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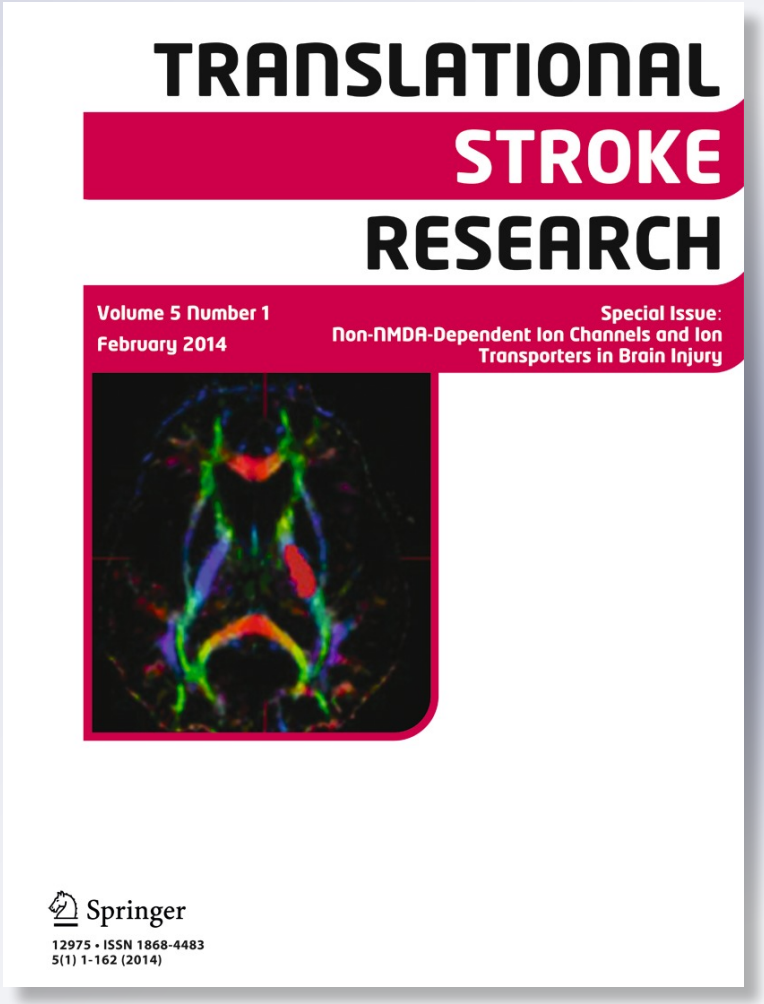

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ORIGINAL ARTICLE

Microglial Voltage-Gated Proton Channel Hv1 in Ischemic Stroke

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Abstract Microglia, resident immune cells in the brain, contribute both to the damage and resolution of ischemic stroke. However, the mechanisms of microglia's detrimental or beneficial role in the disease are poorly understood. The voltage-gated proton channel, Hv1, rapidly removes protons from depolarized cytoplasm, and is highly expressed in the immune system. In the brain, Hv1 is selectively and functionally expressed in microglia but not neurons. Although the physiological function of microglial Hv1 is still not clear, Hv1 is one of major ion channels expressed in resting microglia. Under pathological conditions, microglial Hv1 is required for NADPH oxidase (NOX)-dependent generation of reactive oxygen species (ROS) by providing charge compensation for exported electrons and relieving intracellular acidosis. In a mouse model of cerebral middle artery occlusion, Hv1 knockout mice are protected from ischemic damage, showing reduced NOX-dependent ROS production, microglial activation and neuronal cell death. Therefore, microglial Hv1 aids in NOX-dependent ROS generation, which subsequently induces neuronal cell death and a significant fraction of brain damage after ischemic stroke. These studies illuminate a critical role of microglial Hv1 in ischemic brain injury, providing a rationale for Hv1 as a potential therapeutic target for the treatment of ischemic stroke. The current understanding of Hv1 in ischemic injury through NOX-dependent ROS production may serve as a common model to reveal the deleterious role of microglia in neurological diseases other than ischemic stroke, such as multiple sclerosis, amyotrophic lateral sclerosis, Alzheimer's disease, and neuropathic pain.

Keywords Microglia · Voltage-gated proton channel Hv1 · NADPH oxidase · Reactive oxygen species · Sodium-proton exchanger · Acid-sensing ion channels · Ischemic stroke

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Introduction

Microglia are the principal resident immune response cells in the brain and play important roles in both physiological and pathological brain functions [1, 2]. During ischemic stroke, microglia are the primary initial responders, followed by the recruitment of the circulating immune cells of the inflammatory response [3]. However, the detrimental or beneficial role of microglia in ischemic stroke is still debated. Reactive molecules containing oxygen (reactive oxygen species [ROS]) are produced in ischemic brain and react with lipids, proteins, cofactors and DNA, in turn activating apoptotic pathways [4, 5]. Therefore, oxidative stress produced by damaged neurons and glia is one of the major mechanisms leading to cell death [6, 7]. Among several sources of oxidative stress in the brain, NADPH oxidase (NOX) is a membrane–bound enzyme that is abundantly expressed in phagocytic cells, including microglia [8]. NOX-mediated ROS production in microglia may have evolved as a defense against invading bacteria, but it nonselectively damages bystander cells such as neurons and glia in the brain [9, 10]. Indeed, NOX and ROS are consistently reported to participate in the pathogenesis of cerebral ischemic injury; NOX1, 2 or 4 knockout mice exhibit less brain injury after stroke [11–14]. Thus, reducing NOX-related oxidative stress could ameliorate neuronal damage in ischemic stroke.

During microglial activation in ischemic stroke, NOX generates superoxide by transferring electrons across the membrane, rapidly depolarizing and acidifying these cells. As excessive depolarization and intracellular acidification inhibits further expulsion of electrons, charge-compensating and acidosis-relieving mechanisms are needed to maintain NOX activity [15, 16].

The voltage-gated proton channel, Hv1, a recently cloned ion channel highly expressed in microglia, can rapidly remove protons from the depolarized cytoplasm [17–19]. By sensing both voltage and pH gradients, Hv1 is ideally suited to the task of charge compensation for NOX activation. Hv1 is activated

only upon depolarization, but the amount of depolarization needed is inversely proportional to the pH gradient. Thus, the larger the pH gradient, the less depolarization is needed to activate Hv1. This dual dependence on voltage and pH is a consequence of the interactions between water molecules in a central cleft of Hv1 and its voltage sensor [20], and is the unique feature of Hv1. This means that upon cell acidification, less depolarization from rest is required to allow protons to exit the cell, exactly the conditions under which NOX is active during ischemia.

Indeed, genetic deletion or inhibition of Hv1 greatly reduced NOX–dependent ROS production in leukocytes and bone marrow cells [21, 22]. In a mouse model of ischemic stroke, Hv1 knockout ($Hv1^{-/-}$) mice are protected from ischemic damage, showing reduced microglial activation and neuronal cell death [23]. The mechanisms that involve NOX and Hv1 in brain microglia act in concert to produce ROS via the channel's effectiveness in maintaining cytoplasmic charge balance after NOX-mediated export of electrons. Therefore, experimentally induced ischemia elicits ROS production from microglia that damages neurons and perhaps other brain cells. Hv1 is a crucial component of this response since elimination or inhibition of Hv1 reduces cell damage in cell culture, in brain slices, and in intact animals [23]. In this review, I set out to summarize the recent studies on microglial Hv1 in neuronal damage in ischemic brain injury. Based on these studies, it is proposed that Hv1 could serve as a potential therapeutic target for the treatment of stroke and oxidative stress–related brain disorders.

Microglia in Ischemic Stroke

Microglial activation and inflammation are hallmarks for brain injuries during ischemic stroke. However, the role of microglia in ischemic stroke is hotly debated, as both detrimental and beneficial effects of microglia in stroke brain injury are well documented [3, 24]. Literature has shown the microglia are "the bad guys" or "the good guys" in ischemic stroke using genetic ablation or pharmacological inhibition of microglia. For example, previous studies have highlighted deleterious effects of microglia on neurons in vitro and brain injury during stroke in vivo: (1) Microglia conditioned medium after experimental ischemia, oxygen-glucose deprivation (OGD), induces neuronal cell death in vitro [25]. Similarly, in microglia–neuron co-culture, microglia directly induced neuronal cell death under OGD conditions [23]. (2) Inhibition of microglial activation by minocycline reduces inflammation and protects against both global and focal cerebral ischemia with a wide therapeutic window [26, 27]. In contrast, experiments also showed that microglia are neuroprotective during schemic brain injuries: (1) Ablation of endogenous proliferating microglia increased neuronal cell death and ischemic injury 72 h after middle cerebral artery occlusion (MCAO) in adult mice [28]. Similarly, depletion of phagocytic microglia exacerbates brain damage 72 h after MCAO in neonatal mice [29]. (2) In addition, exogenous microglia injection in brain improved neuronal survival and behavioral function after MCAO in adult rats [30, 31].

Therefore, it is difficult to distill a simple single function for microglia in ischemic stroke. The consensus is that microglia may play different roles at different stages of stroke insult; the pro-inflammatory cytotoxic aspects of activated microglia might be important early in stroke while microglia's anti-inflammatory effects become more prominent later, during tissue repair. However, signals that trigger the phenotypic conversion of neurotoxic microglia to neurotrophic microglia remain to be elucidated. In spite of the controversy, it is clear that microglia play an important role during ischemic stroke and thus are in need of further study. What could be the mechanisms underlying neuroprotective or neurodegenerative functions of microglia in ischemic stroke? We know that microglia are activated in ischemic stroke and release a plethora of microglial factors. Some of these are neurotoxic, including ROS, nitric oxide (NO), glutamate, matrix metalloproteinases, and cytokines, like tumor necrosis factor alpha (TNFα), IL1β, IL6. Microglia also release a number of neurotrophic factors, which include brain-derived neurotrophic factor (BDNF), transforming growth factor beta (TGF-β), IL10, and IL12. These trophic factors released from microglia are critical in tissue repair/remodeling, phagocytosis of cell debris, and in maintaining neuronal integrity after an ischemic insult [3, 24]. In the current review, I will focus on the neurotoxic effects of microglial ROS in ischemic stroke.

Microglia-Derived ROS in Ischemic Brain Injury

ROS are one of the most studied neurotoxic factors that can be released by microglia under ischemic conditions. Various ROS include superoxide, hydrogen peroxide, peroxynitrite, hypochlorous acid, carbonyl radical, and hydroxyl radical. ROS target DNA, membrane lipids, phosphatases/kinases, transcription factors, and ion channels to exert cell toxicity [4]. There are three major sources of ROS production: (1) the mitochondrial electron transport chain, which "leaks" about 1–2% of its electrons; (2) unfolded proteins with disulfide generate ROS during the unfolded protein response; and (3) NOXs, including five NOXs (Nox1–5) and two dual oxidase NOX variants. NOX generates superoxide by transferring electrons across the membrane. NOXs are activated in response to stimulation of associated receptors, such as those for TNFα, platelet-derived growth factor, nerve growth factor, the antibody Fc chain, and complement [8].

OS production by microglia occurs primarily by NOX activation. The NOX complex includes six protein components: two membrane-bound components ($gp9^{phox}$ and $p22^{phox}$) and four cytosolic components ($p67^{phox}$, $p47^{phox}$, $p40^{phox}$, and Rac)

[8]. Free electrons exported by NOX form superoxide in phagocytic compartments and in the extracellular space; these reactive oxygens can be converted to hydrogen peroxide and then to hypochlorous acid [15]. Originally designed to kill invading microbes, NOX-derived ROS play a key role in host defense, which is manifested in chronic granulomatous disease, an inherited disease with most loss-of-function mutations in major NOX subunits [32]. Although effective in clearing infection, the NOX system can be harmful to the host system, inflicting damage to bystander cells, and thus is a key component in neurotoxicity and neuroinflammation. During ischemic conditions, microglial NOX-mediated ROS production is reported to induce cell death of neurons, astrocytes and endothelial cells [23, 33]. In addition, several groups have shown that NOX knockout mice (NOX1, NOX2 and NOX4) or inhibition of NOXs by apocynin have smaller infarcts than do wild-type mice after ischemic stroke [12–14, 34]. Considering the high expression of NOX in microglia, it is plausible that microglial NOX may play a crucial role in brain damage after ischemic stroke. NOXs are also expressed in cell types other than microglia in the brain. Therefore, mice with microglia-specific knockout of NOX are needed to address whether microglia directly account for the protective effect of NOX deletion or inhibition. It is important to note that ROS are not entirely detrimental, as emerging evidence has shown their beneficial effects in tissue repair and remodeling following ischemic injury [28]. In particular, ROS as cerebral vasodilators may be beneficial when they are generated at low levels in the vicinity of the cerebral vascular wall [3, 35]. Therefore, the function of microglial ROS may depend on the temporal and spatial generation of ROS in the ischemic brain.

Microglia Expresses Voltage-Gated Proton Channel Hv1

Voltage-gated proton currents were first described in snail neurons [36], but a gene encoding a protein with voltage-dependent, proton selective flux was only recently identified as Hv1 [17, 18]. The human voltage-gated proton channel, Hv1, contains 273 amino acids. The predicted structure of Hv1 channel protein has four transmembrane domains (S1–S4) without a typical S5–S6 pore domain; both the N and C termini are intracellular. Mutagenesis studies identified that three arginine residues in S4 are responsible for voltage gating, while two histidine residues are required for extracellular inhibition of Hv1 by Zn^{2+} [17]. Hv1 is the most selective ion channel known, showing no detectable permeability to other ions. The ion selectivity filter of Hv1 channel is believed to be comprised of aspartate 112 in S1 and arginine 211 in S4 [37, 38]. Hv1 is a dimer and the cytosolic domain of the channel is necessary and sufficient for dimerization. Interestingly, each subunit of the Hv1 dimer is functional and has a separate permeation pathway with its own pore and voltage sensor [39]. Recent studies showed that the opening of the two pathways in Hv1 channels is highly cooperative, which involves interactions between the two subunits of the Hv1 dimer to initiate the conformational change during activation [40].

Hv1 is highly expressed in the cells of immune system [18]. Not surprisingly, studies have shown that Hv1 is critical for immune function. For example, Hv1 is reported to regulate B cell receptor signaling and antibody production [41], neutrophil migration [42], T-cell homoeostasis [43], basophil histamine release [44], and eosinophil activation-induced cell death [45]. Interestingly, Hv1 also regulates acid secretion from airway epithelium [46] and human spermatozoa [47]. In the mammalian nervous system, whether Hv1 is functionally expressed in neurons and glia is largely unknown. The voltage-gated proton current has been characterized in cultured microglia [48–50], while its presence in situ (i.e., within the brain) is debated [51, 52]. Recent studies found that Hv1 is selectively expressed in microglia but not neurons [21, 23]. Consistently, the electrophysiological results directly show that Hv1 is the sole ion channel mediating the voltage-gated proton current in microglia but not in neurons or astrocytes in the mouse brain (Fig. 1a). The current is largely reduced by Zn^{2+}, the known inhibitor of Hv1, and is completely abolished in mice deficient in *Hv1*$^{-/-}$ microglia (Fig. 1a) [23].

Three points on the presence of Hv1 in the brain are worth noting. (1) Although snail neurons exhibit rapidly activating proton-selective currents [36], the gene encoding this invertebrate channel has not been identified. In contrast, proton currents have rarely been reported in mammalian neurons. An Hv1-like current was reported in cultured hippocampal neurons, but the current was neither blocked by Zn^{2+} [53] nor confirmed in another study using acute hippocampal slices [23]. Therefore, it is unlikely that Hv1 functions as a membrane voltage-gated proton channel in mouse hippocampal neurons. (2) Compared with neurons, microglia express fewer ion channels [54]. Under resting conditions, Hv1 is a major functional ion channel along with K^+ and Cl^- channels. The function of microglial Hv1 is not noticeably regulated by development after birth, as the Hv1-mediated proton current persisted in both neonatal and adult microglia [23], although an immunostaining study suggests the developmental upregulation of microglial Hv1 expression [21]. (3) Hv1 showed surprising species variations. For example, the voltage-gated proton current is much smaller in rat microglia compared with those in mouse microglia [23, 51]. Considering that mouse and rat Hv1 share 89% homology in protein sequence, it would be informative to understand the mechanisms underlying the functional difference between mouse and rat Hv1. In addition, it was also shown that human but not mouse sperm cells express functional Hv1 [47]. Thus, although a voltage-gated proton current was observed in cultured human microglia [23], the existence of functional Hv1 needs to be further confirmed in human brain tissue in situ.

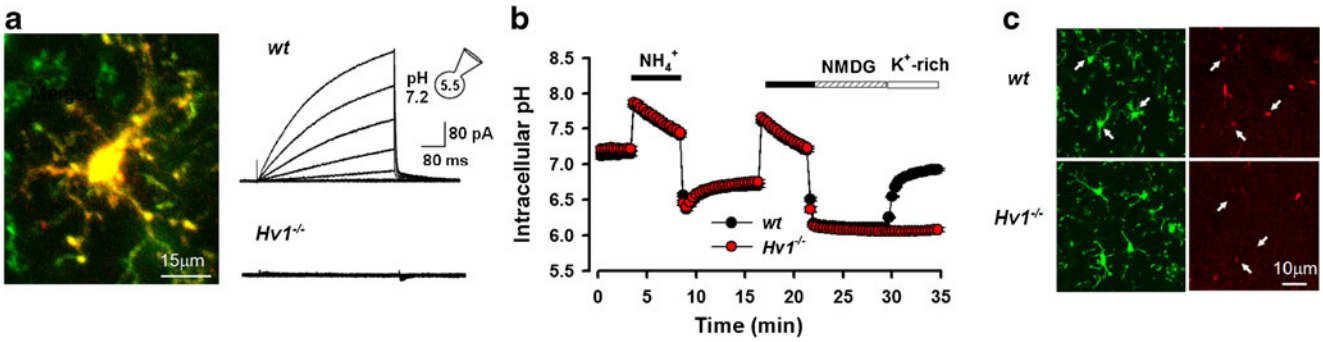

Fig. 1 Cellular function of microglial Hv1 in pH regulation and NOX-dependent ROS production. **a** Whole-cell recordings in brain microglia showing that Hv1 is the sole ion channel mediating voltage-gated proton currents. The recorded microglia (shown as a merged picture, *left*) was labeled with GFP (*green*) and loaded with Alexa Fluor 594 dye (*red*). The voltage-gated proton current was completely absent in $Hv1^{-/-}$ microglia (*right*). **b** Microglial Hv1 relieves intracellular acidosis during membrane depolarization. Intracellular pH (pH_i) is reduced in response to NH_4^+-induced acid load, but cannot recover in Na^+–free solution (Na^+ is replaced by NMDG). Membrane depolarization induced by K^+-rich solution caused rapid pH_i recovery in *wt* but not $Hv1^{-/-}$ microglia. **c** Microglial Hv1 is required for NOX-dependent ROS production. NOX is activated by PMA and ROS is detected by ethidium in the brain slices. Microglia are labeled with GFP in green and ROS production is labeled in red (shown by *arrows*). NOX-dependent ROS production is significantly reduced in $Hv1^{-/-}$ microglia compared with those in *wt* microglia

Microglial Hv1 in pH Regulation and ROS Production

What is the cellular function of Hv1 in brain microglia? The Hv1 channel is activated by membrane depolarization and mediates outward proton current. Therefore, Hv1 affects, and potentially regulates, intracellular pH. As distinct from other proton extrusion pathways, such as the sodium proton exchanger (NHE) and sodium bicarbonate co-transporter [55], the Hv1 channel is unique in that (1) proton extrusion through Hv1 requires not only intracellular acidosis but also membrane depolarization; (2) Hv1-mediated proton extrusion is much more efficient than exchangers or transporters. Due to the requirement of membrane depolarization, Hv1 is not usually required to maintain pH homeostasis under resting condition as wild-type and $Hv1^{-/-}$ microglia show similar basal intracellular pH (Fig. 1b) [23, 42]. Even when there is a dramatic drop in the intracellular pH (~6.5), Hv1 channels are not activated to correct the acidosis. However, when there is membrane depolarization (such as induced by increasing the extracellular K^+ concentration), Hv1 could promptly relieve the acidosis (Fig. 1b) [23]. Therefore, without membrane depolarization, it seems that proton transfer mechanisms other than Hv1 channel, particularly the NHE1, are playing a critical role in pH homeostasis [23, 56]. Together, recent studies indicate that Hv1 is able to function as an efficient pH regulator, but might do so only under conditions of strong membrane depolarization (Fig. 1b). This raises three points: (1) since all the studies were performed in cultured microglia and intracellular acidosis was artificially induced, the hypothesis needs to be confirmed under more physiological conditions from microglia in situ or in vivo; (2) as Hv1 is not ubiquitously expressed, its function in pH homeostasis is assumed to be limited and unique. Obviously, Hv1 is more efficient than NHE1 when both are activated [23, 56]; (3) the requirement of Hv1 activation is quite stringent, requiring both intracellular acidosis and membrane depolarization.

Are there any physiological/pathological conditions that are able to activate Hv1? Interestingly, there is indeed one known condition, NOX activity during respiratory burst, which is associated with both intracellular acidosis and membrane depolarization (Fig. 2b) [15]: (1) NOX activation induces electron transfer across the membrane, which depolarizes the membrane. The best estimate of the membrane depolarization during the respiratory burst was +58 mV in neutrophils [57]. The depolarization could even reach 190 mV within 20 ms if there is no charge compensatory mechanism [16]; (2) during NOX activation, protons are left behind as electrons are transported across the cell membrane. Indeed, NOX activity induces a sharp decrease in cytoplasmic pH in human neutrophils during phagocytosis [58]. Hexose monophosphate shunt activity is believed to be a source of electrons and protons for the respiratory burst [59]. Together, NOX activation associated with membrane depolarization and intracellular acidosis seem sufficient to activate Hv1. On the other hand, NOX is inhibited by membrane depolarization and by intracellular acidosis [15]. Therefore, to maintain NOX activity, particularly under constant activation such as respiratory oxidation, there must be mechanisms to compensate for the charge transfer and to relieve intracellular acidosis. Obviously, Hv1 is ideally suited for this function in cooperation with NOX. Consistent with this idea, accumulated evidence indicates that Hv1 is coupled to NOX-dependent pH regulation, membrane depolarization, or ROS production in a variety of cells, including neutrophils [42], B cells [41], and eosinophils [45]. In microglia, NOX and Hv1 are cooperatively activated; the $Hv1^{-/-}$ microglia show intracellular acidosis when NOX is activated by PMA and other proton extrusion pathways are inhibited. More importantly, Hv1 is required for NOX-dependent ROS production (Fig. 1c) [23].

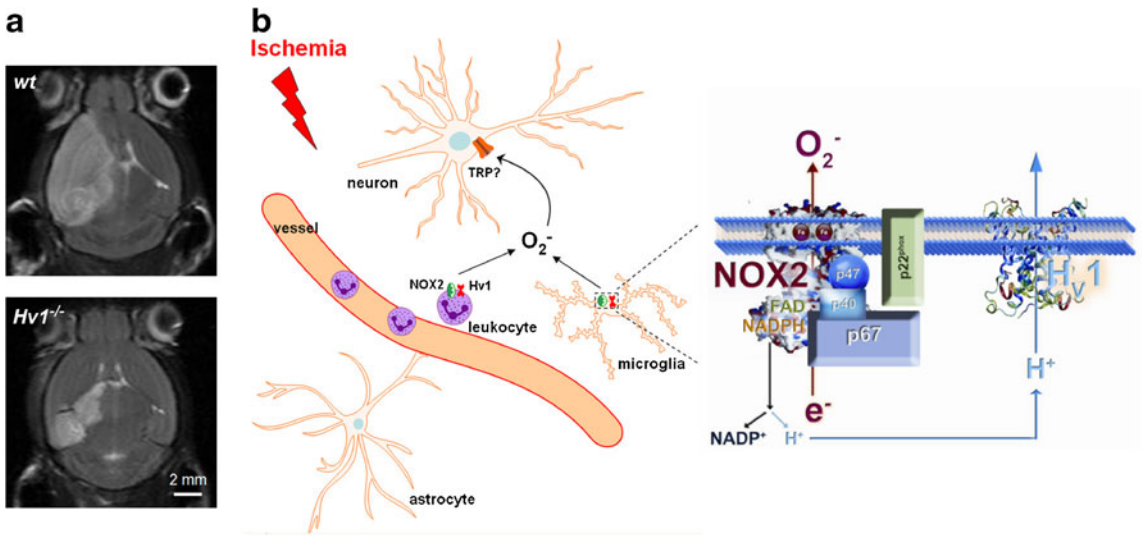

Fig. 2 Microglial Hv1 contributes to ischemic brain damage. **a** Smaller infarct volume in *Hv1*$^{-/-}$ mice 24 h after MCAO compared with that in *wt* mice. Transverse MRI brain images from *wt* and *Hv1*$^{-/-}$ mice after MCAO are shown. **b** Diagram showing Hv1 function in microglial ROS production and neuronal damage after ischemic stroke. Microglia are the first responders during ischemia, preceding leukocyte infiltration. Activated microglia produce ROS through activation of NOX, primarily NOX2. Hv1 is required for full activation of NOX2, by serving a charge compensation function, as well as by removing intracellular H^+. In the mouse brain, Hv1 is highly expressed in microglia but not neurons or astrocytes. Microglial Hv1-related ROS production partially contributes to neuronal cell death in early stages of ischemic stroke

It is evident that Hv1 can provide compensating charge or relive intracellular acidosis for NOX [16, 60, 61]. In the absence of Hv1, however, do other ion channels/transporters also perform this function? Certainly there is a wealth of ion channels that could balance the charge; voltage-gated K^+ channels, Ca^{2+}-activated K^+ channels, and Cl^- channels have all been proposed to act in this regard [62–64]. However, Hv1 has a distinct advantage over other channels for this function, as the operation of other channels would result in osmotic changes with swelling (K^+ influx) or shrinking (Cl^- efflux) while proton current can balance electron flux without net osmotic change [15]. Many studies also suggest that NHE1 functions as a pH regulator during NOX activation in neutrophils and in cultured microglia [56, 58, 65]. However, it is still unclear the respective contributions of Hv1 and other ion channels/transporters (K^+ channels, Cl^- channels, and NHE1) to coupling with NOX function. Nonetheless, although Hv1 is ideally working with NOX, it seems alternative mechanisms exist, which are reasonable given the critical role of the NOX system in immune function.

Microglial Hv1 in Brain Damage in a Mouse Model of Ischemic Stroke

At the cellular level, Hv1 is able to regulate intracellular pH and is coupled with NOX to produce ROS. Then what would be the function of Hv1 in vivo? To answer the question, *Hv1*$^{-/-}$ mice were generated by both the Clapham and Okumura laboratories recently [21, 22]. Most studies focused on Hv1 function in the immune system using the knockout mice. Unexpectedly, *Hv1*$^{-/-}$ mice were able to clear several bacterial infections in vivo including *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Burkholderia cepacia*, despite a significant reduction in superoxide production [22]. The lack of a bacterial infection phenotype in the *Hv1*$^{-/-}$ mice may be due to redundant mechanisms for bacterial clearance. For example, both superoxide- and NO-dependent systems are shown to function synergistically for bacterial clearance in vivo [66]. However, *Hv1*$^{-/-}$ mice showed defective B cell responses and impaired antibody production [41]. The mechanism involves the regulation of B cell receptor-mediated ROS production and its downstream signaling, Syk, Akt and SHP-1 by Hv1 activation. A recent study also found that *Hv1*$^{-/-}$ mice have autoimmune disorder phenotypes, such as splenomegaly, autoantibodies and nephritis [43]. The phenotype might be due to the impaired T-cell homoeostasis, although T cells express very low density of functional Hv1 [67]. Since Hv1 is expressed in almost all cell types in the immune system, future studies employing cell-specific Hv1 deletion are required to dissect the role of Hv1 in immune function.

In the brain, microglial cells are the only resident cell type that highly expresses Hv1. Therefore, it is an advantage to use general *Hv1*$^{-/-}$ mice to study microglial Hv1 function in the brain. Given that Hv1 is one of major ion channels in microglia, it is easy to step back and examine the role of microglia in the brain if we want to understand Hv1 function. Traditionally, microglia were largely implicated in pathological conditions, including ischemic stroke, Alzheimer's disease, Parkinson's disease, human immunodeficiency virus (HIV)-associated

dementia, neuropathic pain, multiple sclerosis, amyotrophic lateral sclerosis, Huntington's disease, and Nasu-Hakola disease [7, 68]. However, recent studies also suggest an interesting role for microglia in nervous system development and maintenance [69, 70]. In theory, microglial Hv1 could be involved in any of these pathological and physiological functions of microglia in vivo. Currently, only microglial Hv1 function in the ischemic stroke has been reported (Fig. 2a) [23], although studies on microglial Hv1 in a mouse model of Alzheimer's disease as well as neuropathic pain are ongoing.

The rationale to study microglial Hv1 in ischemic stroke comes from the role of NOX the in disease [3, 34, 71]. As discussed above, earlier studies have shown that NOX1, 2, 4 knockout mice showed less ischemic brain damage [11–14]. As NOX2 is highly expressed in microglia and Hv1 is coupled to NOX2 activation, it is possible that $Hv1^{-/-}$ mice may be protected in this disease. Indeed, $Hv1^{-/-}$ mice exhibited small infarct volume and better neurological behavior associated with brain injury, compared with those in wild-type mice after MCAO, a commonly used mouse model of ischemic stroke [23]. The protective effects were observed by TTC staining in brain slices as well as in vivo MRI and PET imaging (Fig. 2a). The protection was long lasting, even observed 7 days after MCAO. Also, the reduced brain injury in $Hv1^{-/-}$ mice was confirmed in MCAO, both with permanent occlusion and transient occlusion followed by reperfusion. Further study found decreased neuronal cell death in the penumbra of the ischemic brain from $Hv1^{-/-}$ mice [23]. Together, these results convincingly indicate that $Hv1^{-/-}$ mice are protected in ischemia and show decreased neuronal damage, suggesting a critical role of microglial Hv1 in enhancing neuronal cell death and ischemic brain injury.

Microglial Hv1 in Neuronal Injury in Ischemic Stroke

The next question is how microglial Hv1 activation leads to neuronal cell death and ischemic injury? One major mechanism for ischemia-induced brain damage is NOX activation and ROS generation [3, 71]. Microglia become activated and NOX cytosolic subunits translocate to the membrane to be functional in response to ischemia. Therefore, it is conceivable that microglial Hv1 contributes to ischemic brain damage via the NOX pathway. Indeed, the idea is supported by several lines of evidence [23]. First, NOX-dependent ROS production after experimental ischemia, OGD, was significantly reduced in $Hv1^{-/-}$ microglia compared with wild-type microglia. However, release of NO, glutamate, and cytokines such as TNFα, IL1β, and IL6 is comparable between wild-type and $Hv1^{-/-}$ microglia after OGD. Second, ROS production in brain microglia in situ after stroke was reduced in $Hv1^{-/-}$ mice. Third, reduced neuronal death and brain damage were observed in $Hv1^{-/-}$ mice compared to wild-type mice after stroke. Fourth, OGD-induced neuronal death in microglia ($Hv1^{-/-}$)-neuron co-cultures was less than in wild-type co-cultures. Fifth, both Hv1 and NOX2 are upregulated after MCAO. Finally, ROS scavenger rescue from ischemic damage was less prominent in $Hv1^{-/-}$ mice. Taken together, these results suggest that Hv1 plays a critical role in microglia-derived ROS generation that accounts for a significant amount of brain injury that occurs after experimental stroke in mice [23]. The redox-sensitive targets for ROS in neuronal cell death include lipids, membrane receptors, intracellular kinases and phosphatases, as well as pro-apoptotic transcription factors [4]. Since $Hv1^{-/-}$ mice have normal synaptic transmission, plasticity, and unaltered NMDA receptor function, glutamate neurotoxicity is unlikely to underlie the mechanism for Hv1's contribution to brain damage after stroke [23]. TRPM2 and TRPM7 channels are particularly interesting ROS targets as both ion channels are implicated in stroke-related neuronal cell death [72–74].

Thus, the function of Hv1 is coupled to NOX activation, ROS production, and subsequently neuronal cell death in the ischemic brain [23]. Hv1 proton channel plays two critical roles in cooperating with NOX: compensating electron charge and relieving intracellular acidosis (Fig. 2b). Although Hv1 is ideally suited for this function, an alternative mechanism might be employed in the absence of Hv1. For example, NHE1 is shown to be able to correct NOX-induced intracellular acidosis and thus also is involved in NOX-dependent ROS production [56]. In addition, it is reported that inhibition or partial genetic deletion of NHE1 also protected the brain from ischemic damage, likely via the NOX pathway [75]. During ischemia, cells rapidly acidify; in simple summary, reduced delivery of oxygen to mitochondria impairs ATP production, triggering anaerobic glycolysis that generates H^+. Therefore, a mechanism that reduces cell acidification, such as the Hv1 or NHE1 function, should relieve ischemic damage. However, $Hv1^{-/-}$ mice or $NHE1^{+/-}$ mice are protected from ischemic damage [23, 75]. These results suggest that Hv1 or NHE1's detrimental function in coupling with NOX surpasses their beneficial role in relieving intracellular acidosis. As for Hv1, it could rapidly correct cell acidification, but this requires that the cells depolarize and possess sufficient numbers of Hv1 channels. Microglia and neurons differ greatly in both respects. Neurons frequently depolarize and can acidify, but lack Hv1 channels. Thus, Hv1's deleterious contribution to ischemic damage is not via neurons themselves. Hence, activation of microglial Hv1/NOX may only increase the stress on neurons, without providing benefit in the absence of infection.

Another interesting point is that the activation of microglial Hv1 channels is able to release protons into the extracellular space. This could be a route for activation of neuronal acid-sensing ion channel (ASICs) as: (1) ASICs are proton-gated ion channels that are abundantly expressed in central neurons

and are highly sensitive to extracellular acidosis [76, 77]. (2) Under ischemic conditions, there is intracellular acidification due to anaerobic glycolysis. If Hv1 is activated, a significant amount of protons can be released. (3) The protons released from Hv1 are able to acidify the small extracellular space if the proximity between microglia Hv1 and neuronal ASICs are close. Coincidently, both ASIC1a knockout mice and *Hv1*$^{-/-}$ mice show a reduced brain damage phenotype after ischemic stroke [23, 78]. These results indirectly suggested a possible link between microglial Hv1 and neuronal ASICs. The idea that protons released from microglial Hv1 causes neuronal cell death via activation of neuronal ASICs is provocative. Several caveats need to be addressed. (1) Morphological evidence of the close proximity between microglial Hv1 and neuronal ASIC1a should be provided using electron microscopy. (2) The extracellular acidosis after microglial Hv1 activation should be tested using pH-sensitive dyes or fluorescent proteins. Electrophysiology would be able to directly measure ASIC activation by protons released from microglial Hv1. (3) Considering the limited number of microglia in the brain, it is still questionable whether protons released from microglial Hv1 are the major source for activation of neuronal ASICs in ischemic brain.

Hv1 in Other Cell Types Contributes to Stroke Damage

All cell types in the brain possess NOXs, and mice deficient in NOX1, NOX2 or NOX4 have better stroke outcomes [11–14]. Astrocytes comprise a significant proportion of cells in the brain, perhaps as high as 50% in rodents. Astrocytes express NOX 1, 2 and 3 [79] and toxic stimuli, such as β-amyloid, could activate astrocytic NOX to evoke neuronal death [80]. However, questions remain to be answered about Hv1's potential role in astrocytes. First, it is not known whether ischemic stroke activates astrocytic NOX. Second, the expression of Hv1 in astrocytes has not been confirmed. Considering the low expression of Hv1 in whole brain lysates, it seems unlikely that Hv1 is expressed in astrocytes [23]. Moreover, no measurable Hv1 current was observed in astrocytes in brain slices. Even if there is functional Hv1 in astrocytes, whether Hv1 or other channels may be required for charge compensation for NOX activation in astrocytes must be tested. In contrast, astrocytes may have protective roles in ischemia by reducing neuronal oxidant stress. For example, astrocytes support neuronal glutathione metabolism, release ascorbate, and take up its oxidized form, dehydroascorbate [81].

Circulating phagocytic cells such as neutrophils and macrophages express functional Hv1 and may contribute to ROS generation. Large numbers of circulating blood cells enter the brain tissue at late stages after stroke in mice or humans (~2 days after MCAO) [82–85]. However, the time course and the degree of the recruitment of inflammatory cells may vary depending on different animal stroke models (e.g., permanent or transient MCAO) [86]. Bone marrow transplantation experiments suggest that microglial Hv1, but not leukocyte Hv1, contribute to ischemic brain damage, at least at early time points after MCAO in mice [23]. Interestingly, NOX inhibition has been reported to protect against cerebral damage by alleviating neutrophil infiltration [34]. Migration in response to a bacterial peptide is impaired in *Hv1*$^{-/-}$ neutrophils [42], but *Hv1*$^{-/-}$ microglia have normal ATP-induced chemotaxis and MCP-1- or TNFα-induced migration [23]. Nevertheless, it is unlikely that microglia are the only sources of ROS-mediated ischemic brain damage since ROS are released from all metabolically stressed mitochondria.

Hv1 as Therapeutic Target for Treatment of Ischemic Stroke

Despite tremendous effort in the search for effective therapies, limited treatments for ischemic stroke are available [6]. Given the acceptance of NOX-mediated ROS production as a mechanism for neurotoxicity, its inhibition would likely benefit patients. However, recent studies using NOX inhibitors in experimental stroke show conflicting results, perhaps due to poor NOX selectivity [6, 13]. Hv1 channels may be more tractable targets for prevention of brain injury during ischemia. Hv1 is a relatively simple homodimer [39, 87], whereas NOX is assembled as a complex of diverse proteins [8]. Furthermore, since Hv1 conducts 10–100 protons for every NOX electron transfer, there are presumably many fewer Hv1 channels per NOX complex [15]. The lower number of Hv1 molecules requiring binding for inhibition might translate into lower doses of therapeutics. Notably, Hv1 inhibitors would only target microglia but not neuronal NOX in the brain. Thus, Hv1 represents a new and unique potential target, which could be extended to other ischemic disorders and neurodegeneration, such as multiple sclerosis, amyotrophic lateral sclerosis, Alzheimer's disease, and neuropathic pain. Moreover, developing pharmacologic tools for Hv1 will unravel the physiological and pathological roles of Hv1 in vitro and in vivo.

However, we have to be aware of the beneficial role of both microglia and NOX-dependent ROS in ischemic stroke. For example, microglia may exert important protective effects by producing IL-10 and TGF-β, as well as growth factors in the post-ischemic brain [88]. Similarly, NOX-dependent ROS may also have beneficial effects in tissue repair and remodeling following ischemic injury [28]. The role of microglial ROS probably depends on its temporal and spatial generation in the ischemic brain. Therefore, the dual protective/destructive effects of microglia and ROS should be considered. In addition, a potential side effect of targeting Hv1 in stroke treatment is the possible immunosuppression, considering the critical function of Hv1 in the immune system. Post-

stroke immunodepression and the resulted increase in infections have been considered a major determinant of poor neurological outcome. Although our previous study showed that *Hv1*$^{-/-}$ mice do not have a bacterial infection phenotype, post-stroke infections in these mice should be further investigated when targeting microglial Hv1 for stroke treatment.

Conclusion and Future Directions

Hv1 is a newly discovered ion channel that is mainly expressed in the immune system to support NOX activity in innate immunity. In the brain, Hv1 is highly and selectively expressed in microglia and is one of the major ion channels in resting microglia. The cellular functions of microglial Hv1 include pH regulation and NOX-dependent ROS production. The physiological or pathological significance of Hv1 in vivo is largely dependent on the phenotypes of *Hv1*$^{-/-}$ mice. In the ischemic brain, it is believed that microglial Hv1 is coupled to NOX activation, thereby generating ROS and causing neuronal cell death. However, it is unlikely that the Hv1 or microglia evolved to be detrimental to the brain; the plausible explanation is that microglial Hv1 exert their innate immune response during stroke insults, which is associated with damage to neurons. Microglial Hv1 might also have beneficial roles in tissue repair and remodeling following ischemic injury. Neuroprotective or neurodegenerative function of microglial Hv1 may depend on the cue it receives during stroke progression.

Understanding Hv1 physiology will help elucidate the role of microglia in the brain. In addition, Hv1 might represent a novel therapeutic target for treatment of ischemic stroke and other neurological disorders related to ROS and neuroinflammation. Future studies are needed to address the following critical questions about Hv1's function in the brain. (1) Functional Hv1 expression in human brain. Due to the species differences, it is important to determine the presence of Hv1 in human brain. (2) NOX-independent mechanisms of Hv1 function in microglia. It is unlikely that Hv1 participate only in support of NOX activation in microglia. In addition, Hv1 does not seem to be involved in pH homeostasis under normal conditions. (3) The function of microglial Hv1 in the late stage of ischemic stroke. It is still speculative that microglial Hv1 may play a beneficial role in recovery from stroke. (4) Microglial Hv1 functions in neurological diseases, such as multiple sclerosis, ALS, Alzheimer's disease, and neuropathic pain. Currently, Hv1 function in the brain was only reported in a mouse model of ischemic stroke. Its role in other pathologies needs to be investigated. (5) The role of microglial Hv1 in neurodevelopment and maintenance. Physiological function of microglia in the brain has drawn significant attention in recent years. As a major ion channel in resting microglia, it would be interesting to know whether microglial Hv1 function in neuronal circuits. (6) The development of selective Hv1 inhibitors. Currently, zinc is the mostly used inhibitor for Hv1 research. Recently developed guanidine derivatives (such as 2-gunaidiniumbenzimidazole) are potent for voltage-sensitive domain of Hv1 channel [89]. However, these compounds bind Hv1 channel from the intracellular side of the membrane and thus do not appear to have therapeutic potential. Future studies using high throughout functional screening might be helpful to identify Hv1 inhibitors with higher affinity and selectivity for the channel.

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Conflict of Interest Long-Jun Wu declares that he has no conflict of interest.

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