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# Voltage-Gated Proton Channel $H_V1$ in Microglia

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Long-Jun Wu<sup>1</sup>

## Abstract

Microglia are brain resident immune cells and their functions are implicated in both the normal and diseased brain. Microglia express a plethora of ion channels, including  $K^+$  channels,  $Na^+$  channels, TRP channels,  $Cl^-$  channels, and proton channels. These ion channels play critical roles in microglial proliferation, migration, and production/release of cytokines, chemokines, and neurotoxic or neurotrophic substances. Among microglial ion channels, the voltage-gated proton channel  $H_V1$  is a recently cloned ion channel that rapidly removes protons from depolarized cytoplasm and is highly expressed in the immune system. However, the function of microglial  $H_V1$  in the brain is poorly understood. Recent studies showed that  $H_V1$  is selectively expressed in microglia but not neurons in the brain. At the cellular level, microglial  $H_V1$  regulates intracellular pH and aids in NADPH oxidase-dependent generation of reactive oxygen species. In a mouse model of middle cerebral artery occlusion, microglial  $H_V1$  contributes to neuronal cell death and ischemic brain damage. This review discusses the discovery, properties, regulation, and pathophysiology of microglial  $H_V1$  proton channel in the brain.

## Keywords

voltage-gated proton channel,  $H_V1$ , microglia, NADPH oxidase, ischemic stroke

## Introduction

Microglia are the principal brain immune cells and are actively engaged in physiological and pathological brain functions (Kettenmann and others 2011; Perry and others 2010; Ransohoff and Perry 2009). Recent studies have shown that microglia have remarkably dynamic processes and are frequently interacting with neurons and synaptic elements. Through these interactions, microglia may monitor neuronal/synaptic activities and thus survey the microenvironment in the brain (Eyo and Wu 2013). As resident immune response cells, microglia are also well known to employ their immune functions in the diseased brain, such as during bacterial meningitis, ischemic stroke, Alzheimer's disease, Parkinson's disease, multiple sclerosis, and neuropathic pain (Ransohoff and Perry 2009).

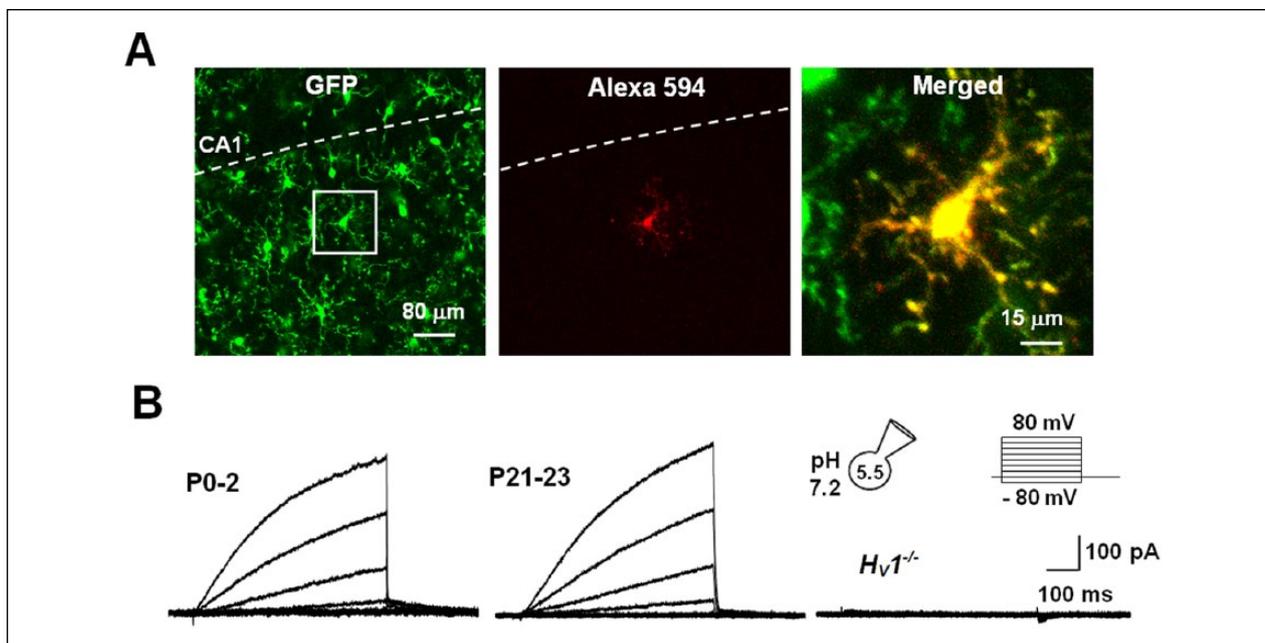
Microglia bear a number of ion channels, including  $K^+$  channels,  $Na^+$  channels, TRP channels,  $Cl^-$  channels, and proton channels. These ion channels may play important functions in proliferation, production of cytokines and cytotoxic substances, morphological changes, and migration of microglial cells (Black and others 2009; Carrithers and others 2007; Craner and others 2005; Eder 1998; Kettenmann and others 2011). The voltage-gated proton current has been well characterized in microglia since 1995 (Eder and DeCoursey 2001), but its molecular

identity as the voltage-gated proton channel  $H_V1$  was only recently discovered (Ramsey and others 2006; Sasaki and others 2006).  $H_V1$  rapidly removes protons from depolarized cytoplasm and is highly expressed in the immune system. Recent studies showed that  $H_V1$  is selectively and functionally expressed in microglia but not neurons in the mouse brain (Wu and others 2012). Microglial  $H_V1$  regulates intracellular pH and aids in NADPH oxidase (NOX)-dependent generation of reactive oxygen species (ROS). In a mouse model of cerebral middle artery occlusion,  $H_V1$  knockout mice were protected from ischemic damage, showing reduced NOX-dependent ROS production, microglia activation, and neuronal cell death (Wu and others 2012). These studies illuminate a critical role of microglial  $H_V1$  in ischemic brain injury, providing a rationale for  $H_V1$  as a potential therapeutic target for the treatment of ischemic stroke. The current understanding of microglial  $H_V1$  in ischemic injury through NOX-dependent ROS production may

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**Figure 1.**  $H_{v1}$  channel mediated voltage-gated proton currents in mouse microglia. (A) A mouse microglia (shown in white box) in the hippocampus was labeled by GFP (green) and loaded with Alexa Fluor 594 (red) during whole-cell recording. The microglia in the white box was magnified as a merged image. Dashed line delineates the CA1 region of the hippocampus. (B) Representative whole-cell voltage-gated proton currents in mouse microglia from acute hippocampal brain slices. Proton currents in response to voltage steps from  $-80$  to  $+80$  mV (shown in inset) were recorded in microglia under conventional whole-cell voltage-clamp mode with extracellular pH at 7.2 and intracellular pH at 5.5. Holding potential was  $-60$  mV. Proton currents were comparable in P0-2 and P21-23 mouse microglia, but were completely abolished in  $H_{v1}^{-/-}$  mouse microglia.

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 diseases, and neuropathic pain. Moreover, understanding microglial  $H_{v1}$  function will unravel the physiological and pathological roles of microglia in the normal and diseased brain (Wu 2013).

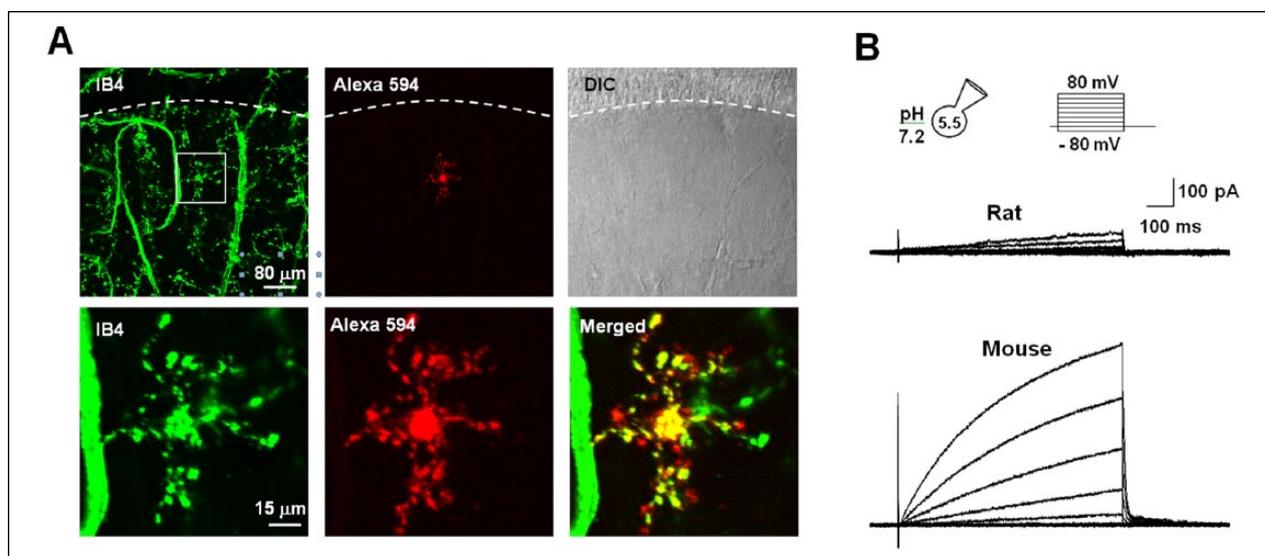
### Voltage-Gated Proton Currents in Microglia

Voltage-gated proton currents were first revealed in snail neurons by Thomas and Meech in 1982 (Thomas and Meech 1982). After they injected HCl into snail neurons, they found that depolarization induced large outward proton currents that were associated with the recovery from intracellular acidosis. In mammalian cells, DeCoursey first recorded the voltage-gate proton currents in rat alveolar epithelial cells in 1991 (DeCoursey 1991). In microglia, the current was first recorded in microglial culture from the murine neonatal brain in 1995 (Eder and others 1995). Subsequently, the voltage-gated current was observed in cultured rat and human microglia as well as microglial cells lines (Eder and DeCoursey 2001).

As microglia are extremely sensitive to the environment, cultured microglia have transformed into amoeboid

cells, which represent an activated form of microglia. These activated microglia showed dramatic differences in membrane properties and electrophysiology compared with the ramified microglia in the acutely isolated slice preparation (Boucsein and others 2000). For example, the inwardly rectified potassium current is normally observed in cultured microglia; however, potassium current is rarely seen in resting microglia from brain slices (Boucsein and others 2000; Wu and others 2007). Similarly, functional AMPA/kainate receptors were observed in cultured microglia but not in microglia from brain slices (Noda and others 2000; Wu and Zhuo 2008). In terms of the voltage-gated proton current, it has been well characterized in cultured microglia. However, there has been a debate about whether microglia in brain slices express functional voltage-gated proton currents, as no current was seen in microglia in P21 rat brain slices whereas the current was observed in microglia in P5-9 mouse brain slices (De Simoni and others 2008; Schilling and Eder 2007).

The controversy on whether microglia in situ express voltage-gated proton current was resolved in a recent study by recording microglia in both rat and mouse brain slices under the same condition (Wu and others 2012) (Fig. 1). Surprisingly, it seems that mouse but not rat microglia in brain slices express functional voltage-gated



**Figure 2.** Small voltage-gated proton currents in rat microglia. (A) A rat microglia in the hippocampus was labeled by IB4 conjugated with Alexa Fluor 488 (green) and loaded with Alexa Fluor 594 (red). Note that IB4 labels both microglia and capillaries. The lower panel shows the magnified area indicated by the white box in the upper panel. (B) Representative whole-cell voltage-gated proton currents in rat and mouse microglia from acute hippocampal brain slices after IB4 staining. Voltage steps were from  $-80$  to  $+80$  mV (shown in inset) and holding potential was  $-60$  mV. Proton currents are extremely small in rat microglia compared with that in mouse microglia.

proton currents (Fig. 2). Considering the close sequence homology between mouse and rat  $H_{V1}$  proton channel, future studies are needed to investigate the mechanism underlying the species difference in microglial voltage-gated proton current. The species differences also raise the concern of the existence of voltage-gated proton currents in human brain tissue in situ, though voltage-gated proton currents were observed in cultured human microglia (McLarnon and others 1997; Wu and others 2012). In addition, the study suggests that the stage of postnatal development may not underlie the differences, as microglial proton current is not noticeably regulated by development after birth and the proton current persisted in both neonatal (P1 and P7) and adult mouse microglia (P21 and P42) (Fig. 1). In contrast to the electrophysiology results, a recent study using immunostaining indicated that the  $H_{V1}$  proton channel is absent in the neonatal mouse brain but present in adult microglia (Okochi and others 2009).

### The Discovery of Voltage-Gated Proton Channel $H_{V1}$

Since the observation of voltage-gated proton currents in immune cells, there has been a debate about whether a component of NOX is the voltage-gated proton channel responsible for the proton currents (DeCoursey 2003). The discovery of the voltage-gated proton channel resolved the dispute and convincingly showed that the voltage-gated proton current results from its own ion

channel. A gene encoding an ion channel with voltage-dependent, proton selective flux was recently identified by two groups independently (Ramsey and others 2006; Sasaki and others 2006). Through sequence homology to the voltage sensor domain of voltage-gated cation channels, the Clapham lab fished out the gene *HVCN1* encoding the voltage-gated proton channel from human genome using bioinformatics searches and named the channel as  $H_{V1}$ . (The H stands for the conducted ionic species,  $H^+$ ; the V subscript indicates voltage sensitivity, and 1 is the first member in the family.) At the same time, using a similar strategy, the Okamura lab identified mouse voltage-gated proton channel, mVSOP (mouse voltage-sensor domain-only protein) (Sasaki and others 2006), which is a homologue of the first voltage-sensitive lipid phosphatase discovered by the same group previously from *Ciona intestinalis* (Murata and others 2005). Subsequently, voltage-gated proton channels have been identified in coccolithophores (Taylor and others 2011) and a dinoflagellate (Smith and others 2011). Genes that are homologous to *HVCN1* are present in many species, from green alga, zebrafish, to monkey (DeCoursey 2013), and thus future studies are needed to confirm functional voltage-gated proton channels in these species.

The  $H_{V1}$  channel belongs to the voltage-gated ion channel superfamily but has surprising and remarkable features. Human  $H_{V1}$  proton channel protein has four transmembrane domains (S1-S4) without a typical S5-S6 pore domain required for ion conduction; it contains 273

amino acids with intracellular N- and C-termini. Mutagenesis studies identified that three arginine residues in S4 are responsible for voltage gating, whereas two histidine residues are required for extracellular inhibition of  $H_V1$  by  $Zn^{2+}$  (Ramsey and others 2006).  $H_V1$  is the most selective ion channel known, showing no detectable permeability to other ions. The ion selectivity filter of  $H_V1$  channel is believed to be composed of aspartate 112 in S1 and arginine 211 in S4 (Berger and Isacoff 2011; Musset and others 2011).  $H_V1$  is a dimer, and the cytosolic domain of the channel is necessary and sufficient for dimerization. Interestingly, each subunit of the  $H_V1$  dimer is functional and has a separate permeation pathway with its own pore and voltage sensor (Tombola and others 2008). Recent studies showed that the opening of the two pathways in  $H_V1$  channels is highly cooperative, which involves interactions between the two subunits of the  $H_V1$  dimer to initiate the conformational change during activation (Qiu and others 2013).

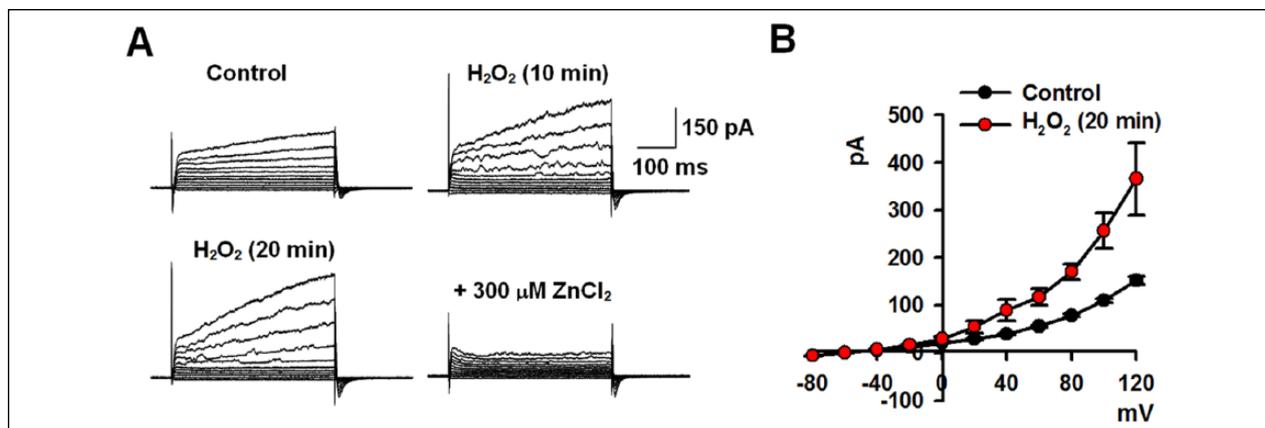
In mammals, the  $H_V1$  is enriched in immune tissues such as the lymph node, bone marrow, and spleen (Ramsey and others 2006; Sasaki and others 2006). Not surprisingly,  $H_V1$  is also expressed in microglia, the principal immune cells in the brain (Wu and others 2012).  $H_V1$  is expressed in many species, and thus its functions are very diverse, from triggering bioluminescent flash in dinoflagellates (Fogel and Hastings 1972), facilitating calcification in coccolithophores (Taylor and others 2011), bacterial killing by macrophages (Ramsey and others 2009), to sperm activation (Lishko and others 2010). Interestingly, a naturally occurring mutation producing the M91T mutant  $H_V1$  proton channel was found in human airway epithelium cells (Iovannisci and others 2010). The M91T channel activates around 30 mV more positive than the wild-type (*wt*)  $H_V1$  channel. Consequently, the activation of acid secretion in epithelia expressing M91T  $H_V1$  channel required 0.5 unit higher extracellular pH than *wt* epithelia (Iovannisci and others 2010). The properties and potential functions of the  $H_V1$  proton channel in biological systems have been reviewed thoroughly (DeCoursey 2013). The current review will mainly discuss the properties, regulation, and pathophysiology of  $H_V1$  proton channel in microglia.

### **$H_V1$ Mediates Voltage-Gated Proton Current in Microglia but Not in Neurons or Astrocytes**

As we mentioned above, voltage-gated proton currents have been characterized in cultured human, mouse, and rat microglia (Eder and DeCoursey 2001). In addition, the voltage-gated proton current was reported in microglia from mouse acute brain slices but not in rat brain slices (De Simoni and others 2008; Schilling and Eder 2007;

Wu and others 2012). To test whether the  $H_V1$  channel mediated voltage-gated proton currents in microglia from mouse brain slices, multidisciplinary methods including biochemical, electrophysiological, pharmacological, and genetic approaches were used (Wu and others 2012). (1) Quantitative PCR results showed that *Hvcn1* mRNA is expressed in the brain and cultured microglia. Consistently,  $H_V1$  protein expression in cultured microglia was confirmed. However,  $H_V1$  protein cannot be detected in whole brain lysate, which might be because of the limited number of microglia in the brain. (2) Using transgenic mice where microglia are selectively labeled with GFP, whole-cell recording were performed in microglia in acute brain slices. Depolarization-induced slowly activating outward currents were observed in microglia and were shown to possess pH dependence and proton selectivity, suggesting the presence of voltage-gated proton currents in mouse microglia. (3) The voltage-gated proton current in microglia is largely reduced by  $Zn^{2+}$ , the known inhibitor of  $H_V1$ . (4) More important, the voltage-gated proton current in microglia is completely abolished in microglia from  $H_V1^{-/-}$  mice (Fig. 1). These results demonstrated that  $H_V1$  mediated voltage-gated proton currents in microglia. Particularly, the results suggest that  $H_V1$  is the only channel that is responsible for voltage-gated proton currents in mouse microglia. Interestingly, under the same condition, the voltage-gated proton current is 10 times smaller in rat microglia compared with those in mouse microglia (De Simoni and others 2008; Wu and others 2012) (Fig. 2). Considering that mouse and rat  $H_V1$  share 89% identity in protein sequence, it would be informative to understand the mechanisms underlying the functional difference between mouse and rat  $H_V1$ .

The voltage-gated proton current was first reported in snail neurons (Thomas and Meech 1982), which prompted investigation to test whether  $H_V1$  is also expressed mouse neurons. Indeed, an  $H_V1$ -like current was reported in cultured hippocampal neurons (Cheng and others 2008). However, several lines of evidence suggest that  $H_V1$  is unlikely to function as a membrane voltage-gated proton channel in mammalian neurons. (1) The  $H_V1$ -like current in the cultured hippocampal neurons cannot be blocked by  $Zn^{2+}$  (Cheng and others 2008). (2) Immunostaining results have shown that  $H_V1$  is selectively expressed in microglia but not neurons (Okochi and others 2009). (3) Western blot cannot detect  $H_V1$  expression in the whole brain lysate or cultured neurons. Also, the expression of *Hvcn1* gene in neurons is extremely low compared with that in microglia (Ramsey and others 2006; Wu and others 2012). (4) Electrophysiological recordings showed that the  $H_V1$ -like current in hippocampal pyramidal neurons cannot be abolished in  $H_V1^{-/-}$  mice nor is it affected by changing the proton gradient (Wu and others 2012).



**Figure 3.** Microglial voltage-gated proton currents were enhanced by H<sub>2</sub>O<sub>2</sub>. (A) In acute mouse hippocampal slices, microglial voltage-gated proton currents were increased after H<sub>2</sub>O<sub>2</sub> (1 mM) perfusion. Proton currents were recorded using perforated patch clamp method to preserve physiological intracellular conditions. Voltage steps from  $-80$  to  $+80$  mV were used to activate proton currents in microglia. Holding potential was  $-60$  mV. Zn<sup>2+</sup> inhibited the proton current. (B) Voltage–current relationships showing increased voltage-gated proton currents 20 minutes after H<sub>2</sub>O<sub>2</sub> perfusion.

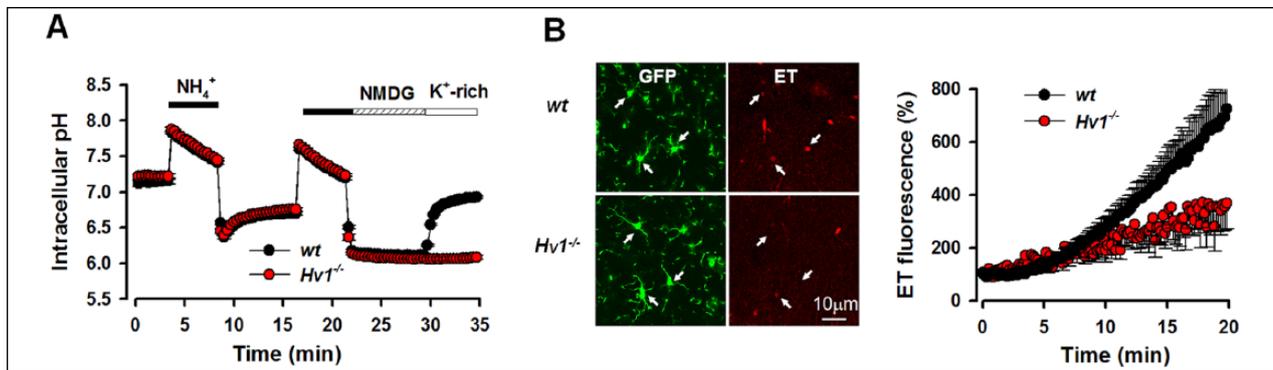
Similarly, low expression of H<sub>V</sub>1 and no functional H<sub>V</sub>1 current was observed in astrocytes. Together, these results suggest that neither hippocampal pyramidal neurons nor astrocytes express functional H<sub>V</sub>1 proton currents in the mouse brain. Whether H<sub>V</sub>1 is expressed in certain types of neurons (such as GABAergic interneurons) or in different nuclei (such as respiratory chemoreceptor areas of the brainstem) in the brain is still unknown.

### Microglial Activation and H<sub>V</sub>1 Proton Channel Function

In contrast to neurons or astrocytes, microglia undergo dramatic alterations in the morphology when they are activated in many neurological diseases, such as ischemic stroke, Alzheimer's disease, and neuropathic pain (Ransohoff and Perry 2009). It is not surprising that microglial ion channels are associated with different activation status of microglia. For example, both outward and inward rectifying K<sup>+</sup> channels are up-regulated during microglial activation under nerve injury or epilepsy (Boucein and others 2000; Menteyne and others 2009). Under resting conditions, H<sub>V</sub>1 is a major functional ion channel in microglia along with K<sup>+</sup> channels and TRP channels (Schilling and Eder 2007; Wu and others 2012). The voltage-gated proton current in cultured microglia is reported to be regulated by microglial activation status. An early study showed that when cultured microglia treated with astrocyte condition medium, which deactivate microglia, proton currents are decreased and the kinetics of current activation are slowed (Klee and others 1999). Interestingly, activated microglia by LPS treatment exhibited reduced proton currents (Klee and others 1999). It is still unknown how deactivation by astrocyte condition

medium and activation by LPS have a similar modulation on voltage-gated proton currents in microglia.

In the ischemic brain, after middle cerebral artery occlusion (MCAO), activated microglia in the ipsilateral brain showed enhanced H<sub>V</sub>1 proton currents compared with control microglia in the contralateral brain (Wu and others 2012). Several different mechanisms may underlie the increased H<sub>V</sub>1 proton current in microglia after ischemic stroke. First, it seems that the H<sub>V</sub>1 protein expression is up-regulated in the brain after stroke (Wu and others 2012). However, whether the increased H<sub>V</sub>1 expression is because of the increased number of microglia after proliferation/infiltration or H<sub>V</sub>1 up-regulation in activated microglia is still unclear. Second, the increased H<sub>V</sub>1 proton current could be because of the modulation by inflammatory mediators, such as arachidonic acid or reactive oxygen species (ROS), which are released by neurons or glial cells in ischemic stroke (Chan 2001). Arachidonic acid can robustly increase proton conductance in neutrophil and macrophage (DeCoursey and Cherny 1993). In addition, ROS like H<sub>2</sub>O<sub>2</sub> can significantly enhance the H<sub>V</sub>1 current in brain microglia (Wu and others 2012) (Fig. 3). However, the mechanism by which these inflammatory mediators regulate H<sub>V</sub>1 channel function is largely unknown. A recent study showed that H<sub>V</sub>1 channel can be phosphorylated at the site of Thr29 in N-terminal by protein kinase C, which increased proton channel gating and conductance of the H<sub>V</sub>1 channel (Musset and others 2010). It would be interesting to test whether arachidonic acid or ROS may enhance the H<sub>V</sub>1 current through activation of kinases and thereby H<sub>V</sub>1 channel phosphorylation. Third, it is possible that there is an increase in the number of membrane H<sub>V</sub>1 channels because of the trafficking in activated microglia in the ischemic brain. Early studies using



**Figure 4.** Microglial  $H_V1$  channel in pH regulation and NOX-dependent ROS production. (A) Microglial  $H_V1$  relieves intracellular acidosis during membrane depolarization. Intracellular pH ( $pH_i$ ) was 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  $H_V1^{-/-}$  microglia. (B) Microglial  $H_V1$  is required for NOX-dependent ROS production. NOX was activated by PMA and ROS was detected by ethidium (ET) in the brain slices. Microglia were labeled with GFP in green and ROS production was labeled in red (shown by arrows, left). NOX-dependent ROS production was gradually accumulated in wt microglia but not in  $H_V1^{-/-}$  microglia (right).

cultured microglia showed reduced proton current after chronic but not acute treatment of cytoskeletal disruptive agents, such as cytochalasin or colchicine (Klee and others 1998), suggesting that the trafficking  $H_V1$  channels might be important for the microglial proton current. Nevertheless, although many studies have shown that the trafficking of ion channels/receptor is critical in their physiological or pathological functions (Krugers and others 2010), the trafficking of  $H_V1$  channels in microglia is a largely uncharted area.

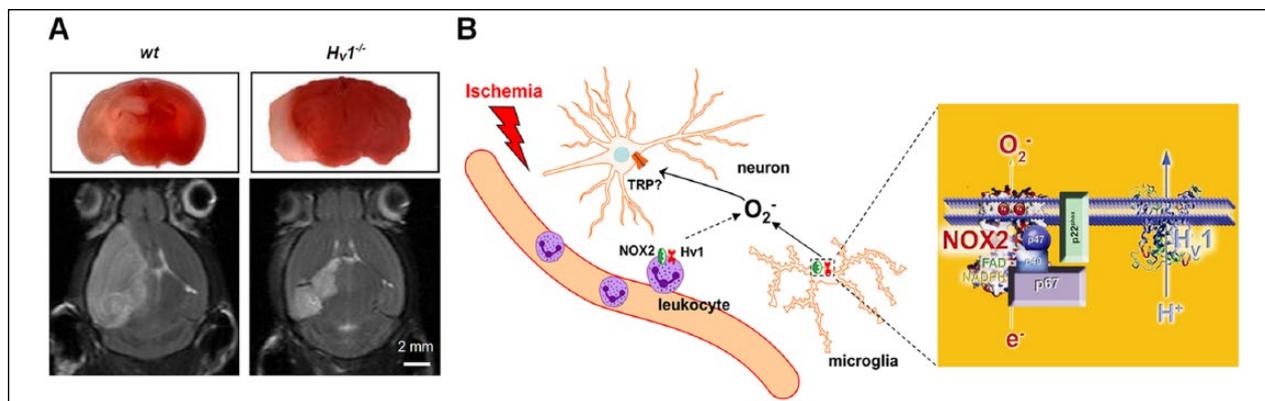
Does  $H_V1$  contribute to microglial activation? Activation of microglia is accompanied by changes in morphology, proliferation, up-regulation of immune surface antigens, and the production of cytotoxic or neurotrophic molecules (Ransohoff and Perry 2009). Under normal conditions,  $H_V1^{-/-}$  microglia seem to have similar morphology compared with wt microglia. However, the enlarged cell bodies after stroke were attenuated in  $H_V1^{-/-}$  microglia compared with wt microglia, suggesting that  $H_V1$  contributes to microglial activation after ischemia (Wu and others 2012). In the neutrophil,  $H_V1$  is reported to participate in the  $Ca^{2+}$  signaling and neutrophil migration (El Chemaly and others 2010). It is still unknown whether the attenuated microglial activation in  $H_V1^{-/-}$  mice after stroke results from the impaired  $Ca^{2+}$  influx or indirectly from the impaired ROS production/pH homeostasis as we will discuss below.

### Cellular Functions of Microglial $H_V1$ : pH Homeostasis and ROS Production

The  $H_V1$  proton channel is activated by membrane depolarization and mediates outward proton currents.

Therefore, there are two consequences to the cells with  $H_V1$  activation: one is the hyperpolarization of plasma membrane and the other is the alkalization of intracellular cytosol. The  $H_V1$ -mediated membrane hyperpolarization might be important for  $Ca^{2+}$  entry through store-operated  $Ca^{2+}$  channels (El Chemaly and others 2010). The  $H_V1$ -mediated intracellular alkalization potentially regulates pH homeostasis that was intensively studied in neutrophils (El Chemaly and others 2010; Morgan and others 2009).

In brain microglia, it has been shown that the activation of  $H_V1$  drastically adjusts intracellular acidification under certain conditions such as during microglial depolarization (Wu and others 2012). As  $H_V1$  activation requires membrane depolarization, it seems that the sodium-proton exchanger (NHE1) but not  $H_V1$  channels maintain basal intracellular pH under resting condition (Liu and others 2010; Wu and others 2012). Even when there is a dramatic drop in the intracellular pH ( $\sim 6.5$ ),  $H_V1$  channels are not activated to correct the acidosis because of the high voltage threshold for  $H_V1$  activation. Microglial membrane depolarization, however, could activate  $H_V1$  and promptly relieve the intracellular acidosis (Fig. 4A). Compared with NHE1-dependent proton extrusion,  $H_V1$ -mediated pH recovery is several times faster than that of NHE1 in microglia. Therefore, microglial  $H_V1$  is able to function as an efficient pH regulator but might do so only under conditions of strong membrane depolarization, such as induced by increased extracellular  $K^+$  concentration or NOX activation. However, future in vivo studies are needed to determine whether  $H_V1$  indeed regulates pH homeostasis under physiological conditions, considering the fact that the requirement of  $H_V1$  activation is quite stringent. Consistent with this



**Figure 5.** Microglial  $H_{v1}$  channel contributed to ischemic brain damage. (A) Smaller infarct volume in  $H_{v1}^{-/-}$  mice 24 hours after MCAO compared with that in wt mice. TTC staining of coronal brain slices (upper) and transverse MRI brain images (lower) from wt and  $H_{v1}^{-/-}$  mice after MCAO are shown. (B) Diagram showing  $H_{v1}$  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.  $H_{v1}$  is required for full activation of NOX2, by serving a charge compensation function, as well as by removing intracellular  $H^+$ . In the mouse brain,  $H_{v1}$  is highly expressed in microglia but not neurons or astrocytes. Microglial  $H_{v1}$ -related ROS production partially contributes to neuronal cell death in early stages of ischemic stroke.

notion,  $H_{v1}$  channels do not participate in ATP-induced chemotaxis in microglia (Wu and others 2012), although NHE1-mediated intracellular alkalization is known to be critical for fibroblast and neutrophil migration (Casey and others 2010). We have known that ATP activates P2Y receptor-coupled  $K^+$  channel and thus might hyperpolarize microglia (Boucsein and others 2003; Wu and others 2007). Therefore,  $H_{v1}$  cannot be activated and participate in the ATP-induced chemotaxis.

Proton currents were proposed to function cooperatively with NOX activities during respiratory burst, which is associated with both intracellular acidosis and membrane depolarization (DeCoursey 2003): (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 the neutrophil. The depolarization could even reach 190 mV within 20 ms if there is no charge compensatory mechanism (DeCoursey and others 2003). (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 (Morgan and others 2009). Together, NOX activation associated with membrane depolarization and intracellular acidosis seems sufficient to activate  $H_{v1}$ . On the other hand, NOX is inhibited by membrane depolarization and by intracellular acidosis (DeCoursey 2003). 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,  $H_{v1}$  is ideally suited for this function in cooperation with NOX

(Fig. 5B). Consistent with this idea, accumulating evidence indicates that  $H_{v1}$  is coupled to NOX-dependent pH regulation, membrane depolarization, and ROS production neutrophils (El Chemaly and others 2010), B cells (Capasso and others 2010), and eosinophils (Zhu and others 2013).

In brain microglia,  $H_{v1}$  and NOX (particularly NOX2) are highly expressed. The  $H_{v1}^{-/-}$  microglia show intracellular acidosis when NOX is activated by phorbol myristate acetate (PMA), suggesting that NOX and  $H_{v1}$  are cooperatively activated (Wu and others 2012). In addition,  $H_{v1}$  is required for NOX-dependent ROS production and  $H_{v1}^{-/-}$  microglia accumulate significantly less ROS compared with that of wt microglia in brain slices (Fig. 4B). More important, microglial  $H_{v1}$  is also critical for NOX-dependent ROS production in ischemia conditions both in vitro and in vivo. Therefore, similar to that in other immune cells, the  $H_{v1}$  proton channel in microglia aids in NOX activation and subsequent ROS production probably under disease conditions. However, we have to keep in mind that NOX-dependent ROS production is not completely abolished in  $H_{v1}^{-/-}$  microglia. These results suggest that NHE1 and other ion channels may serve as alternative mechanisms to compensate NOX activities, even though the  $H_{v1}$  channel seems to be the ideal channel coupled to NOX.

### Physiological and Pathological Function of Microglial $H_{v1}$

To study the pathophysiological function of  $H_{v1}$  in vivo,  $H_{v1}^{-/-}$  mice were generated by both the Clapham and Okumura laboratories recently (Okochi and others 2009;

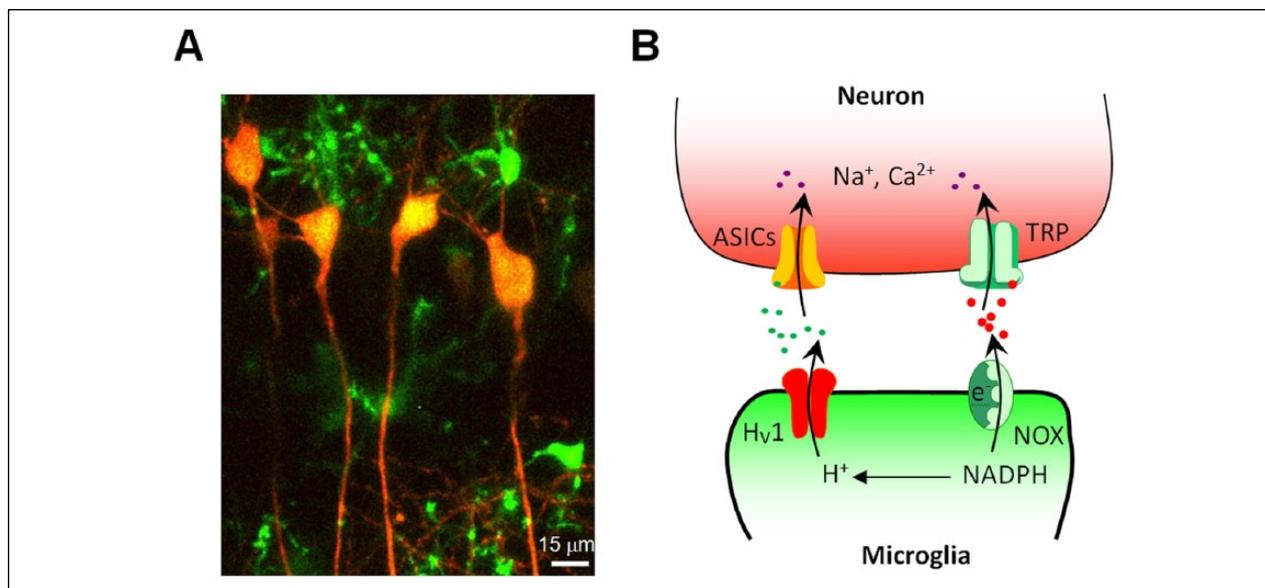
Ramsey and others 2009). Most studies focused on  $H_V1$  function in the immune system using the knockout mice. Unexpectedly,  $H_V1^{-/-}$  mice were able to clear several bacterial infections in vivo including *S. aureus*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia*, despite a significant reduction in superoxide production (Ramsey and others 2009). The lack of a bacterial infection phenotype in the  $H_V1^{-/-}$  mice may be because of redundant mechanisms for bacterial clearance. For example, both superoxide- and nitric oxide-dependent systems are shown to function synergistically for bacterial clearance in vivo (Shiloh and others 1999). However,  $H_V1^{-/-}$  mice showed defective B cell responses and impaired antibody production (Capasso and others 2010). The mechanism involves the regulation of B cell receptor-mediated ROS production and its downstream signaling, Syk, Akt, and SHP-1 by  $H_V1$  activation. A recent study also found that  $H_V1^{-/-}$  mice have autoimmune disorder phenotypes, such as splenomegaly, autoantibodies, and nephritis (Sasaki and others 2013). The phenotype might be because of the impaired T-cell homeostasis, although T cells express very low density of functional  $H_V1$  channel (Schilling and others 2002). Because  $H_V1$  is expressed in almost all cell types in the immune system, future studies employing cell-specific  $H_V1$  deletion are required to dissect the role of  $H_V1$  in immune function.

In the brain, microglial cells are the only resident cell type that highly expresses  $H_V1$ . Therefore, it is advantageous to use general  $H_V1^{-/-}$  mice to study microglial  $H_V1$  function in the brain. Microglial  $H_V1$ 's function in ischemic stroke has recently been reported (Wu and others 2012). The rationale to study microglial  $H_V1$  in ischemic stroke comes from the earlier studies that NOX1, 2, 4 knockout mice showed less brain damage during the pathology (Bedard and Krause 2007; Wu 2013). As NOX2 is highly expressed in microglia and  $H_V1$  is coupled to NOX2 activation, it is possible that  $H_V1^{-/-}$  mice may be protected in this disease. Indeed,  $H_V1^{-/-}$  mice exhibited smaller infarct volumes and better neurological behaviors associated with brain injury, compared with those in *wt* mice after MCAO (Fig. 5A). The protection was long lasting, even observed 7 days after MCAO. Further study found decreased neuronal cell death in the penumbra of the ischemic brain from  $H_V1^{-/-}$  mice (Wu and others 2012). Additional evidence demonstrated that microglial  $H_V1$  contributes to neuronal death and ischemic brain damage via the NOX pathway using a microglia-neuron co-culture system. These results suggest that  $H_V1$  plays a critical role in microglial-derived ROS generation that accounts for a significant amount of neuronal cell death and brain injury that occurs after experimental stroke in mice (Wu and others 2012) (Fig. 5B). ROS target DNA, membrane lipids, phosphatases/kinases, proapoptotic transcription factors, and ion channels to exert

cell toxicity (Nathan and Ding 2010). TRPM2 and TRPM7 channels are particularly interesting ROS targets, as both ion channels are implicated in stroke-related neuronal cell death (Wu and others 2010) (Fig. 5B). Alternatively, a NOX-independent  $H_V1$  function in cell death might involve neuronal acid-sensing ion channel (ASICs) (Fig. 6B), because ASICs are proton-gated ion channels abundantly expressed in central neurons and are highly sensitive to extracellular acidosis (Wu and others 2004). Coincidentally, both ASIC1a knockout mice and  $H_V1^{-/-}$  mice show a reduced brain damage phenotype after ischemic stroke (Wu and others 2012; Xiong and others 2004).

Recent studies using NOX inhibitors in experimental stroke show conflicting results, perhaps due to poor NOX selectivity (Bedard and Krause 2007).  $H_V1$  channels may be more tractable targets for prevention of brain injury during ischemia, because (1)  $H_V1$  is a relatively simple homodimer (Tombola and others 2008), whereas NOX is assembled as a complex of diverse proteins (Bedard and Krause 2007). (2) Because  $H_V1$  conducts 10 to 100 protons for every NOX electron transfer, there are presumably many fewer  $H_V1$  channels per NOX complex (DeCoursey 2003). The requisite of lower numbers of  $H_V1$  molecules binding for inhibition might translate into lower doses of therapeutics. (3)  $H_V1$  inhibitors would target only microglial but not neuronal NOX in the brain. However, currently there is no available selective  $H_V1$  antagonist that holds therapeutic potential (Hong and others 2013). In addition, it is highly improbable that microglial  $H_V1$  evolved to be detrimental to the brain because only benign and advantageous mutations endure the process of natural selection. The more plausible explanation is that microglial  $H_V1$  exert their innate immune response to stroke insults, which is associated with damage to neurons. Microglial  $H_V1$  might also have beneficial roles in tissue repair and remodeling following ischemic injury. Neuroprotective or neurodegenerative functions of microglial  $H_V1$  may depend on the stimuli/signal it receives as well as temporal and spatial generation of microglial ROS in the ischemic brain. In addition, a potential side effect of targeting  $H_V1$  in stroke treatment is possible immunosuppression, considering the critical function of  $H_V1$  in the immune system. Although our previous study showed that  $H_V1^{-/-}$  mice do not have a bacterial infection phenotype, poststroke infections in these mice should be further investigated when targeting microglial  $H_V1$  for stroke treatment.

Given that  $H_V1$  is one of the major ion channels in microglia, stepping back and examining the general role of microglia in the brain is a practical approach to understanding  $H_V1$  function. Traditionally, microglia were largely implicated only in pathological conditions, including ischemic stroke, Alzheimer's disease,



**Figure 6.** H<sub>v</sub>1 channel in microglia-neuron communication. (A) A live fluorescent image showing close interactions between microglia (green) and neurons (yellow) in mouse hippocampus. Double transgenic mice **with** microglia labeled with GFP and neuron labeled with YFP were used. The image was taken from 300 μm acute hippocampal slices by a two-photon microscope. (B) Diagram showing the possible routes for H<sub>v</sub>1's function in mediating microglia-neuron communication in the brain. Microglial H<sub>v</sub>1 channel aids NOX in the production of ROS, which is able to activate neuronal TRP channels, such as TRPM2 and TRPM7. In addition, microglial H<sub>v</sub>1 channel releases protons, which can activate neuronal acid-sensing ion channels (ASICs). Activation of neuronal TRP channels and ASICs may mediate microglial H<sub>v</sub>1 function in the normal and diseased brain.

neuropathic pain, epilepsy, bacterial meningitis, multiple sclerosis, amyotrophic lateral sclerosis, Parkinson's disease, HIV dementia, Huntington's disease, and Nasu-Hakola disease (Perry and others 2010). In theory, microglial H<sub>v</sub>1 could potentially be involved in any of these pathological conditions. Using H<sub>v</sub>1<sup>-/-</sup> mice, our recent results suggest that spinal microglial H<sub>v</sub>1-dependent ROS is critical for neuropathic pain, whereas brain microglial H<sub>v</sub>1 is involved in the phagocytosis of β-amyloid in a mouse model of Alzheimer's disease (Wu and others, unpublished data). In addition, H<sub>v</sub>1<sup>-/-</sup> mice are being tested in other disease models, such as Parkinson's disease, multiple sclerosis, and spinal cord injury by several research groups. The resulting characterizations of microglial H<sub>v</sub>1 in these various neurological diseases will enrich our understanding of microglia and H<sub>v</sub>1 channel function in the brain as well as in peripheral systems. Recent studies also suggest that microglia play interesting roles under physiological conditions, particularly in nervous system development and maintenance (Eyo and Wu 2013; Schafer and others 2013). Indeed, microglia have close interactions with neurons observed *in vivo* or *in situ* (Fig. 6A). It is possible that H<sub>v</sub>1 could potentially exert a central role in microglia-neuron communication by releasing proton and ROS production. The microglia-derived protons and ROS can target neuronal ASICs and TRP channels,

respectively (Fig. 6B). Future studies are needed to test these interesting ideas.

## Conclusion and Future Directions

In summary, H<sub>v</sub>1 is a newly discovered ion channel primarily expressed in the immune system to support NOX activity in innate immunity. In the brain, H<sub>v</sub>1 is highly and selectively expressed in microglia and is one of the major ion channels in resting microglia. The cellular functions of microglial H<sub>v</sub>1 include pH regulation and NOX-dependent ROS production. The physiological or pathological significance of H<sub>v</sub>1 *in vivo* is largely dependent on the phenotypes of H<sub>v</sub>1<sup>-/-</sup> mice. In the ischemic brain, microglial H<sub>v</sub>1 is thought to be coupled to NOX activation, thereby generating ROS and causing neuronal cell death. H<sub>v</sub>1 might represent a novel therapeutic target for the treatment of ischemic stroke and other neurological disorders related to ROS and neuroinflammation, such as Alzheimer's disease, neuropathic pain, epilepsy, bacterial meningitis, multiple sclerosis, amyotrophic lateral sclerosis, Parkinson's disease, HIV dementia, Huntington's disease, and Nasu-Hakola disease (Perry and others 2010). Future studies are needed to determine the role of microglial H<sub>v</sub>1 in these diseases. Particularly, the beneficial role of microglial H<sub>v</sub>1 as well as NOX-independent mechanisms of H<sub>v</sub>1 function in microglia in

the normal brain (such as during neurodevelopment and maintenance) and diseased brain should be investigated.

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