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Proton-sensitive cation channels and ion exchangers in ischemic brain injury: new therapeutic targets for stroke?

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Abstract

Ischemic brain injury results from complicated cellular mechanisms. The present therapy for acute ischemic stroke is limited to thrombolysis with the recombinant tissue plasminogen activator (rtPA) and mechanical recanalization. Therefore, a better understanding of ischemic brain injury is needed for the development of more effective therapies. Disruption of ionic homeostasis plays an important role in cell death following cerebral ischemia. Glutamate receptor-mediated ionic imbalance and neurotoxicity have been well established in cerebral ischemia after stroke. However, non-NMDA receptor-dependent mechanisms, involving acid-sensing ion channel 1a (ASIC1a), transient receptor potential melastatin 7 (TRPM7), and Na⁺/H⁺ exchanger isoform 1 (NHE1), have recently emerged as important players in the dysregulation of ionic homeostasis in the CNS under ischemic conditions. These H⁺-sensitive channels and/or exchangers are expressed in the majority of cell types of the neurovascular unit. Sustained activation of these proteins causes excessive influx of cations, such as Ca²⁺, Na⁺, and Zn²⁺, and leads to ischemic reperfusion brain injury. In this review, we summarize recent pre-clinical experimental research findings on how these channels/exchangers are regulated in both *in vitro* and *in vivo* models of cerebral ischemia. The blockade or transgenic knockdown of these proteins was shown to be neuroprotective in these ischemia models. Taken together, these non-NMDA receptor-dependent mechanisms may serve as novel therapeutic targets for stroke intervention.

Keywords

Acidosis; ASIC; Calcium; NHE1; TRPM7; Zinc

1. Introduction

A stroke occurs when blood flow to the brain is disrupted by an obstruction (i.e., ischemic stroke) or hemorrhage (i.e., hemorrhagic stroke). Strokes are the leading cause of death in the United States, affecting approximately 800,000 people each year (Roger et al., 2011).

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They are also a major cause of long-term disabilities, with 20% of all stroke survivors requiring long-term institutional care and 15%–30% of them being permanently disabled, unable to resume work and other daily activities (Goldstein et al., 2011; Roger et al., 2011).

The current treatment for acute ischemic stroke is limited to restoring the blood supply to the affected area. Reperfusion therapy consists of administering the thrombolytic agent recombinant tissue plasminogen activator (rtPA), and endovascular mechanical clot extraction (Nesbit et al., 2004). However, rtPA has a narrow therapeutic timeframe of 3–4.5 h (Hacke et al., 2004; Wardlaw et al., 2012) because of the high risk of intracranial hemorrhage after thrombolysis beyond the window, especially in patients with severe strokes or increased age (van der Worp and van Gijn, 2007). Thus, only approximately 5% of stroke patients can benefit from rtPA treatment. Combination therapies with neuroprotective agents have been extensively investigated to prevent delayed neuronal death in stroke. Unfortunately, almost all neuroprotective agents that showed great promise in pre-clinical experimental studies in the past three decades failed in clinical trials (O'Collins et al., 2006). Some clinical trials demonstrated protection in acute ischemic stroke by blocking N-methyl-D-aspartate (NMDA)-mediated neurotoxicity, however, the required early treatment time (immediately after endovascular repair procedure) limits its clinical application (Hill et al., 2012; Kaste, 2012). Therefore, continued effort is needed to better understand the complex processes of stroke-induced brain injury and to identify novel therapeutic targets for stroke intervention.

During a stroke, the disruption of blood flow to the brain deprives cells of energy and disturbs the ionic homeostasis of the cells (Siesjo, 1992). Inhibition of oxidative phosphorylation and depletion of ATP result in the loss of ATP substrate for $\text{Na}^+\text{-K}^+\text{-ATPase}$, which leads to the dissipation of transmembrane K^+ and Na^+ gradients and subsequent membrane depolarization (Lipton, 1999). Sustained depolarization causes excessive Ca^{2+} entry through voltage-sensitive Ca^{2+} channels, which initiates an excessive release of the neurotransmitter glutamate (Benveniste et al., 1984; Nicholls and Attwell, 1990) and, subsequently, the excessive stimulation of NMDA receptors. The resulting Ca^{2+} overload (Choi, 1988; Choi, 1992; Simon et al., 1984) then triggers secondary signal cascades, activating proteases and phospholipases, and producing free radicals (Puyal et al., 2013). This is the well-known excitotoxicity mechanism that contributes to the cerebral ischemia-induced neuronal injury (Lai et al., 2011).

In addition to NMDA-dependent mechanisms, recent studies have shown that other Ca^{2+} permeable channels, such as the acid-sensing ion channel 1a (ASIC1a), transient receptor potential melastatin 7 (TRPM7), and $\text{Na}^+\text{/H}^+$ exchanger isoform 1 (NHE1), contribute to neuronal injury after ischemia and reperfusion. Under ischemic conditions, hypoxia enhances glycolysis, resulting in the buildup of lactic acid and subsequent tissue acidosis. Extracellular pH in the brain typically drops to below 6.5 during ischemia under normoglycemic conditions (Nedergaard et al., 1991). However, with hyperglycemia, the concentration of lactate in the brain could rise to 25 $\mu\text{mol/g}$, causing the pH of the ischemic brain to drop to approximately 6.0 (Rehncrona, 1985). The notion that acidosis exacerbates ischemic brain injury first rises from the observations that ischemic outcomes are worsened in the case of incomplete ischemia or glucose-infused subjects, where additional glucose is delivered to the tissue during the ischemic insult (Siesjo, 1988). Indeed, excessive lactate accumulation can cause edema, BBB dysfunction, and extensive tissue necrosis in part by inhibition on glutamate uptake (Swanson et al., 1995), impairment of brain energetics (Swanson et al., 1997), and oxidative stress (Ying et al., 1999). However, it is notable that recent research also suggests protective effects of mild intracellular pH reduction against NMDA-mediated neuronal toxicity via inhibiting the NADPH oxidase (Lam et al., 2013).

Apart from these, acidosis can activate homomeric ASIC1a, causing a large influx of Na^+ and Ca^{2+} , leading to neuronal injury (Xiong et al., 2004; Yermolaieva et al., 2004). Thus, deleting ASIC1a or inhibiting its activation is potentially neuroprotective. Importantly, the effective therapeutic time window for ASIC1a inhibition is longer than 5 h in animal models of stroke (Pignataro et al., 2007). On the other hand, in response to intracellular acidosis, NHE1 is activated to extrude H^+ and to maintain intracellular H^+ homeostasis (Putney et al., 2002). Sustained stimulation of NHE1 activity during reperfusion – in conjunction with the reversal of $\text{Na}^+/\text{Ca}^{2+}$ exchange – contributes to Na^+ and Ca^{2+} overload and causes injury during reperfusion after ischemia (Luo and Sun, 2007). Pharmacological inhibition or transgenic knockdown of NHE1 significantly reduces the volume of the infarct and edema in animal models of focal ischemia (Luo et al., 2005; O'Donnell et al., 2013). In addition, increases in oxidative stress and the production of reactive oxygen species during cerebral ischemia may activate another nonselective cation channel, the TRPM7 channel (Aarts et al., 2003). TRPM7 is highly permeable to both Ca^{2+} and Zn^{2+} (Harteneck, 2005; Monteilh-Zoller et al., 2003), and Zn^{2+} toxicity is largely mediated by the activation of TRPM7 (Inoue et al., 2010). Extracellular acidosis can potentiate TRPM7 and, thus, might also contribute to TRPM7-mediated neuronal injury. Most importantly, these channels and exchangers are expressed in the components of the “neurovascular unit”, including neurons, astrocytes, endothelial cells, pericytes, and microglia (Figure 1). In this review, we focus on the recent advances in the understanding of pH-sensitive cation channels and H^+ exchangers in ischemic brain injury. These channels and exchangers may present as novel targets for developing effective neuroprotective agents for stroke treatment.

2. Acid-sensing ion channels (ASICs)

2.1. Distribution

Acid-sensing ion channels (ASICs) are proton-gated, voltage-insensitive, cationic channels that are distributed throughout the central and peripheral nervous systems. To date, six ASICs isoforms encoded by four genes have been described in mammals: ASIC1a, ASIC1b (β), ASIC2a, ASIC2b, ASIC3, and ASIC4 (Krishtal, 2003) (Figure 2A). ASIC1a, ASIC2a, and ASIC2b are abundantly distributed in both the central and peripheral nervous systems (Duan et al., 2011; Lingueglia et al., 1997; Waldmann et al., 1997b; Wemmie et al., 2002), while ASIC3 and ASIC1b are predominantly distributed in the peripheral nervous system (Bassler et al., 2001; Chen et al., 1998; Waldmann et al., 1997a). Some studies have indicated that ASIC3 is distributed in the central nervous system. For example, ASIC3 mRNA and/or protein have been detected in rat (Meng et al., 2009) and human brain tissues (Babinski et al., 1999). Recent evidence also suggests that ASICs are present in some non-neuronal cells, such as astrocytes (Huang et al., 2010), vascular smooth muscle cells (Grifoni et al., 2008; Jernigan et al., 2012), and glioma cells (Berdiev et al., 2003; Kapoor et al., 2009; Vila-Carriles et al., 2006).

2.2. Structure

ASICs belong to the degenerin/epithelial sodium channel (DEG/ENaC) superfamily (Alvarez et al., 2000; Waldmann et al., 1997b) and share the overall structure of the DEG/ENaC family, with two transmembrane domains surrounding a large extracellular loop containing conserved cysteine residues (Alvarez et al., 2000; Saugstad et al., 2004; Waldmann et al., 1997b) (Figure 2B). ASICs form homomeric or heteromeric channels with trimeric assembly of identical or different subunits (Jasti et al., 2007). The low-pH crystal structure of the chicken ASIC1 at a 1.9 Å resolution shows a chalice-shaped homotrimer, with each subunit composed of short amino and carboxy termini, two transmembrane helices, and multidomain extracellular regions enriched in acidic residues (Jasti et al., 2007).

The domain arrangement in each ASIC1 subunit resembles an upright forearm and clenched hand. The extracellular domain protrudes around 80 Å from the membrane plane and consists of the palm, β -ball, knuckle, finger, and thumb domains (Jasti et al., 2007). The palm domain is the central feature of each subunit. It contains a 7-strand sheet with β -strands 1 and 12 connecting to transmembrane 1 (TM1) and transmembrane 2 (TM2) domains and β -strands 9 and 10 connecting to the thumb domain. Above the palm domain are the knuckle and finger domains (Jasti et al., 2007). The knuckle domain is composed of two short helices ($\alpha 6$ and $\alpha 7$), and the finger domain is composed of a few helices and non- α and non- β structures. The thumb domain is enriched in disulfide bonds, which touch the tip of the finger domain. The small 5-strand β -ball domain located in the center of the “clenched hand” is surrounded by the palm, knuckle, finger, and thumb domains (Jasti et al., 2007).

Within the extracellular domain, there is a highly negatively charged acidic pocket formed by the residues from the palm, β -ball, finger, and thumb domains. Two pairs of unusually close carboxyl-carboxylate interactions between the side chains of aspartate or glutamate residues are pivotal for the molecule's ability to sense pH. For example, neutralization of Asp350 in the Asp238–Asp350 pair and Asp346 in the Glu239–Asp346 pair has profound effects on either the $\text{pH}_{0.5}$ or Hill Coefficient (Jasti et al., 2007). In addition, Glu-79 and Glu-416, located in the lower palm domain, play a role in proton gating and desensitization (Della Vecchia et al., 2013). Proton binding in the extracellular domain is followed by the displacement of the $\beta 9$ - $\alpha 4$ loop of the thumb, leading to the rotation of the TM1 domain to stabilize the channel in the closed state (Swain and Bera, 2013).

The structure of the transmembrane domain resembles an hourglass, with each of the transmembrane domains (TM) defined by two long α -helices. TM1 and TM2 are each composed of three subunits related by the three-fold axis of crystallographic symmetry (Gonzales et al., 2009). Most of the contact of the TM1 helices is within the lipid bilayer, whereas the TM2 helices line the putative ion channel pore (Gonzales et al., 2009). All six transmembrane helices are arranged to form an ion-selective pore. A V-shaped fenestration extends 5–10 Å into the lipid bilayer, which connects the bulk solution to the pore's extracellular vestibule and is most likely one route by which ions gain access into the channel pore. The interior of the ion channel pore is defined primarily by residues of the TM2 domain, especially in the extracellular vestibule. The overall negative electrostatic potential of the interior of ASIC1 likely contributes to its cation selectivity. Information on the structure of ASIC1 is invaluable for studying its functions as well as for designing drugs to target ASIC1.

2.3. Electrophysiology

ASICs vary in their sensitivity to decreases in extracellular pH depending on their subunit composition. ASIC1a is highly sensitive to protons with an activation threshold close to pH 7.0 and a $\text{pH}_{0.5}$ (half maximal activation) of ~6.2 (Grunder and Chen, 2010; Waldmann et al., 1997b). ASIC3 has two current components, with a $\text{pH}_{0.5}$ of ~6.2 for the peak component and ~4.3 for the sustained component (de Weille et al., 1998; Waldmann et al., 1997a). ASIC1b is similar to ASIC1a in that it has a $\text{pH}_{0.5}$ of ~6.0 (Chen et al., 1998; Ugawa et al., 2001). ASIC2a has the lowest sensitivity to acidic pH, with a $\text{pH}_{0.5}$ of ~4.4 (Waldmann et al., 1999). Interestingly, the homomeric ASIC2b and ASIC4 channels do not respond to stimulation by protons (Akopian et al., 2000; Grunder et al., 2000; Lingueglia et al., 1997), suggesting the possible existence of unknown endogenous ligands for these receptors (Page et al., 2005; Sole-Magdalena et al., 2011; Wemmie et al., 2006). ASICs predominantly conduct Na^+ ; however, the homomeric ASIC1a and heteromeric ASIC1a/2b channels are also permeable to Ca^{2+} , providing new voltage-independent pathways for Ca^{2+} entry into neurons (Hoagland et al., 2010; Waldmann et al., 1997b; Xiong et al., 2004).

The response of ASICs to protons is complex. Protons activate ASICs; however, the amplitude of the current generated by the protons decays with their continued presence, resulting in a desensitization of the channel. This response is especially true for the ASIC1a channels. The kinetics of the desensitization and recovery of the ASICs depend largely on the composition of their subunits. For example, ASIC currents in DRG neurons from ASIC2a null mice recovered more slowly from desensitization than neurons from wild-type mice. Thus, incorporation of ASIC2a accelerates recovery from desensitization (Benson et al., 2002). Similarly, in hippocampal or spinal cord neurons, ASIC2 knockout significantly slows down the recovery of ASIC current from desensitization (Askwith et al., 2004; Baron et al., 2008). As for ASIC3, when the extracellular pH drops below 5.0, two components of current are induced: a fast desensitized component and a slow non-desensitized component (Sutherland et al., 2001; Waldmann et al., 1997a). Under experimental conditions, homomeric ASIC1a channels are activated only by rapid decreases in pH. Pre-exposure of an ASIC1a channel to small decreases in pH (e.g., from 7.4 to 7.2) that do not activate the channel result in potent inactivation of the channel (Babini et al., 2002; Chen et al., 2005). However, the channel does not respond to subsequent large decreases in pH. A summary of the properties and functions of ASIC1a is presented in Table 1.

In addition to changes in pH, the desensitization and inactivation of ASICs can also be modulated by a number of endogenous signaling molecules associated with brain ischemia (Chu et al., 2011; Chu and Xiong, 2013). These are discussed in the next section.

2.4. Pharmacology

ASICs are modulated by a variety of metal ions, including Zn^{2+} , Ca^{2+} , Mg^{2+} , Cu^{2+} , Ba^{2+} , Pb^{2+} , Cd^{2+} , Ni^{2+} , and Gd^{3+} (Babini et al., 2002; Babinski et al., 2000; Baron et al., 2001; Staruschenko et al., 2007a; Wang et al., 2006). Although most of the divalent cations listed inhibit the ASICs, Zn^{2+} exerts a complex modulatory function on ASIC1a- and ASIC2a-containing channels. At nanomolar concentrations, extracellular Zn^{2+} inhibits ASIC1a-containing channels with an IC_{50} of ~10 nM (Chu et al., 2004b). However, at high micromolar concentrations, it potentiates ASIC2a-containing channels with an EC_{50} of ~120 μM (Baron et al., 2001).

Several naturally occurring venom peptides inhibit or activate ASICs. For example, PcTx1, a peptide purified from the venom of the South American tarantula, *Psalmopoeus cambridgei*, potently inhibits the homomeric ASIC1a current at ~1 nM, without affecting the homomeric ASIC1b, ASIC2a, or ASIC3 currents (Escoubas et al., 2000). A recent study of the crystal structure of chicken ASIC1 in complex with PcTx1 revealed that PcTx1 molecules bind to the proton-sensitive acidic pockets of the ASIC1 channels (Dawson et al., 2012). Considering the high selectivity of PcTx1 for ASIC1, the PcTx1/ASIC1 complex structure could serve as an invaluable template for the future design of new molecules with the aim of conserving the high selectivity for ASIC1 but have the better permeability than PcTx1 in crossing the blood-brain-barrier.

The toxin APETx2, isolated from the venom of the sea anemone *Anthopleura elegantissima*, inhibits homomeric and heteromeric ASIC3 channels with an IC_{50} of ~63 nM and 2 μM , respectively (Chagot et al., 2005; Diochot et al., 2004). In contrast to PcTx1 and APETx2, which inhibit ASICs, MitTx, a toxin from the Texas coral snake, *Micrurus tener tener*, has been shown to produce pain by activating ASICs (Bohlen et al., 2011). MitTx evokes robust ASIC1a- and ASIC1b-like currents with an EC_{50} of ~9 and ~23 nM, respectively (Bohlen et al., 2011). In contrast, mambalgins, toxins from the black mamba snake, *Dendroaspis polylepis polylepis*, are able to abolish pain by inhibiting ASICs (Diochot et al., 2012). Mambalgins inhibit ASIC1b and ASIC1a/ASIC1b channels, which are specific to sensory

neurons, with an IC_{50} of 192 and 72 nM, respectively (Diochot et al., 2012). These toxins have provided valuable pharmacological tools for the studies of ASICs.

In addition to metal ions and peptide toxins, small molecules, such as amiloride, a potassium-sparing diuretic, and some of its derivatives, reversibly inhibit ASIC currents at micromolar concentrations (Bassilana et al., 1997; Chen et al., 2002; Waldmann et al., 1997b). Interestingly, amiloride inhibits transient ASIC3 currents but potentiates, paradoxically, sustained ASIC3 currents (Benson et al., 1999; Waldmann et al., 1997a). Non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen and flurbiprofen, at concentrations that are therapeutic for inflammation (IC_{50} of ~92–350 μ M), also inhibit ASIC currents (Voilley et al., 2001).

Aromatic diamidines are synthetic anti-protozoan small molecule compounds (Neidle, 2001). Chen and colleagues found that, in cultured hippocampal neurons, diarylamidines 4', 6-diamidino-2-phenylindole (DAPI), diminazene, hydroxystilbamidine (HSB), and pentamidine inhibited the ASIC current with IC_{50} values of 2.8 μ M, 0.29 μ M, 1.5 μ M, and 38 μ M, respectively (Chen et al., 2010b). In addition to reducing the peak current amplitude, diminazene shortened the desensitization time constant from ~1.7 to 0.4 sec (Chen et al., 2010b), suggesting that diminazene may promote the closure of ASICs. Diminazene is the most potent small-molecule inhibitor of ASICs found so far (Chen et al., 2010b); however, its effect on ASIC-related neurological disorders has not yet been tested.

Recent studies have shown that local anesthetics, such as lidocaine and tetracaine, also inhibit ASICs. Lidocaine, for example, reversibly inhibits the ASIC1a current without affecting the ASIC2a current (Lin et al., 2011). Tetracaine inhibits the ASIC1a and ASIC3 currents but not the ASIC2a current. Unlike amiloride, tetracaine inhibits both the transient and sustained ASIC3 currents (Leng et al., 2013). The inhibition of ASICs by lidocaine and tetracaine may indicate a novel mechanism underlying the analgesic effect of these local anesthetics. A summary of the pharmacological modulations of ASIC1a is presented in Table 1.

2.5. The role of ASIC1a in stroke

ASICs have been demonstrated to play critical roles in physiological processes, such as nociception (Deval et al., 2008; Duan et al., 2007; Ugawa et al., 2002), mechanosensation (Price et al., 2000), fear behavior (Coryell et al., 2007; Gregoire and Matricon, 2009; Wemmie et al., 2003), and synaptic plasticity (Wemmie et al., 2002), as well as in pathological conditions, including brain ischemia (Pignataro et al., 2011; Xiong et al., 2004), seizure (Ziemann et al., 2008), multiple sclerosis (Friese et al., 2007), and tumor cell migration (Kapoor et al., 2009; Li and Xiong, 2011). In this review, we discuss the recent evidence for the contribution of ASICs to ischemic brain injury.

Under normal physiological conditions, extracellular and intracellular pH are generally maintained at ~7.3 and ~7.0, respectively, through the Na^+/H^+ and Cl^-/HCO_3^- exchange systems (Chesler, 1990; Nedergaard et al., 1991). Local fluctuations in brain pH may occur during physiological processes, for example, during synaptic transmission induced by the release of acidic vesicles (Chesler and Kaila, 1992). However, dramatic reductions in brain pH are more commonly observed in pathological conditions, such as stroke (Rehncrona, 1985), seizure (Somjen, 1984; Wang and Sonnenschein, 1955), and brain trauma (Deval et al., 2010).

During a stroke, for example, the deprivation of oxygen switches glucose metabolism to glycolysis in the affected brain region, resulting in a buildup of lactic acid and subsequent acidosis. As a result of this metabolic change, the loss of energy makes the Na^+/H^+ and Cl^-

HCO₃⁻ exchanger systems unable to maintain the extracellular and intracellular H⁺ gradient, which further contributes to the acidosis. Ischemia typically drives the brain pH to decrease to below 6.5 under normoglycemic conditions. Hyperglycemia may further exacerbate the situation, causing the extracellular pH in the ischemic core region to drop to as low as 6.0 (Rehncrona, 1985). Extracellular acidosis in the brain activates inward currents in neurons, which are largely mediated by ASICs. Thus, the high responsiveness to decreases in pH and the demonstrated permeability to Ca²⁺ of ASIC1a makes it a critical sensor of acidosis during stroke (Benveniste and Dingledine, 2005). Indeed, a number of recent studies have demonstrated strong evidence that ASIC1a activation plays an important role in acidosis-mediated neuronal injury (Gao et al., 2005; Pignataro et al., 2011; Xiong et al., 2004).

In most CNS neurons, lowering the extracellular pH to the level commonly observed in an ischemic brain induces membrane depolarization and an increase in intracellular Ca²⁺ ([Ca²⁺]_i) (Varming, 1999; Xiong et al., 2004; Yermolaieva et al., 2004). Activation of ASICs as a result of acidosis or ischemia induces neuronal injury occurring either in the absence or presence of blockers of voltage-gated Ca²⁺ channels and glutamate receptors (Sherwood and Askwith, 2009; Xiong et al., 2004; Yermolaieva et al., 2004). This finding implies that acidosis can cause neuronal injury independent of the activation of voltage-gated Ca²⁺ channels or glutamate receptors. The acid-induced neuronal injury can be ameliorated by the inhibition of ASICs and by a reduction in the concentration of extracellular Ca²⁺, suggesting that Ca²⁺ permeable ASIC1a, and likely ASIC1a/2b channels (see below) play a vital role in ischemic neuronal injury. Intracellular Ca²⁺ is important for a number of physiological processes; however, excessive amounts of Ca²⁺ can abnormally activate a series of enzymes including phospholipases A2, nNOS, which result in production of excessive ROS and reactive nitrogen species (RNS) and causes subsequent neuronal injury (Emerit et al., 2004; Lipton and Rosenberg, 1994). Oxidative stress damages DNA, which triggers the activation of poly (ADP-ribose) polymerase-1 (PARP-1) and resultant release of apoptosis-inducing factor (AIF), leading to initiate the cell death (Erdelyi et al., 2005; Yu et al., 2006). Thus, oxidative stress might play an important role in neuronal injury/apoptosis downstream of ASIC1a over-activation and Ca²⁺ overload. In focal ischemia models, the pharmacological inhibition of the ASIC1a activity or disruption of the ASIC1a gene protects the brain from ischemic injury (Duan et al., 2011; Pandey et al., 2011; Pignataro et al., 2007; Xiong et al., 2004).

As anticipated, agents that potentiate ASIC1a activity, such as spermine, big dynorphin, and nitric oxide, have been shown to exacerbate acidosis-mediated neuronal injury and/or ischemic outcomes (Duan et al., 2011; Jetty et al., 2010; Sherwood and Askwith, 2009). It has also been demonstrated that increasing the ASIC1a surface expression, e.g., through an inhibition of ASIC1a internalization, exacerbates acidosis-induced neuronal injury (Zeng et al., 2013).

In a mouse model of focal ischemia or a newborn piglet cardiac arrest model of global ischemia, the activation of either ASICs or glutamate receptors both contributed to brain injury (Pignataro et al., 2007; Yang et al., 2011). Interestingly, a recent study in a rat cardiac model of global ischemia showed that the ASIC inhibitor amiloride, but not the NMDA receptor blocker memantine, reduced neurodegeneration (Tai and Truong, 2013). This result might suggest that, in some models of brain ischemia, ASICs play a more important role than NMDA receptors in the mediation of neuronal injury.

In addition to homomeric ASIC1a, heteromeric ASIC2b/1a has been shown to be Ca²⁺ permeable and to contribute to acidosis-induced neuronal injury (Sherwood et al., 2011). Although homomeric ASIC2b fails to form functional channels, heteromeric ASIC2b/1a can produce proton-gated currents (Sherwood et al., 2011). When extracellular sodium was

substituted by calcium, only a slight shift of the reversal potential was observed, implying that ASIC2b/1a heteromeric channels are calcium permeable (Sherwood et al., 2011). ASIC2b/1a heteromeric channels are indistinguishable from ASIC1a homomeric channels in activation and inactivation kinetics and in PcTx1 sensitivity (Sherwood et al., 2011). These findings suggest that, like homomeric ASIC1a channels, ASIC2b/1a heteromeric channels might contribute to acidosis-mediated neuronal injury.

The role of ASIC2b/1a in acidosis-induced neuronal injury is further supported by the use of barium, an inhibitor that has different potencies for the inhibition of heteromeric ASIC2b/1a, homomeric ASIC1a, and heteromeric ASIC2a/1a channels (Sherwood et al., 2011). At 10 mM, barium potently inhibits the ASIC2b/1a current with no effect on the ASIC1a current and shows only a slight inhibition on the ASIC2a/1a current (Sherwood et al., 2011). Barium reduces the acidosis-induced injury of cultured hippocampal neurons from wild-type but not ASIC2 knockout mice, indicating that barium-mediated neuroprotection is ASIC2 dependent. As only the ASIC2b subunit confers significant barium sensitivity, these results support the involvement of ASIC2b/1a channels in acid-induced neuronal injury.

2.6. Modulation of ASICs by ischemia-related signals

The typical ASIC1a response is characterized by a transient inward current. In most electrophysiological recordings *in vitro*, the ASIC1a current is desensitized within a few seconds (Waldmann et al., 1997b). In addition, pre-exposure of ASIC1a to a small decrease in pH that is insufficient to activate the channel inhibits the channel activation upon subsequent larger pH drops. These findings raise an important question as to how the activation of ASIC1a channels contributes significantly to ischemic brain injury. One explanation is that endogenous modulators exist and may prevent the desensitization of ASICs and/or potentiate their responses. In this regard, a number of endogenous molecules that can dramatically modulate the properties of ASICs have been identified recently (Chu et al., 2011; Xiong and Xu, 2012):

Lactate—Following ischemia, anaerobic metabolism of glucose leads to an increase in the production of lactic acid (Schurr, 2002; Schurr and Rigor, 1998). Although the specific mechanism for this was unclear, the concentration of lactate and its accompanying acidosis correlated well with the degree of brain injury (Siesjo et al., 1996). Immke & McCleskey first demonstrated, in sensory neurons that innervate the heart, that the addition of lactate at concentrations similar to that experienced during ischemia dramatically increased the amplitude of the ASIC current activated by a moderate pH drop in pH to ~7.0 (Immke and McCleskey, 2001). Addition of the same concentration of lactate at pH values that do not activate ASICs (e.g., pH 8.0 or pH 7.4) induced no response. Thus, lactate acts by potentiating, but not activating, the ASICs. In COS-7 expressing ASICs, both ASIC3 and ASIC1a currents were potentiated by lactate (Immke and McCleskey, 2001). Because lactate has the ability to chelate divalent cations, which are known to modulate ion channels (Hess et al., 1986; Xiong and MacDonald, 1999; Zhou and Jones, 1995), it was hypothesized that the potentiation of the ASICs was mediated by the lactate chelation of divalent cations. This hypothesis was supported by the finding that increasing the divalent concentrations of Ca^{2+} and Mg^{2+} eliminated the effect of lactate on the ASICs, whereas reducing the divalent concentrations mimicked the effect of lactate (Immke and McCleskey, 2001). In addition to cardiac sensory neurons, potentiation of the ASIC current by lactate has been reported in other types of neurons, such as cerebellar Purkinje cells (Allen and Attwell, 2002).

Arachidonic acid—Arachidonic acid (AA) is one of the most abundant fatty acids in the brain. Following ischemia, $[\text{Ca}^{2+}]_i$ accumulation activates phospholipase A2, leading to increased production of AA (Farooqui and Horrocks, 2006; Muralikrishna and Hatcher,

2006;Rehncrona et al., 1982). Allen and Attwell were the first to demonstrate that AA enhances the activity of ASICs (Allen and Attwell, 2002). Addition of 5 or 10 μM AA resulted in a large potentiation of the ASIC current in Purkinje cells from the rat cerebellum. In addition to the peak amplitude, AA enhanced or induced a sustained ASIC current (Allen and Attwell, 2002). One potential explanation for this phenomenon is that the insertion of AA into the cell membrane stretches the membrane, thus potentiating the ASICs. This explanation is supported by the finding that the treatment of neurons with a hypotonic solution, which causes cell swelling and membrane stretch, has a similar effect as AA (Allen and Attwell, 2002).

Spermine—Spermine is an endogenous polyamine. Following ischemia, the enhanced activity of ornithine decarboxylase, a rate-limiting enzyme in the synthesis of polyamines, results in an increase in the production of spermine (Kindy et al., 1994). Babini et al. first reported a potentiating effect of spermine on the activities of ASICs (Babini et al., 2002). Subsequent studies by Duan et al. showed that extracellular spermine exacerbates ischemic neuronal injury by sensitizing ASIC1a to acidosis (Duan et al., 2011). Indeed, blocking or deleting ASIC1a reduces the potentiating effect of spermine on ischemic neuronal injury both in neuronal cell culture and in a mouse model of focal ischemia. On the one hand spermine inhibits the desensitization of ASIC1a in the open state, and on the other hand it accelerates its recovery from desensitization in response to repeated stimulation by acid (Duan et al., 2011).

Proteases—During brain injury, blood-derived proteases, including thrombin, plasmin, and tissue plasminogen activator, can gain access to the interstitial spaces of the central nervous system due to a compromised blood-brain barrier (Gingrich and Traynelis, 2000; Vivien and Buisson, 2000). Poirot and colleagues were the first to show that the activity of ASIC1a can be modulated by serine proteases (Poirot et al., 2004). Exposure of CHO cells stably expressing ASIC1a to trypsin, proteinase K, or chymotrypsin shifted the pH dependence of activation and steady-state inactivation of the ASIC1a channels to more acidic pH values. As a consequence, protease exposure decreased ASIC1a activity when the currents were induced by a pH drop from 7.4. Interestingly, protease exposure increased the ASIC1a activity when the current was induced from a basal pH of 7.0, a condition more relevant to ischemia. In addition, protease treatment accelerated the recovery of ASIC1a from desensitization (Poirot et al., 2004). Since tPA is also a serine protease, whether it has a similar effect on ASIC1a warrants future investigations.

CaMKII— Ca^{2+} /calmodulin (CaM)-dependent protein kinase II (CaMKII) is the most abundant multi-functional kinase isoform found in the brain and is particularly enriched in neurons. Activation of glutamate receptors triggers an increase in intracellular Ca^{2+} , leading to autophosphorylation and the activation of CaMKII. Gao et al. demonstrated that a coupling between the NMDARs/CaMKII signaling pathway and the ASIC1a channel significantly contributed to ASIC1a mediated neuronal cell death (Gao et al., 2005). An increased phosphorylation of ASIC1a by CaMKII at Ser478 and Ser479 was observed in global brain ischemia in rats. This phosphorylation increases the sensitivity of ASIC1a to low pH, resulting in increased Ca^{2+} influx and resultant cell death. Suppression of the CaMKII phosphorylation of ASIC1a with KN-93 or the mutation of ASIC1a at Ser478 and Ser479 provided neuroprotection. Thus, phosphorylation of ASIC1a by CaMKII plays an important role in ischemic brain injury.

Nitric oxide (NO)—During ischemia, the accumulation of intracellular Ca^{2+} results in the activation of the neuronal isoform of nitric oxide synthase (nNOS), leading to an increased production of NO (Bolanos and Almeida, 1999; Schulz et al., 1997). NO can also be released

by activated microglia (Boje and Arora, 1992). Increased NO production by nNOS is known to increase neuronal injury (Boje and Arora, 1992). Although increased generation of the strong oxidant OONO^- contributes to neuronal injury, other mechanisms may also participate in this process. Cadiou and colleagues were the first to suggest that ASIC1 might be a target of NO. They demonstrated that the NO donor S-nitroso-N-acetylpenicillamine (SNAP) potentiates proton-gated currents in neurons and in CHO cells (Cadiou et al., 2007). Consistent with these findings, subsequent studies by Jett et al. demonstrated that acid-induced cell injury is potentiated by a NO donor (Jett et al., 2010). At a pH of 6.1, the death rate of Neuro2A cells expressing ASIC1 channels was significantly higher than in cells that did not express ASICs. Blocking ASICs with amiloride protected the cells from acid-injury. Sodium nitroprusside, another NO donor, potentiated the ASIC currents and increased the amount of acid-induced cell death.

Dynorphins—Dynorphins are endogenous opioid neuropeptides expressed abundantly in the CNS. Under pathophysiological conditions in which the levels of dynorphins are substantially increased, these peptides can cause excitotoxicity in neurons (Hauser et al., 1999). Sherwood and Askwith reported that high concentrations of dynorphin A and big dynorphin potentiate acid-activated currents in cortical neurons and in CHO cells expressing homomeric ASIC1a (Sherwood and Askwith, 2009). Furthermore, the potentiation of the ASIC1a activity was mediated by a limitation of the steady-state desensitization of the channel. As expected, the reduction in the steady-state desensitization of ASIC1a by dynorphins enhanced neuronal injury by prolonged acidosis.

2.7. Future perspective

ASIC1a may represent a promising therapeutic target for intervention after stroke (Figure 3). The physiological functions of ASIC1a channels are less important than those of the NMDA receptors. For example, the ASIC1a knockout, unlike NMDA receptor knockout, is not lethal to embryonic development (Wemmie et al., 2002). Thus, suppressing the activation of ASIC1a during stroke may have few side effects.

ASIC1a has been implicated in synaptic plasticity (Wemmie et al., 2002), complete inhibition of these channels may impair learning and memory; however, the role of ASIC1a in learning and memory remains to be confirmed. In contrast to the findings by Wemmie and colleagues (2002), a recent study by Wu and colleagues suggested that ASIC1a is not required for hippocampal LTP and spatial memory (Wu et al., 2013). From a therapeutic perspective, small molecule ASIC1a inhibitors may represent promising pharmacological agents for combating stroke, owing to their relatively high permeability across the blood brain barrier compared to large molecule peptide toxins. However, the currently available small molecule inhibitors of ASICs are poorly selective for ASIC1a channels. In addition to screening novel ASIC1a inhibitors, structure modifications of the known ASIC inhibitors may help to develop highly selective and potent small molecule ASIC1a inhibitors.

3. Transient receptor potential melastatin (TRPM) channels

3.1. Structure and Distribution

The transient receptor potential (TRP) forms a superfamily of non-selective cation-permeable channels that are widely expressed in mammalian tissues (Venkatachalam and Montell, 2007). As illustrated in Figure 4, all TRP channel subunits have six putative transmembrane spans (TM), with intracellular amino and carboxyl termini and a pore region formed by the loop between the TM5 and TM6 domains (Venkatachalam and Montell, 2007). Currently, there are seven subfamilies of TRP channels, including TRPC, TRPV, TRPM, TRPA, TRPN, TRPP, and TRPML (Venkatachalam and Montell, 2007). TRP

channels play vital roles in the perception of a wide range of physical and chemical stimuli and in the initiation of multiple fundamental cellular responses and functions (Venkatachalam and Montell, 2007). In this review, we focus on TRPM7, which is one of the eight members of the transient receptor potential melastatin (TRPM) subfamily and has been shown to play an important role in ischemic brain injury. TRPM7 is a channel, which, like its close relative TRPM6, has a kinase domain in its C-terminal region (Figure 4). This C-terminal kinase domain seems to contribute to the regulation of TRPM7 by intracellular Mg^{2+} (Demeuse et al., 2006; Schmitz et al., 2003). A complete crystal structure for TRPM7 has not been resolved; however, the crystal structure of a portion of the rat TRPM7 C-terminus has been reported and revealed a coiled-coil assembly domain that is critical for the formation of tetramers (Fujiwara and Minor, Jr., 2008). TRPM7 is ubiquitously expressed in mouse, while in human, a more concentrated expression is observed in the heart, pituitary gland, and bone (Fonfria et al., 2006).

3.2. Electrophysiology

TRPM7 channels have a single-channel conductance of ~40 pS and show outward rectification in whole-cell recordings with a reversal potential close to 0 mV (Runnels et al., 2001). As a non-selective cation channel, TRPM7 is highly permeable to divalent cations, with the following order of permeability: $Zn^{2+} \approx Ni^{2+} > Ba^{2+} > Co^{2+} > Mg^{2+} > Mn^{2+} > Sr^{2+} > Cd^{2+} > Ca^{2+}$ (Monteilh-Zoller et al., 2003) (Figure 4). The presence of divalent cations, however, decreases the permeability of TRPM7 to monovalent cations. On the other hand, deprivation of divalent cations induces a larger current with an increased permeability of TRPM7 to K^+ and Na^+ (Runnels et al., 2001). Under physiological conditions, the TRPM7 current is inhibited by intracellular Mg^{2+} /ATP (Kozak and Cahalan, 2003; Langeslag et al., 2007), suggesting that it plays an important role in cellular monitoring of Mg^{2+} homeostasis and metabolic status.

TRPM7 has several electrophysiological characteristics that make it a unique target for stroke intervention: (1) its current is potentiated by a decrease in extracellular divalent cations such as Ca^{2+} and Mg^{2+} (Aarts et al., 2003; Nadler et al., 2001), or (2) is potentiated by an increase in extracellular protons (Jiang et al., 2005). (3) It is highly permeable to divalent cations, including Ca^{2+} and Zn^{2+} (Clapham et al., 2001; Monteilh-Zoller et al., 2003), and (4) it is activated by oxidative stress (Aarts et al., 2003). During cerebral ischemia, excessive activation of voltage-gated calcium channels and NMDA receptors and an influx of Ca^{2+} into neurons produce a decrease in the extracellular Ca^{2+} level (MacDonald et al., 2006). The extracellular concentration of Mg^{2+} is similarly affected in the ischemic brain (Lin et al., 2004). This reduction in extracellular Ca^{2+} and Mg^{2+} causes a disinhibition of the TRPM7 channel, further enhancing the overload of intracellular Ca^{2+} . The increased production of oxidants and protons, which is a common feature of tissue ischemia, also activates or potentiates the action of TRPM7 channels. Furthermore, Ca^{2+} entry through TRPM7 may reinforce the production of reactive oxygen/nitrogen species, resulting in a further activation of TRPM7 and the development of a positive feedback loop that leads to neuronal injury (Aarts et al., 2003). As in the case of Ca^{2+} , the intracellular accumulation of Zn^{2+} is involved in neuronal injury after stroke (Chen et al., 2009; Frederickson et al., 2005b; Koh et al., 1996), and the correlation between Zn^{2+} accumulation and cell viability is striking (Frederickson et al., 2005a; Sensi et al., 2009; Shuttleworth and Weiss, 2011).

Extracellular acidosis is also a common feature of stroke and has been demonstrated to enhance the TRPM7 current in expressed in HEK-293 cells, with an approximate 10-fold increase in the TRPM7 current at pH 4.0 and a 1–2-fold increase at pH 6.0 (Jiang et al., 2005). The potentiation of TRPM7 by protons might contribute to the calcium overload and

subsequent neuronal injury during stroke. A summary of the properties of TRPM7 channels is presented in Table 2.

3.3. Pharmacology

There have been promising findings for the suppression of TRPM7 in both *in vitro* and *in vivo* models of cerebral ischemia; however, the lack of selective pharmacological tools that specifically inhibit TRPM7 channels creates obstacles for the investigation of the physiological and pathophysiological functions of TRPM7 channels. Some compounds, including 2-aminoethyl-diphenylborinate (2-APB), gadolinium (Gd^{3+}), Lanthanum (La^{3+}), SKF-96365, spermine, and carvacrol, can inhibit TRPM7; however, their selectivity is poor (Aarts et al., 2003; Kerschbaum et al., 2003; Li et al., 2006; Parnas et al., 2009). Waixenicin A, a naturally occurring compound from the soft coral *Sarcothelia edmondsoni*, has been shown to inhibit TRPM7 without significantly affecting other TRPM channels, such as TRPM2 or TRPM4 (Zierler et al., 2011). Thus, it would be interesting to evaluate the potential neuroprotective effect of Waixenicin A in cerebral ischemia, although the selectivity of this compound on other TRP channels remains to be elucidated. A summary of the pharmacological modulation of TRPM7 channels is presented in Table 2.

3.4. The role of TRPM7 in stroke

TRPM7 has been implicated in important biological processes, such as Mg^{2+} homeostasis (Schmitz et al., 2003; Venkatachalam and Montell, 2007) and neurotransmitter release (Krapivinsky et al., 2006), and in pathological conditions, such as cancer cell growth and proliferation (Jiang et al., 2007). Its role in ischemia-mediated neuronal injury has also been demonstrated (Aarts et al., 2003; Jiang et al., 2008; Lipski et al., 2006; Sun et al., 2009). For example, Aarts and colleagues first demonstrated that when cultured primary cortical neurons were challenged by a prolonged oxygen-glucose deprivation (OGD), they showed an increase in Ca^{2+} influx and neuronal cell death, even in the presence of the glutamate receptor inhibitors and voltage-gated Ca^{2+} channel blockers (Aarts et al., 2003). This glutamate-independent Ca^{2+} toxicity was inhibited by scavengers of reactive oxygen species (ROS) and by TRPM7 siRNA (Aarts et al., 2003), providing *in vitro* evidence that TRPM7 channels may be involved in ischemic brain injury. Recent studies by Sun and colleagues have provided solid *in vivo* evidence that TRPM7 knockdown is neuroprotective in a cardiac arrest model of brain ischemia (Sun et al., 2009). Cardiac arrest victims may suffer from transient brain ischemia resulting in delayed neuronal cell death, particularly in the hippocampal CA1 region. Sun et al. also demonstrated in adult rats that inhibiting the expression of TRPM7 in CA1 neurons by local injection of viral vectors carrying TRPM7 shRNA to the hippocampus made the neurons resistant to brain ischemia induced neuronal injury. Additionally, it protected against ischemia-induced LTP impairment and preserved the memory related performance including fear-associated and spatial-navigational memory tasks. These findings suggest that regional suppression of TRPM7 is a feasible option for preventing delayed neuronal death *in vivo*.

In addition to Ca^{2+} toxicity, the activation of TRPM7 channels has recently been shown to mediate Zn^{2+} toxicity in neurons (Inoue et al., 2010). For example, in cultured mouse cortical neurons, the addition of zinc at a concentration similar to that found in ischemic brain tissues (e.g., 30–100 μM) produced significant neuronal injury. This Zn^{2+} -mediated neurotoxicity was reduced by the non-specific TRPM7 channel blocker Gd^{3+} and by knockdown of the TRPM7 protein with siRNA. Zn^{2+} -mediated neuronal injury under OGD conditions was also diminished by TRPM7 knockdown (Inoue et al., 2010). In contrast, over-expression of TRPM7 in HEK-293 cells led to an increase in intracellular Zn^{2+} and subsequent Zn^{2+} -mediated cell injury (Inoue et al., 2010).

3.5. Future perspective

Like ASIC1a, TRPM7 provides a novel pathway for $[Ca^{2+}]_i$ overload (Figure 3). Both *in vitro* and *in vivo* studies support an important role for TRPM7 channels in ischemic brain injury. Thus, targeting these channels is a promising therapeutic strategy for treating stroke patients. Considering the ubiquitous expression of TRPM7, the inhibition of these channels may produce some side effects, which were observed with NMDA receptor antagonism. However, because localized knockdown of TRPM7 in adult rat hippocampal CA1 neurons had no clear effect on animal survival, neuronal and dendritic morphology, neuronal excitability, or synaptic plasticity (Sun et al., 2009), the suppression of TRPM7 may be feasible in the adult central nervous system. The CNS-targeted administration of a TRPM7 inhibitor may be beneficial for stroke treatment with a lower probability of causing severe side effects (compared with NMDA receptor antagonist). However, further investigations into the potential peripheral side effects of TRPM7 inhibitors are still needed. Future development of highly selective TRPM7 inhibitors would be beneficial for understanding the physiological and pathological functions of the TRPM7 channel and for therapeutic intervention in stroke patients.

4. The sodium/hydrogen exchangers

4.1. Structure and physiological functions

The sodium/hydrogen exchangers (NHEs) are a family of membrane proteins that mediate the secondary active electroneutral translocation of one Na^+ for one H^+ and play a crucial role in maintaining intracellular pH (pH_i) and cell volume homeostasis. Ten NHE isoforms (NHE1–NHE10) have been identified, of which NHE isoform 1 is the most abundantly expressed one in the central nervous system (Lee et al., 2008; Malo and Fliegel, 2006). NHE1 resides predominantly on the cell surface, but is also expressed in discrete microdomains of the plasma membrane in different cell types (Orlowski and Grinstein, 2004), such as the fibroblast lamellipodia (Grinstein et al., 1993), the epithelial basolateral membrane (Biemesderfer et al., 1992), and the cardiac myocyte intercalated disks and t-tubules (Petrecca et al., 1999). The distribution of other NHE isoforms is restricted to specific tissues with more specialized functions. For example, NHE-2 and NHE-3 are mainly expressed in the gastrointestinal tract and kidney, while NHE-5 is predominantly distributed in the brain (Zachos et al., 2005). NHE-6 and NHE-7 are expressed in intracellular organelles, such as the mitochondria and trans-Golgi, mainly in tissues with high metabolic rates, such as the brain, heart, and skeletal muscle (Brett et al., 2002; Numata and Orlowski, 2001). NHE-8 and NHE-9 are also located in the kidney, stomach, and intestine (Goyal et al., 2003; Nakamura et al., 2005), while NHE10 is restricted to osteoclasts (Lee et al., 2008). The main focus of the following review is on NHE1.

All characterized NHE isoforms are composed of approximately 600–900 amino acids with about 40% homology in amino acids. NHE1 has 815 amino acids, and both its N- and C-termini are in the cytosol. The N-terminal consists of 12 transmembrane (TM) domains, which are highly conserved among most NHEs, and is responsible for the exchange of Na^+ and H^+ (Figure 5). TM6 and TM7 have 95% amino acid homology and play a central role in cation translocation (Putney et al., 2002), while TM4 and TM9 are possibly associated with the sensitivity of NHE1 to inhibitors (Wang et al., 1995). TM11 is important for “pH sensing” and is also involved in the targeting of the NHE1 protein to the cell surface (Wakabayashi et al., 2000). On the other hand, the distal C-terminal tail of NHE1 is less conserved and contains the main regulatory sites for NHE1 activation, which could be modified by binding of regulatory factors in the subdomains or phosphorylation as described below (Figure 5).

4.2. Regulation of NHE1 activity

NHE1 is activated by various stimuli, including intracellular acidification, osmotic shrinkage, growth factors and hormones, hypoxia, and mechanical stress (Luo and Sun, 2007). Different hormones and growth factors may stimulate NHE1 via the activation of G protein-coupled receptors and receptor tyrosine kinases (Wakabayashi et al., 1992; Winkel et al., 1993). The activation of receptor tyrosine kinases regulates NHE1 activity through a common mitogen-activated protein kinase (MAPK) pathway, which activates the extracellular signal-related kinase (ERK)-p90 ribosomal S kinase (p90^{RSK}). For example, the C-terminal Ser 703 residue of NHE1 is phosphorylated by p90^{RSK} in response to cardiac ischemia/reperfusion (Moor et al., 2001), and the Thr 717, Ser 722, Ser 725, and Ser 728 residues can be directly phosphorylated by p38 MAPK activation (Khaled et al., 2001). NHE1 activity is also regulated by the G-proteins Gαq and Gα13 (Kitamura et al., 1995). The downstream mechanisms of NHE1 activation require the participation of PKC and GTPase RhoA, which are involved in the activation of ROCK and the direct phosphorylation of the C-terminal serine residues of NHE1 (Tominaga et al., 1998).

NHE1 has two fundamental functions: the regulation of pH_i and cell volume. First, NHE1 is the major mechanism to alkalinize pH_i in many cell types; together with the bicarbonate transporting systems (such as the Na⁺-HCO₃⁻ cotransporters, Cl⁻/HCO₃⁻ exchangers, and Na⁺ driven Cl⁻/HCO₃⁻ exchangers). NHE1 functions to counteract excessive intracellular acidification and maintain the acid-base balance in cytoplasm (Orlowski and Grinstein, 1997). These acid regulatory mechanisms have been examined in different neurons of the CNS (Chesler, 2003), of which the Na⁺/H⁺ exchange is the most prevalent mechanism in all neurons. It was reported that in various CNS cell cultures, NHE1 plays a dominant role in pH_i regulation, including cortical neurons (Luo et al., 2005), cortical and hippocampal astrocytes (Cengiz et al., 2013; Kintner et al., 2004), as well as primary microglia (Liu et al., 2010). NHE1 is highly sensitive to pH_i changes, and reduction of pH_i allosterically increases its protein activity (Aronson et al., 1982). Second, Na⁺/H⁺ exchange is a major contributor for Na⁺ influx, and functions in regulatory volume increase following hyperosmolality stress-induced cell shrinkage (Rotin and Grinstein, 1989). Due to its critical roles in regulating pH_i and cell volume, NHE1 is involved in several important physiological processes, including cell growth, differentiation, proliferation, migration, and cell death (Fliegel, 2005). In the CNS, NHE1 is expressed at a high level and important in the regulation of pH_i and cell volume in both neurons and glial cells (Douglas et al., 2001; Pedersen et al., 1998).

4.3. NHE inhibitors

There are two major classes of pharmacological reagents used to inhibit NHE1 activity. One class consists of amiloride and its analogues, including dimethylamiloride (DMA) and ethylisopropylamiloride (EIPA). The other class includes the benzoylguanidines and their derivatives, such as HOE 642 (cariporide), HOE 694 and EMD-85131 (eniporide) (Masereel et al., 2003). Amiloride shows different inhibitory efficacy among NHE isoforms. Both NHE1 and NHE-2 are sensitive to amiloride derivatives; however, other isoforms, such as NHE-3 and NHE-4, are insensitive. Moreover, amiloride inhibition is non-specific among NHE isoforms, while guanidine derivatives are more selective and efficient inhibitors of NHE1 (Masereel et al., 2003). Of all derivatives, cariporide is the most potent one, showing ~10⁴ to 10⁵ times more specificity for NHE1 than for NHE-3 and NHE-5 (Putney et al., 2002). A summary of the inhibitory potency of common NHE inhibitors on different NHE isoforms is displayed in Table 3 (Masereel et al., 2003).

4.4. NHE1 in ischemic brain damage

4.4.1. NHE1 in ischemic neuronal death—Neurons are sensitive to glutamate-induced toxicity and thus are susceptible to injury from ischemia, hypoxia, or metabolic dysregulation (Rothman and Olney, 1986). In addition, intracellular acidosis affects the gating of ion-channels and has an impact on neuronal excitability (Takahashi and Copenhagen, 1996). Therefore, efficient H^+ efflux mechanisms are important for normal neuronal function. NHE1 activity has been identified in almost all types of neurons and plays a crucial role for physiological functions of neurons. Primary cortical neuronal culture obtained from transgenic NHE1 knockout mice exhibited decreased steady-state pH_i as well as abolished H^+ extrusion when challenged with intracellular acidification (Luo et al., 2005). CA1 pyramidal neurons acutely dissociated from NHE1 null mouse brains displayed similar characteristics (Yao et al., 1999), suggesting the vital role of NHE1 in regulating neuronal pH_i . On the other hand, over-stimulation of NHE1 following cerebral ischemia could have deleterious effects. During hypoxia/ischemia, intracellular acidosis stimulates NHE1 activity, causing an increase in Na^+ influx. Elevated intracellular Na^+ ($[Na^+]_i$) then triggers an accumulation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) via the reversal of the Na^+/Ca^{2+} exchanger (NCX) and promotes the Ca^{2+} -mediated signaling cascade of deleterious events. Three hours of oxygen and glucose deprivation (OGD) followed by 1 hour of reoxygenation (REOX) caused a 7-fold elevation in $[Na^+]_i$ and a 1.5-fold elevation in $[Ca^{2+}]_i$ in primary mouse cortical neuronal culture (Luo et al., 2005). Neuronal death was increased to $68 \pm 10\%$ after 21 hours of REOX (Luo et al., 2005). Both pharmacological blockade of NHE1 with HOE 642 and genetic knockdown of NHE1 protein expression reduced OGD/REOX-induced neuronal death and attenuated the overload of intracellular Na^+ and Ca^{2+} (Luo et al., 2005). Inhibition of NHE1 has also been reported to be protective against ischemia/hypoxia-mediated injury in different tissue types, such as cardiomyocytes (An et al., 2001), endothelial cells and smooth muscle cells (Besse et al., 2006).

Inhibition of NHE1 was neuroprotective against glutamate-mediated toxicity. NHE1 inhibitor KR-33028 significantly reduced glutamate-induced release of LDH in cultured cortical neurons (Lee et al., 2009). KR-33028 had an anti-apoptotic effect and reduced TUNEL-positive staining as well as activity of caspase-3. Another non-specific NHE inhibitor, SM-20220, reduced neuronal death induced by glutamate and ameliorated the cell swelling in a dose-dependent manner (Matsumoto et al., 2004). SM-20220 also suppressed glutamate-induced elevation of $[Ca^{2+}]_i$.

Activation of NHE1 is largely due to the regulation of its activity by phosphorylation by several kinases. Among these regulators, the ERK signaling cascade is one of the most important ones in response to pathological insults (Chu et al., 2004a). After activation, ERK 1/2 phosphorylates several downstream targets, such as Elk-1 and $p90^{RSK}$ and plays a vital role in the activation of NHE1 in neurons following OGD/REOX (Luo et al., 2007). NHE1 activity was significantly elevated in cortical neurons following 2 hours of OGD and 1 hours of REOX. Ten min after REOX, elevated NHE1 phosphorylation was detected with a concurrent increase in $p90^{RSK}$ phosphorylation (Luo et al., 2007). In conclusion, NHE1 inhibition ameliorated glutamate or ischemia-induced neuronal death and cell swelling, through the reduction of both the Na^+ and Ca^{2+} influx. These studies suggest that overstimulation of the NHE system may exacerbate neuronal damage and edema after cerebral ischemia.

4.4.2. NHE1 and ischemic glial damage—Kintner et al. showed that NHE1 is the major pH_i regulator in cultured cortical astrocytes, and genetic knockdown of NHE1 protein in these cells attenuates the ionic regulation disruption and cell swelling following ischemic insults (Kintner et al., 2004). NHE1 null astrocytes had lower resting pH_i levels compared to

wild type astrocytes, and inhibition of NHE1 with HOE 642 significantly acidified NHE1^{+/+} astrocytes. In NHE1 null astrocytes, the pH recovery after acidification was impaired (Kintner et al., 2004).

Astrocytes are the major cell type that uptakes extracellular glutamate in the nervous system. NHE1 activity in astrocytes also functions to maintain an optimal pH_i during this process. The transmembrane gradient of H⁺ concentration is important for effective glutamate uptake, since the glutamate transporters EAAT1 and EAAT2 require cotransport of H⁺ and Na⁺ together with glutamate (Danbolt, 2001). Intracellular acidosis impairs glutamate uptake in astrocytes after oxidative stress, and NHE1-mediated H⁺ extrusion may contribute to maintain pH_i during this process (Daskalopoulos et al., 2001). On the other hand, overstimulation of NHE1 activity may cause astrocyte Na⁺ overload and cell swelling, thereby counteracting glutamate uptake. In cultured mouse cortical astrocytes, OGD/REOX significantly stimulates NHE1 activity, leading to accumulation of intracellular Na⁺ and Ca²⁺ (Kintner et al., 2004). In mouse hippocampal astrocytes, 5 h REOX caused intracellular Na⁺ to reach 35 mM and significantly impaired glutamate uptake (Cengiz et al., 2013). NHE1 inhibition or genetic knockdown significantly attenuated the [Na⁺]_i rise and cell swelling (Kintner et al., 2004), and facilitated glutamate uptake by astrocytes.

4.4.3. NHE1 and the blood brain barrier—The blood brain barrier (BBB) is a physical and metabolic barrier between the blood vessels and surrounding brain parenchyma. It plays a crucial role in the maintenance of CNS normal functions and homeostasis. Microvascular endothelial cells (EC) are the anatomical basis of the BBB, and tight junctions (TJs) are important players in maintaining BBB functions (Harhaj and Antonetti, 2004). Shortly after the onset of cerebral ischemia, neurovascular dysfunction is manifested by BBB disruption. EC are converted into a proinflammatory state, and an increased expression of matrix metalloproteinase (MMP) further facilitates neuroinflammation and BBB disruption (Zhang et al., 2012b). On the other hand, pericytes contract in response to the ischemic insult, causing a persistent constriction of the microvasculature (Yemisci et al., 2009). This constriction, coupled with a loss of β1-integrin expression in the EC and astrocytes, further contributes to an increased cerebrovascular permeability (Zhang et al., 2012a).

NHEs have been shown to regulate pH_i homeostasis in brain capillary EC, and EIPA treatment acidifies these cells (Sipos et al., 2005). Both NHE1 and NHE-2 expression are detected in EC in the BBB. Hypoxia, aglycemia, and arginine vasopressin (AVP), which are typical factors present during cerebral ischemia, significantly increase cerebral microvascular endothelial NHE activity (Lam et al., 2009), suggesting that NHE in EC may be stimulated during ischemia. The role of NHEs in ischemic BBB disruption and edema formation was validated in *in vivo* studies where the application of an NHE inhibitor, SM-20220, reduced edema formation and brain damage following rat middle cerebral artery occlusion (MCAO) (Suzuki et al., 2002). HOE 642 was also shown to reduce brain edema and Na⁺ uptake during the early hours in a rat permanent MCAO model (O'Donnell et al., 2013). In addition, NHE1 may play a role in the dysfunction of the BBB by affecting its permeability and tight junction function. Blocking NHE1 activity with sabiporide significantly alleviates ischemia/aglycemic hypoxia-induced leakage of Evans Blue dye, showing protection against BBB hyperpermeability. Sabiporide also reduced the ischemia-induced disruption of the tight junction proteins occludin and zonula (Park et al., 2010). Given the broad expression of NHE1 in different cell types of the neurovascular unit, the protective effects of NHE1 inhibition on BBB damage *in vivo* may result from its effects on EC or indirect effects from surrounding parenchymal cells. Future studies using EC-specific NHE1 knockout mice will be able to better elucidate these mechanisms.

In summary, preserving the structural and functional integrity of EC in the BBB is crucial to therapeutic approaches intended to ameliorate ischemic brain damage. The protective effects of blocking NHE1 in key components of the neurovascular system (i.e., BBB endothelium as well as astrocytes and neurons) demonstrate its potential as a novel therapeutic target for cerebral ischemic injury therapy.

4.4.4. NHE1 in *in vivo* ischemic brain damage—The role of NHE1 in ischemic neuronal injury has been investigated in different *in vivo* model systems, such as focal cerebral ischemia, global cerebral ischemia, and neonatal hypoxia-ischemia (HI). Activation of ERK1/2-p90^{RSK} pathways following *in vitro* ischemia results in the phosphorylation of NHE1 and an increase in its activity, which in turn leads to neuronal damage (Back et al., 2001). This response was later confirmed in *in vivo* models of ischemia (Manhas et al., 2010) in which ERK and p90^{RSK} were stimulated 3 min after 60 min of MCAO and contributed to NHE1 phosphorylation. In addition, both the ERK inhibitor U0126 and the p90^{RSK} inhibitor FMK were shown to reduce infarct volume following MCAO (Manhas et al., 2010; Namura et al., 2001), which may result, in part, from blocking the activity of NHE1. Direct inhibition of NHE1 with HOE 642 was also reported to reduce infarct volume when administered 5 min prior to a 2 hr MCAO treatment and 24 hr reperfusion (Luo et al., 2005). Genetic ablation of NHE1 decreased the release of mitochondrial cytochrome c and ischemia-induced cell apoptosis following a 30 min MCAO treatment and 24 hr reperfusion (Wang et al., 2008). The nuclear translocation of apoptosis-inducing factor (AIF), the number of TUNEL-positive cells, and caspase-3 activation, were significantly attenuated in NHE1^{+/-} mice (Wang et al., 2008). The non-selective NHE inhibitor, EIPA, significantly reduced the loss of CA1 pyramidal neurons following global cerebral ischemia in adult gerbils when administered prior to ischemia (Hwang et al., 2008).

The neuroprotective effects of HOE 642 have also been reported in an HI immature brain injury model. HI caused ionic homeostasis disruption, which leads to brain injury. Intracellular alkalosis following neonatal HI in infants has been reported to correlate with the severity of brain injury (Robertson et al., 2002). More severe brain injury at 2 weeks after birth and neurodevelopment deficit at 1 year of age were observed in infants with more alkaline brain pH_i (Ott et al., 2002). Excessive activation of NHE is considered to contribute to the persistent intracellular alkalosis. A non-selective NHE inhibitor, N-methyl-isobutyl-amiloride (MIA), protects against neonatal brain injury in a mouse HI model (Kendall et al., 2006). Another study by Cengiz et al. reports that inhibition of NHE1 with HOE 642 attenuates neurodegeneration induced by HI and preserves hippocampal structures in mouse. HOE 642 not only reduced acute brain injury after HI, but it also improved the long-term striatum-dependent motor learning and spatial learning in mice after 8 weeks of HI (Cengiz et al., 2011). In summary, NHE1 plays an important role in the disruption of ionic homeostasis and neuronal damage after neonatal HI.

NHE1 also plays an important role in the regulation of astrocyte function after *in vivo* ischemia. Reactive astrogliosis occurs following an ischemic insult, where surviving astrocytes adjacent to the injured brain tissue undergo hypertrophy, proliferation, and upregulation of glial fibrillary acidic protein (GFAP) (Sofroniew and Vinters, 2010). As early as 1–3 hr post-ischemia, hypertrophic astrocytes could be detected in non-infarct areas (Petito and Babiak, 1982). Eventually, a glial scar is formed by the meshwork of glial cytoplasmic processes around the area of necrosis. NHE1 is up-regulated in hippocampal astrocytes following global cerebral ischemia (Cengiz et al., 2011; Hwang et al., 2008). Astrocyte activation is attenuated in the hippocampal CA1 region of gerbils by the inhibition of NHE1 (Hwang et al., 2008). However, NHE1 inhibition with HOE 642 had no effects on astrogliosis following global or focal cerebral ischemia (Cengiz et al., 2011; Shi et al., 2011). Despite the absence of direct effects of NHE1 inhibition on astrogliosis after ischemia,

NHE1 activation in reactive astrocytes may alter their functions. This view is supported by a recent study by Cengiz et al. that there is increased NHE1 expression and activation in cultured reactive hippocampal astrocytes following OGD (Cengiz et al., 2013). Inhibition of NHE1 ameliorates astrocyte intracellular Na^+ and Ca^{2+} overload, and reduces release of gliotransmitter and pro-inflammatory cytokines (Cengiz et al., 2013). In addition, inhibition of NHE1 also reduced glutamate release from cultured hippocampal astrocytes and improved glutamate uptake (Cengiz et al., 2013). Thus, it is possible that the effect of NHE1 inhibition on *in vivo* astrogliosis may result from indirect actions through adjacent cell types. Additional studies with cell-type specific NHE1 knockout animal models will help reveal roles of NHE1 in altering functions of reactive astrocytes following *in vivo* ischemia.

4.4.5. NHE1 in microglial-mediated inflammation—Following focal cerebral ischemia, a robust inflammatory reaction is initiated that is characterized by influx of peripheral leukocytes into the cerebral parenchyma as well as the activation of resident microglia (Jin et al., 2010). Microglia are CNS resident macrophages and serve as neurological sensors. They are important mediators of inflammatory responses during cerebral ischemic injury (Liesz et al., 2009). How microglia are activated in response to ischemic insults is not completely understood, but signaling via CD14 and TLR4 may be important in this process (Beschoner et al., 2002; Lehnardt et al., 2003). Whether microglia play a damaging role in cerebral ischemia is not clear. Microglia/macrophages are highly plastic cells, and local environmental inputs can alter their phenotypes and properties (Mosser and Edwards, 2008; Stout and Suttles, 2004). In particular, stimulation with LPS or the proinflammatory cytokine interferon- γ (IFN- γ) induces “classically activated” M1 microglia/macrophages that typically release deleterious proinflammatory mediators and reactive oxygen species (Gordon, 2003; Saijo and Glass, 2011). Conversely, cytokines, such as interleukin-4 (IL-4) or IL-10, can skew activated macrophages toward an “alternatively activated” M2 phenotype that generates high levels of anti-inflammatory cytokines and contributes to the repair or remodeling of tissues (Ponomarev et al., 2007; Varin and Gordon, 2009). M2 phenotype is not a homogeneous population and consists of three different subsets, M2a, M2b, and M2c (David and Kroner, 2011), with particular polarizing signals and differing functional properties. The “alternatively activated” M2 microglia secrete neurotrophic factors that support neuronal survival and growth and clean up cell debris, thus contributing to tissue repairing (Liang et al., 2010).

“Classically activated” M1 microglia may exacerbate brain injury by initiating cytotoxic proinflammatory responses (Yenari et al., 2010; Yoshioka et al., 2010) by, for example, producing IL-1 β , TNF- α , ROS, and NO (Jin et al., 2010). Several studies have suggested that microglial activation could contribute to ischemic injury. A free radical scavenger, edaravone, reduced activation of microglia, decreased brain infarct volume and improved the neurological functions in ischemic mice (Zhang et al., 2005). Minocycline, an antibiotic of the tetracycline family, was reported to be neuroprotective following ischemia via inhibiting activation and proliferation of microglia (Yrjanheikki et al., 1998). However, simply ablating proliferating microglial cells exacerbates ischemic brain injury (Lalancette-Hebert et al., 2007). Whether microglia are beneficial or detrimental may depend on the stage and regions of the ischemic brain injury. A recent study by Hu et al. showed that the local microglia and newly recruited macrophages displayed the M2 phenotype at an early stage of ischemic stroke but were gradually turned into the M1 phenotype in peri-infarct tissues at a later stage (Hu et al., 2012). Therefore, investigation of the spatial and temporal specific microglial activation phenotype following cerebral ischemia is warranted for improving the understanding of the inflammatory response with ischemic brain injury and for developing new therapeutic targets for stroke.

NADPH oxidase (NOX) plays a crucial role in microglial activation and is involved in many microglial functions, including phagocytosis and the “respiratory burst”. NOX catalyzes the reduction of molecular oxygen to the superoxide anion using NADPH as an electron donor and is thus the major source of microglial ROS production (Bedard and Krause, 2007). The by-product H^+ accumulates inside microglia in this process, resulting in depolarization and cytoplasmic acidification (De Vito, 2006). Because NOX is sensitive to pH_i with an optimal pH_i of 7.2 (Henderson et al., 1988), its function may be impaired by intracellular acidosis. Therefore, NHE1 is involved in maintaining an optimal pH_i for NOX and sustaining the “respiratory burst” of microglia. In microglial cultures, NHE1 is found to be the major regulator of the baseline pH_i as well as the recovery pH_i after an intracellular acid load (Liu et al., 2010). Moreover, microglial activation *in vitro* in response to several proinflammatory stimuli, such as lipopolysaccharide (LPS), PMA, and OGD/REOX, stimulates NHE1 activity and strongly depends on NHE1-mediated H^+ homeostasis (Liu et al., 2010). Blockade of NHE1 activity with HOE 642 not only impairs pH_i regulation in microglia but also reduces production of ROS as well as the proinflammatory cytokines IL-6, IL-1 β , and TNF- α , which are induced by LPS or by OGD/REOX (Liu et al., 2010). Similar results were observed in *in vivo* ischemia where the activation of microglia following transient MCAO in mouse is significantly reduced with NHE1 inhibitors or genetic ablation (Shi et al., 2011). Blockade of NHE1 also reduced NOX activation and production of proinflammatory cytokines (Shi et al., 2011). Because activation of microglia lasts for days after stroke onset, these protective effects of NHE1 blockade provides a promising therapeutic target with a prolonged treatment window from the current treatment modality.

Another important component of microglial activation is migration to the injury or site of inflammation (Kreutzberg, 1996). Ischemic and traumatic brain tissues release ATP and ADP to extracellular space, which stimulate microglial migration (Honda et al., 2001). Other chemotactic factors, including morphine (Takayama and Ueda, 2005), epidermal growth factor (Nolte et al., 1997), cannabinoids (Walter et al., 2003), and bradykinin (Ifuku et al., 2007), are also released and stimulate the migration of microglia toward the injury sites. Microglia have been demonstrated to move along the chemokine gradients in *in vitro* and *in vivo* injury models for experimental autoimmune encephalitis, Alzheimer’s disease, and cerebral ischemia (Cartier et al., 2005). The expression and localization of NHE1 in distinct subdomains of the plasma membrane of different cell types suggests that it plays specialized roles in other cell functions, such as cell migration. NHE1 is reported to play a vital role in the remodeling of the cortical actin cytoskeleton and changes in cell shape of fibroblasts through its interactions with the cytoskeletal-associated proteins ezrin, radixin, and moesin (ERMs) at the C-terminal tail (Denker et al., 2000); thus, NHE1 regulates cell morphology, adhesion, and directed movement. In our recent study, NHE1 was shown to be expressed in primary mouse microglia, especially in the lamellipodia (Shi et al., 2013), which is the dynamic surface protrusion of microglia responsible for directed cell movement. Under stimulation by the chemoattractant bradykinin (BK) (Albert-Weissenberger et al., 2013), microglia developed more motile lamellipodia with more dynamic changes, which were abolished when NHE1 activity was inhibited by HOE 642. NHE1 activity was elevated upon BK stimulation, especially in the local lamellipodial area, causing an alkaline pH_i in the microglial lamellipodia (Shi et al., 2013). How this alkaline pH_i facilitates cell migration is unknown, but several studies have indicated that the important actin binding protein cofilin favors an alkaline pH_i to promote actin accumulation at the leading edge of a moving cell (Carrier et al., 1997; Frantz et al., 2008; Ghosh et al., 2004). In addition to pH_i regulation, NHE1 activity was also coupled with NCX_{rev} function in microglia, causing a significant rise in $[Ca^{2+}]_i$ which may be involved in the regulation of cell movement. Furthermore, the NHE1 protein structurally interacts with the cytoskeleton protein ezrin, which provide an anchoring point for actin elongation inside the cell (Shi et al., 2013). A summary of the mechanisms of NHE1 in microglial activation and migration is presented in Figure 6.

4.5. Future perspective

NHE1 has long been considered to be therapeutic targets for myocardial ischemia and reperfusion injury. The protective effects of NHE inhibitors in cardiac ischemia reperfusion injury have been demonstrated in numerous experimental studies (see review by Karmazyn et al.) (Karmazyn et al., 1999). The mechanisms underlying NHE inhibition-afforded protection are primarily through the attenuation of NHE1-mediated intracellular Na^+ overload and coupled activation of sarcolemmal $\text{Na}^+/\text{Ca}^{2+}$ exchange (Avkiran, 2001). In light of the new findings from the studies of ischemic brain injury, it can be concluded that NHE1-mediated ischemic damage present a common mechanism in all ischemic reperfusion diseases.

Given the significant neuroprotective effects of NHE1 inhibitors in animal models of cerebral ischemia, these inhibitors may serve as promising therapeutic drugs for stroke treatment. To date, no clinical trials on NHE1 inhibitors have been conducted for the treatment of ischemic stroke. The efficacy of NHE1 inhibitors for cardioprotection has been assessed. HOE 642 showed protective effects when administered at the time of reperfusion in patients with acute anterior myocardial infarction that were treated with percutaneous transluminal coronary angioplasty in a small (100 patients) multicenter, randomized, placebo-controlled clinical trial (Rupprecht et al., 2000). However, in two larger studies performed later, the ESCAMI study (Evaluation of the Safety and Cardioprotective effects of eniporide in Acute Myocardial Infarction, Phase 2, 1,389 patients) and the GUARDIAN study (GUARD During Ischemic Against Necrosis, Phase 2/Phase 3, 11,590 patients), the NHE1 inhibitors eniporide and HOE 642 failed to improve clinical outcome compared to placebo (Boyce et al., 2003; Theroux et al., 2000; Zeymer et al., 2001). In the EXPEDITION study (sodium-hydrogen Exchange inhibition to Prevent coronary Events in acute cardiac CONDITION, Phase 3, 5,761 patients), HOE 642 was shown to significantly reduce nonfatal myocardial infarction. However, increased mortality due to an increased incidence of cerebrovascular events of thromboembolic origin was observed in the patients receiving HOE 642 (Mentzer, Jr. et al., 2008). This result was speculated to result from an abrupt withdrawal of HOE 642 and the rapid NHE activation and hyperactivity of platelets (Mentzer, Jr. et al., 2008); blocking platelet NHE1 has been previously shown to inhibit platelet activation (Roskopf, 1999). A recent analysis suggests that the significant side-effects observed in the EXPEDITION trial may also have resulted from overdosing of HOE 642, which was continuously administered with nearly double the highest dose used in the GUARDIAN study (Karmazyn, 2013). Taken together, NHE1 remains as a promising target for the treatment of ischemia/reperfusion injury of the myocardium and brain tissues. Further studies to develop more selective NHE1 inhibitors and improve the clinical trial designs are warranted and will benefit future clinical trial studies.

5. Summary and conclusions

In summary, recent preclinical research findings have demonstrated that the H^+ -sensitive, non-NMDA-dependent ion channels and ion exchangers, such as the ASICs, TRPM7 channels, and NHE1, play an important role in brain injury following ischemic reperfusion therapy. These channels and exchangers are expressed in all cell types of the neurovascular units of the brain, and sustained stimulation of their activity contributes to ischemic reperfusion brain damage. Blocking these proteins is neuroprotective in multiple ischemia models and effective in a relatively long therapeutic time window. An improved understanding of the molecular and cellular pathways that regulate these proteins in cerebral ischemia will be helpful for developing more selective pharmacological agents and translating the current findings to future clinical trial studies for stroke treatment.

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Abbreviations

AA	arachidonic acid
ADF	actin depolymerizing factor
AIF	apoptosis-inducing factor
ASIC	acid-sensing ion channel
BBB	blood-brain barrier
BK	bradykinin
CAMKII	Ca ²⁺ /calmodulin (CaM)-dependent protein kinase II
CHP	calcineurin homologous protein
DEG/ENaC	degenerin/epithelial sodium channel
ERK	extracellular signal-regulated kinase
ERM	ezrin radixin moesin
GFAP	glial fibrillary acidic protein
HI	hypoxia-ischemia
IL	interleukin
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MCAO	middle cerebral artery occlusion
MMP	matrix metalloproteinase
NCX	Na ⁺ /Ca ²⁺ exchanger
NHE	sodium/hydrogen exchanger
NIK	Nck-interacting kinase
NMDA	N-methyl-D-aspartate
NOX	NADPH oxidase
OGD	oxygen and glucose deprivation

p90^{RSK}	p90 ribosomal S kinase
p160ROCK	p160 Rho-associated kinase
pH_i	intracellular pH
PIP2	phosphatidylinositol 4, 5-bisphosphate
REOX	reoxygenation
ROS	reactive oxygen species
rtPA	recombinant tissue plasminogen activator
TRPM	transient receptor potential melastatin

Highlights

Roles of H⁺-sensitive ion channels ASIC1a and TRPM7 in ischemic brain damage

Roles of H⁺-sensitive NHE1 in ischemic brain damage

Prospects of blocking H⁺-sensitive channels and NHE1 as potential stroke therapeutic targets

Figure 1. Pathophysiological changes in the neurovascular unit following cerebral ischemia and the participating non-NMDA proton-sensitive ion channels/exchangers

Cerebral ischemia deprives neurons of energy required to maintain ionic homeostasis. Homomeric acid-sensing ion channel 1a (ASIC1a), transient receptor potential melastatin 7 (TRPM7), and Na⁺/H⁺ exchanger isoform 1 (NHE1) are proton-sensitive channels/exchangers that are activated during this process and contribute to ischemic neuronal death. Glial cells that support and interact with neurons are also affected by the activity of exchangers. Endothelial cell channels/exchangers play an important role in BBB disruption and brain edema formation. Thus, these proton-sensitive channels/exchangers contribute to ischemic brain injury via various cell types and mechanisms, providing novel therapeutic targets for stroke intervention.

Figure 2. Structure and ionic permeability of ASIC channels

A. Phylogeny tree illustrates the subfamily members of ASIC channels and their distribution in the central nervous system (CNS) and peripheral nervous system (PNS).

B. Predicted structural topology of ASIC channel. ASIC channel has a large extracellular domain, two putative transmembrane spans and two short intracellular termini. Functional ASICs are trimers, which predominantly conduct Na⁺. ASIC1a is the only homomeric ASIC that is substantially permeable to Ca²⁺. ASIC2b and ASIC4 do not form functional proton-gated homomeric channels. For more details see review (Grunder and Chen, 2010).

Figure 3. Contributions of NMDAR, ASICs, and TRPM7 channels to intracellular Ca^{2+} overload and neuronal injury in cerebral ischemia

During cerebral ischemia, the loss of $\text{Na}^{+}\text{-K}^{+}\text{-ATPase}$ activity due to the shortage of ATP causes depolarization of neurons. Increased depolarization triggers uncontrolled release of the excitatory neurotransmitter glutamate. Excessive release of glutamate over-stimulates NMDA receptors, causing Ca^{2+} overload and neuronal injury. In addition, deprivation of oxygen makes anaerobic glycolysis become the primary source for ATP production. Anaerobic glycolysis causes the buildup of lactic acid, resulting in tissue acidosis. Extracellular acidosis activates ASIC1a, causing Ca^{2+} overload and neuronal injury. Following brain ischemia, increased generation of ROS, reduced intracellular $\text{Mg}^{2+}/\text{ATP}$ content, and reduction of extracellular divalent cations all facilitate the activation of TRPM7 channels, contributing to Ca^{2+} and Zn^{2+} entry and deteriorating the ischemia outcome. In addition to activating ASIC1a, extracellular acidosis may potentiate the activation of TRPM7 channels and subsequent $\text{Ca}^{2+}/\text{Zn}^{2+}$ toxicity.

Figure 4. Structure and ionic permeability of TRPM channels

A. Phylogeny tree illustrates the subfamily members of TRPM channels, based on reference (Nilius and Owsianik, 2011).

B. Predicted structural topology of TRPM7 channel. TRPM7 channel has six putative transmembrane spans (TM) and a cation-permeable pore region formed by the loop between TM5 and TM6. TRPM7 is permeable to Na^+ and divalent cations including Ca^{2+} and Mg^{2+} , and trace metal ions Zn^{2+} . TRPM7 is a channel with an atypical C-terminal α -kinase domain.

Figure 5. Structure and regulatory sites of the NHE1 protein

NHE1 is composed of 813–822 amino acids, with 12 transmembrane (TM) segments and cytoplasmic N- and C-terminal domains. The cytoplasmic domains that are important in regulation or protein-protein interaction, the positions of reentrant loops, and the membrane-associated segments are illustrated. The associated factors, phosphatidylinositol 4, 5-bisphosphate (PIP2), ERM protein family (ezrin, radixin and moesin), calmodulin (CaM), and calcineurin homologous protein (CHP) are also shown in their approximate known binding sites. “P” indicates the approximate site for phosphorylation of the cytosolic tail of the protein mediated by extracellular signal related-kinase (ERK1/2), p90 ribosomal S kinase (p90^{RSK}), Nck-interacting kinase (NIK), or p160 Rho-associated kinase (p160ROCK) (This figure was published in Luo and Sun, 2007. Copyright © 2013 Bentham Science Publishers).

Figure 6. The role of NHE1 in microglial activation and migration following cerebral ischemia
Following cerebral ischemia, NHE1 functions to maintain microglial pH_i homeostasis and to sustain NOX function and “respiratory burst” in microglia. In turn, activated microglia release proinflammatory substances and participate in ischemic neuroinflammation and cell death. On the other hand, NHE1 also regulates microglial migration. NHE1 interacts with ERM proteins and functions as an anchoring point for actin filaments, contributing to membrane protrusion and microglial movement. NHE1 activity also maintains an alkaline pH_i in lamellipodia, which facilitates the pH_i-sensitive actin binding proteins actin depolymerizing factor (ADF)/cofilin function during microglial movement. In addition, NHE1-mediated Na⁺ influx triggers the NCX_{rev} operation and [Ca²⁺]_i rise, which further facilitates ERM activation and actin accumulation. Taken together, NHE1 is an important regulator in microglial function and microglial-mediated inflammatory responses.

Table 1

Summary of ASIC1a properties and functions

Electrophysiology	Pharmacology	Physiology	Associated pathology
Activation $\text{pH}_{0.5 \text{ act}} \sim 6.2$ (Waldmann et al., 1997b) Na^+ , H^+ , and Ca^{2+} permeable (Waldmann et al., 1997b)	Inhibition: Zn^{2+} : $\text{IC}_{50} \sim 7 \text{ nM}$ (Chu et al., 2004b); Pb^{2+} : $\text{IC}_{50} \sim 3.7 \mu\text{M}$ (Wang et al., 2006); Ca^{2+} : $1\text{--}10 \text{ mM}$ (Zhang et al., 2006); (Wang et al., 2006); Ni^{2+} : $\text{IC}_{50} \sim 0.58 \text{ mM}$ (Staruschenko et al., 2007b) Amiloride: $\text{IC}_{50} \sim 10 \mu\text{M}$ (Waldmann et al., 1997b); Flurbiprofen/ibuprofen: $\text{IC}_{50} \sim 350 \mu\text{M}$ (Voilley et al., 2001); Diminazene: $\sim 39\%$ inhibition at 3 uM (Chen et al., 2010b); Lidocaine: $\sim 50\%$ inhibition at 10 mM (Lin et al., 2011); Tetracaine: 3 mM use-dependent inhibition (Leng et al., 2013); PcTx1 : $\text{IC}_{50} \sim 1 \text{ nM}$ (Escoubas et al., 2000); Mambalgins: $\text{IC}_{50} \sim 55 \text{ nM}$ (Diochot et al., 2012); Oxidizing reagent DTNB: 0.5 mM (Chu et al., 2006); Trypsin (Vukicevic et al., 2006); CaMKII (Gao et al., 2005) Potentiation: Ca^{2+} , Mg^{2+} : $\text{EC}_{50} \sim 2.0 \text{ mM}$ ($\text{pH} 7.4$); Ba^{2+} : $\text{EC}_{50} \sim 2.9 \text{ mM}$ (Babini et al., 2002); (Wang et al., 2006); Lactate: 15 mM (Immke and McCleskey, 2001); Arachidonic acid: $5 \mu\text{M}$ (Smith et al., 2007); Spermine $250 \mu\text{M}$ (Babini et al., 2002; Duan et al., 2011); Nitric Oxide (Cadiou et al., 2007); Reducing reagent DTT, glutathione and cysteine: 1 mM (Cho and Askwith, 2007; Chu et al., 2006); MitTx: $\text{EC}_{50} \sim 9.4 \text{ nM}$ (Bohlen et al., 2011); Dynorphins: $\text{EC}_{50} \sim 26\text{--}32 \text{ nM}$ (Sherwood and Askwith, 2009); FMRFamide: $\text{EC}_{50} (\text{sustained}) \sim 33 \mu\text{M}$ (Askwith et al., 2000)	Fear conditioning (Coryell et al., 2007; Wemmie et al., 2003; Ziemann et al., 2009); Synaptic Plasticity/ Learning and memory (Coryell et al., 2008; Wemmie et al., 2003; Wemmie et al., 2002; Wu et al., 2013; Zha et al., 2006); Visceral function (Page et al., 2004); Gastrointestinal mechanosensory function (Page et al., 2005); Retinal function (Ettaiche et al., 2006)	Seizure (Biagini et al., 2001; Ziemann et al., 2008); Pain (Diochot et al., 2012; Duan et al., 2007; Holland et al., 2012; Sluka et al., 2003; Walder et al., 2010); Depression (Coryell et al., 2009); Cerebral ischemia (Duan et al., 2011; Gao et al., 2005; Pignataro et al., 2011; Pignataro et al., 2007; Xiong et al., 2004; Yang et al., 2011); Multiple sclerosis (Friese et al., 2007); Glioma (Kapoor et al., 2009; Kapoor et al., 2011); Osteoclastogenesis (Li et al., 2013a); TBI (Yin et al., 2013); Irritable bowel syndrome (Matricon et al., 2013)

Table 2

Summary of TRPM7 properties and functions

Electrophysiology	Pharmacology	Physiology	Associated pathology
Activation Constitutive activation; Removal of external divalent (Nadler et al., 2001); Swelling and stretch (Numata et al., 2007b) $Zn^{2+}/Mg^{2+}/Ca^{2+}/Na^{+}$ permeable cation channel, outward rectification, 40~105 pS (Li et al., 2006;Runnels et al., 2001;Ryazanov, 2002)	Potentiation: Proton: EC ₅₀ pH 3.4–5.1 (Jiang et al., 2005); Mechano-stress (Numata et al., 2007a); 2-APB: >1 mM (Li et al., 2006) Inhibition: Gd ³⁺ : IC ₅₀ ~2.4 μM (Aarts et al., 2003); [Mg ²⁺]: 1–3 mM (Kozak and Cahalan, 2003;Nadler et al., 2001); La ³⁺ : 2 mM (Runnels et al., 2001;Zholos, 2010); Hydrolysis of PIP(2) (Runnels et al., 2002); Waixenicin A: IC ₅₀ ~16 nM-7 μM (Zierler et al., 2011); NS8593: IC ₅₀ ~1.6 μM (Chubanov et al., 2012); 2-APB: IC ₅₀ ~178 μM (Li et al., 2006); Spermine: 0.2–20 μM (Kerschbaum et al., 2003); Carvacrol: 500 μM (Parnas et al., 2009)	Cellular Mg ²⁺ homeostasis (Nadler et al., 2001;Ryazanova et al., 2010;Schmitz et al., 2003); Inhibit endothelial cell proliferation and function (Baldoli et al., 2013;Inoue and Xiong, 2009); Skeletogenesis (Elizondo et al., 2005); Cell volume regulation (Numata et al., 2007b); Neurotransmitter release (Brauchi et al., 2008;Krapivinsky et al., 2006); Ventricular function, conduction and repolarization (Sah et al., 2013); Cell proliferation/migration/ adhesion/differentiation/survival (Abed et al., 2011;Abed and Moreau, 2007;Abed and Moreau, 2009;Chen et al., 2014;Clark et al., 2006;Cui et al., 2013;Desai et al., 2012;Kuras et al., 2012;McNeill et al., 2007;Nadler et al., 2001;Sahni et al., 2010;Su et al., 2006;Su et al., 2011;Wykes et al., 2007;Zhang et al., 2012c); Embryonic development (Jin et al., 2008;Jin et al., 2012;Liu et al., 2011); Touch-evoked escape in zebrafish (Low et al., 2011); Fluid flow or shear stress (Gurney, 2006;Oancea et al., 2006)	Cerebral ischemia/anoxic neuronal death (Aarts et al., 2003;Sun et al., 2009); (Coombes et al., 2011;Jiang et al., 2008;Zhang et al., 2011) Zinc toxicity (Inoue et al., 2010); Cancer cell proliferation/migration/ invasion/metastasis/apoptosis (Chen et al., 2010a;Gao et al., 2011;Guilbert et al., 2009;Guilbert et al., 2013;Hanano et al., 2004;Jiang et al., 2007;Kim, 2013;Meng et al., 2013a;Middelbeek et al., 2012;Rybarczyk et al., 2012;Sun et al., 2013b;Visser et al., 2013;Yee et al., 2011;Zierler et al., 2011); Guamanian amyotrophic lateral sclerosis (ALS-G) and parkinsonism dementia (PD-G) (Hermosura et al., 2005); Hyperglycemia mediated injury of vascular endothelial cells (Sun et al., 2013a;Wuensch et al., 2010); Hypertension (Li et al., 2013b;Touyz et al., 2006;Yogi et al., 2011); Kidney injury in renal transplantation (Meng et al., 2013b;Meng et al., 2012); Fibrogenesis/fibrosis (Du et al., 2010;Sontia et al., 2008); Aromatase excess syndrome (Tiulpakov et al., 2005)

Table 3

Inhibitory potency of NHE inhibitors on different NHE isoforms

Drug	NHE1	NHE2	NHE3	NHE4	NHE5	NHE7
Amloride	<i>1-1.6*</i>	<i>1.0**</i>	<i>>100*</i>		<i>21</i>	
	5.3*		100-309*	813*		>2000
EIPA	<i>0.01-0.02**</i>	<i>0.08-0.5**</i>	<i>2.4*</i>		<i>0.42</i>	
	25.1*		3.3*	>10*	1.53	
DMA	<i>0.023*</i>	<i>0.25*</i>	<i>14*</i>			
HOE 694	<i>0.085*</i>		<i>640*</i>		<i>9.1</i>	
HOE 642	0.03-3.4	4.3-62	1->100			>30
Emiporide	0.005-0.38	2-17	100-460	>30		

Values are IC50 or *K_i* (in *Italic*) in μM.

* from rat.

** from rabbit. (This table was published in Masereel et al., 2003, Copyright © 2013 Elsevier Masson SAS. All rights reserved.)