroglial reactivity, suggesting a mediating role of TRPV4 in ischemic insult. The role of the TRPC channel family in Ca²⁺ homeostasis regulation related to cell survival and growth has received more and more attention [101–103]. An *in vivo* study on TRPC channel knockout mice demonstrated that the activity of TRPC1/4 and TRPC5 channels are

involved in pilocarpine-induced seizure and secondary excitotoxicity in the brain [104]. Opening of TRPC1/4 channels contributes to the depolarizing plateau potential in some neurons and TRPC5 is sensitive to and inhibited by intracellular ATP [99]. These studies suggest TRPC1/4 and TRPC5 may play a role in epilepsy and stroke. TRPC6 channels are critical for neuronal survival by mediating the trophic action of brain-derived neurotrophic factor (BDNF) and Ca^{2+} influx as growth signals [105]. The expression level of TRPC6 protein is much lower than TRPC4 and TRPC5 in rodent brain [99]. A recent study reported that neuronal TRPC6 proteins are degraded in ischemic brain via a NMDA receptor-dependent manner [106]. The authors found that preventing the TRPC6 degradation reduced infarct size and activating TRPC6 channels prevented neuronal death. It was concluded that the TRPC6 channel mediated moderate Ca^{2+} influx but not Ca^{2+} overload, which is beneficial for cell survival via activation of the cAMP response element-binding protein (CREB).

Collectively, the paradigm for the role of TRP channels in ischemic stroke is not very clear because of TRP family member diversity, no specific antagonists for distinguishing each member, unrevealed expression patterns in the brain and paradoxical action on intracellular Ca^{2+} increase. Much more work needs to be done to delineate the significance of TRP channel family members in stroke injury.

KCNQ2/3 channels and apoptosis

In addition to the discussion earlier about NMDA and AMPA receptors, the pro-apoptotic K^+ efflux and intracellular K^+ loss are most likely mediated via over-activation of selective and non-selective K^+ channels [29, 30]. For instance, ischemia associated membrane depolarization activates the delayed rectifier K^+ channels such as the Kv2.1 channel, and increased intracellular Ca^{2+} can activate Ca^{2+} -activated K^+ channels [29, 107, 108]. All of these commonly seen events can result in excessive K^+ efflux and intracellular K^+ depletion [29]. Since our report in 1997 [28], increasing types of K^+ channels have been identified as mediators in the pro-apoptotic K^+ efflux [29, 36, 109, 110].

Among many K^+ channels that may participate in AVD, KCNQ channels, specifically the KCNQ1 channel, in cardiomyocytes was shown to mediate the regulatory volume decrease [111]. Based on the expression pattern and the non-inactivating kinetics of the KCNQ channels, we examined the hypothesis that KCNQ2/3 channels, the molecular basis for the M-type K^+ current, may contribute to apoptosis in CNS neurons [112]. KCNQ2/3 channel openers *N*-ethylmaleimide (NEM) and flupirtine caused dose-dependent K^+ efflux, intracellular K^+ depletion, caspase activation and cell death in hippocampal neurons. The NEM-induced cell death was antagonized by co-applied KCNQ channel inhibitor XE991, or by elevated extracellular K^+ concentration. More specifically, expression of KCNQ2 or KCNQ2/3 channels in Chinese hamster ovary (CHO) cells initiated caspase-3 activation. NEM increased the expression of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), induced mitochondria membrane depolarization, cytochrome *c* release, formation of the apoptosome complex, and apoptosis-inducing factor (AIF) translocation into the nucleus. All of these events were attenuated by blocking KCNQ2/3 channels [99].

In a study on several Kv7 channels, Gamper et al. showed that oxidative stress markedly enhanced the currents carried by Kv7.2, Kv7.3, Kv7.2/7.3, Kv7.4, and Kv7.5 (corresponding to KCNQ2, KCNQ3, KCNQ2/3, KCNQ4, and KCNQ5, respectively) channels expressed in CHO cells [99]. They further showed that blocking KCNQ channels during a 30-min OGD worsened the outcome of the insult, suggesting a protective action of these channels. This protection from upregulated K^+ channel activity during the acute phase of excitotoxicity is well-known. It is due to a hyperpolarization effect that reduces excitability and Ca^{2+}

influx ^[113]. On the other hand, this protective effect would disappear if persistent channel activation leads to disruption of the K⁺ homeostasis. The consequences after a longer duration of KCNQ channel activation, however, were not tested in this report.

Na⁺/K⁺-ATPase, K⁺ homeostasis and hybrid cell death

Under physiological conditions when sufficient energy supply is available, up-regulation of K⁺-permeable channels/receptors may not cause detrimental consequences because K⁺ efflux can be well balanced by activated Na⁺/K⁺-ATPase. Given that energy depletion occurs in ischemia and the key role for Na⁺/K⁺-ATPase in ionic homeostasis especially in K⁺ homeostasis, a Na⁺/K⁺-ATPase failure plays a critical role in ischemic brain damage. Indeed, ATP depletion and dysfunction of the Na⁺ pump are indispensable event in ischemic pathology. Besides its dependence on a supply of energy, our data suggest that Na⁺/K⁺-ATPase activity is also highly sensitive to block by ROS production ^[15]. To better understand the consequence of the Na⁺ pump failure, we tested an in vitro model of cultured cortical neurons using ouabain as the selective Na⁺/K⁺-ATPase inhibitor ^[99]. Ouabain caused cell swelling as shown by previous investigations after an acute exposure to ouabain ^[99]. Somewhat unexpected at the time, several hours after initial cell swelling there was marked and enduring cell shrinkage, accompanied by intracellular K⁺ depletion, cytochrome c release, and caspase-3 activation ^[114]. Electron microscopic examination revealed the concurrent existence of ultrastructural features of apoptosis and necrosis in the same cells. This mixed cell death was named hybrid death, characterized by concurrent necrotic and apoptotic components in a given cell. According to recent investigations and the ultrastructural features of injured cells, such as the appearance of multiple vacuoles in the cytoplasm, ischemia-induced hybrid cell death may additionally include autophagic cell injury ^[115–118].

It is apparent that cells in the ischemic brain face insults which are far more complex than the controlled condition in any cytotoxicity experiment in vitro. Thus, it is not surprising that morphological and cellular evidence from in vivo studies often does not support the existence of typical or pure apoptotic alterations that can be seen in cultured cells ^[119, 120]. In adult rats subjected to focal barrel cortex ischemia, we specifically examined the phenotypes of cell death. Early cell death was detected several hours after ischemia while delayed cell death in the peri-infarct region and secondary cell death in the ventrobasal (VB) thalamus was seen 2–3 days later ^[99]. TUNEL positive neurons were found in these two regions, but with striking morphological differences, designated as type I and type II TUNEL positive cells. The type I TUNEL positive cells in the ischemic cortex underwent necrotic changes. The type II TUNEL positive cells in the thalamus and the cortex penumbra region underwent a hybrid death, featuring concurrent apoptotic and necrotic alterations in individual cells. This included marked caspase-3 activation, nuclear condensation/fragmentation, swollen cytoplasm, damaged organelles, and deteriorated membranes. Similar mixed cell death was also evident after ischemic stroke in the developing brain of neonatal rats ^[99]. The concept of mixed or hybrid cell death has gradually gained popularity with various modifications such as the identification of necroptosis ^[2, 42, 117, 121–132]. More recent evidence suggests that autophagy may also contribute as a component of hybrid cell death after ischemia ^[133, 134]. Future investigations are necessary to better understand the interplay between different cell death mechanisms and the implication in the treatment of stroke.

Concluding Remarks

During the acute stage of cerebral ischemia, excessive Na⁺ and Ca²⁺ influx and cell swelling mediated by multiple receptors/channels is the earliest evidence of excitotoxicity in neuronal

and non-neuronal cells. Recently identified cation channels activated by acidosis and ATP depletion in ischemic conditions have provided new targets for neuroprotective treatments of stroke. However, an efficient treatment for stroke may rely on a combination therapy targeting multiple receptors/ion channels and regulatory mechanisms. In addition to focusing on apoptosis, the existence of hybrid cell death, identified after Na⁺/K⁺-ATPase blockade in vitro or energy depletion in vivo, suggests that a more comprehensive approach for ischemic stroke therapy should target the parallel pathways which are involved in the hybrid cell death and promote Na⁺/K⁺-ATPase function.

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Figure 1. Ionic mechanism of ischemia-induced cell death

The graphic diagram illustrates a simplified model of ischemia-induced neuronal cell death. Excessive activation of Ca²⁺ and Na⁺ permeable channels and receptors leads to intracellular Ca²⁺ and Na⁺ accumulation. The resulted cell swelling and destructive consequences are characteristics of necrosis. On the other hand, ischemia can cause over-activation of K⁺ permeable channels and receptors that mediate pro-apoptotic K⁺ efflux. The resulted intracellular K⁺ reduction consequently induces caspase activation and apoptotic cell death. Concurrent activation of these cell death events likely occurs after an ischemic insult and may results in hybrid cell death features in the same cells. The dysfunction of Na⁺/K⁺ pump plays a critical role in the development of hybrid cell death. Recent data suggest that excessive autophagy also contributes to ischemic cellular damage and is an integrated component of hybrid cell death.