Microglial voltage-gated proton channel Hv1 in spinal cord injury

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From the Contents

Abstract

After spinal cord injury, microglia as the first responders to the lesion display both beneficial and detrimental characteristics. Activated microglia phagocyte and eliminate cell debris, release cytokines to recruit peripheral immune cells to the injury site. Excessively activated microglia can aggravate the secondary damage by producing extravagant reactive oxygen species and pro-inflammatory cytokines. Recent studies demonstrated that the voltage-gated proton channel Hv1 is selectively expressed in microglia and regulates microglial activation upon injury. In mouse models of spinal cord injury, Hv1 deficiency ameliorates microglia activation, resulting in alleviated production of reactive oxygen species and pro-inflammatory cytokines. The reduced secondary damage subsequently decreases neuronal loss and correlates with improved locomotor recovery. This review provides a brief historical perspective of advances in investigating voltage-gated proton channel Hv1 and home in on microglial Hv1. We discuss recent studies on the roles of Hv1 activation in pathophysiological activities of microglia, such as production of NOX-dependent reactive oxygen species, microglia polarization, and tissue acidosis, particularly in the context of spinal cord injury. Further, we highlight the rationale for targeting Hv1 for the treatment of spinal cord injury and related disorders.

Key Words: Hv1 proton channel; ion channels; microglia; NADPH oxidase; pH regulation; reactive oxygen species; spinal cord injury

Introduction

Traumatic spinal cord injury (SCI) is a severe damage to the spinal cord, leading to a life-altering neurological disorder. The pathophysiology of SCI has two phases, a primary phase involving the initial mechanical injury directly disrupting spinal cord tissue, and a secondary injury phase including vascular dysfunction, excitotoxicity, inflammation, free radical production, and cell death (Venkatesh et al., 2019). As resident innate immune cells accounting for approximately 10% of cells in the central nervous system (CNS) (Colonna and Butovsky, 2017), microglia are one of the first responders to the SCI, playing both protective and detrimental roles. Under normal conditions, microglia exhibit a ramified morphology, actively surveying their surrounding area with processes (Liu et al., 2019). Upon SCI, microglia quickly activate and transform to amoeboid shape in response to the lesion site in hours. Activated microglia phagocyte and eliminate microbes and cell debris, release cytokines to recruit peripheral immune cells to infiltrate, and form an astrocyte-immune interface to limit the lesion. However, excessively activated microglia may also aggravate the secondary damage by releasing reactive oxygen species (ROS) and pro-inflammatory cytokines (Kroner and Rosas Almanza, 2019). Therefore, effective regulation of microglial activities would be aid in treatment of SCI.

Ion channels regulate and shape the physiological functions of microglia. Indeed, ion flux regulates a variety of microglia activities, including but not limited to proliferation, migration, morphological alternations, cytokine release, and ROS production (Thei et al., 2018). In this regard, recently there has been focus on a voltage-gated proton channel, Hv1, which is selectively expressed in microglia in the CNS (Wu et al., 2012). To date, a well-studied function of Hv1 is optimizing NADPH oxidase (NOX) activity (Decoursey, 2003, 2016). Knockout of Hv1 impedes NOX activities, resulting in remarkable ROS reduction. After traumatic CNS injuries, Hv1 deficient (*Hv1*$^{-/-}$) mice show neuroprotective effect compared to wild-type mice (WT) (Wu et al., 2012; Liu et al., 2015; Li et al., 2020a, b, c, 2021; Murugan et al., 2020; Ritzel et al., 2021). Deficiency of Hv1 directly alters microglial activation, resulting in alleviated production of ROS and pro-inflammatory cytokines, and subsequently reduced neuronal loss and improved locomotor recovery. In this review, we provide a brief historical perspective on the discovery of the voltage-gated proton channel Hv1 and advances made in investigating microglial Hv1. We then summarize the documented roles of Hv1 activation in pathophysiological activities of microglia, particularly in the context of SCI. Further, we highlight findings from recent animal studies that indicate the detrimental role of microglial Hv1, thereby, suggesting that Hv1 is an ideal target for reducing ROS, alleviating tissue damage, and improving locomotor recovery following SCI.

Search Strategy

The following PubMed search was used to collect literature - "microglia" AND "hv1" AND "spinal cord injury" from 1900 to 2021 for this comprehensive report on microglial Hv1.

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Voltage-Gated Proton Channel Hv1

The voltage-gated proton channel, Hv1, is a unique ion channel with high selectivity for protons. The first description of voltage-gated proton currents was in snail neurons in the early 1980s (Thomas and Meech, 1982). However, it was not until 2006, that the gene encoding Hv1 was identified independently and concurrently by two research teams (Ramsey et al., 2006; Sasaki et al., 2006). Using a bioinformatics approach in pursuit of identifying novel voltage-sensitive ion channels, Clapham lab identified the gene *HVCN1* from the human genome and named the channel Hv1 (Ramsey et al., 2006). "H" stands for the conducted ionic species, "v" indicates its voltage sensitivity, and "1" denotes the family's first member (Wu, 2014b). At the same time, Okamura lab used an amino acid sequence of the voltage-sensor domain of *Ciona intestinalis* VSP as a query for bioinformatics search and identified the mouse cDNA for Hv1, and called it mouse voltage-sensor domain–only protein (Sasaki et al., 2006). Soon after the identification of the gene encoding the Hv1 channel, Hvcn1 knockout mice ($Hv1^{-/-}$) was generated by inserting β-galactosidase/neomycin fusion protein into an intron following exon 2, which contains the Hv1 translation initiation codon (Okochi et al., 2009; Ramsey et al., 2009). Since the availability of knockout mice, progress of investigations on the Hv1 channel function *in vivo* has accelerated.

Hv1 channel has unique structural and functional properties compared to classical voltage-gated cation channels. Structurally, Hv1 assembles as a dimer of two voltage-sensor domains without a pore domain (Ramsey et al., 2006; Sasaki et al., 2006; Koch et al., 2008; Lee et al., 2008; Tombola et al., 2008). While each monomer containing a pore, a voltage sensor (S4), and a gate (S1) can function autonomously, the opening of the two subunits in the dimer is cooperatively regulated by the S1–S4 dimer interface, resulting in slowing of channel opening and closure compared to the monomer (Gonzalez et al., 2010; Tombola et al., 2010; Fujiwara et al., 2012; Mony et al., 2020). Hv1 has no detectable permeability to other ions, and its cation selectivity filter is determined by aspartate 112 in S1 and arginine 211 in S4 (Berger and Isacoff, 2011; Musset et al., 2011). The Hv1 channel is mainly expressed in immune cell populations including macrophages (Subramanian Vignesh et al., 2013), neutrophils (Okochi et al., 2009; Ramsey et al., 2009; El Chemaly et al., 2010), eosinophils (Zhu et al., 2013), plasmacytoid dendritic cells (Montes-Cobos et al., 2020), B cells (Capasso et al., 2010), and some subset of T cells (Sasaki et al., 2013; Asuaje et al., 2018). In the mouse CNS, Hv1 is selectively expressed in microglia but not in neurons or astrocytes (Wu et al., 2012). The gating of Hv1 is dependent on both membrane depolarization and intracellular acidosis, which means it is activated most likely when cells are facing extreme challenges, as seen in pathological conditions.

To date, in all phagocytic immune cells studied, Hv1 is linked to optimizing NADPH oxidase (NOX) activity by restoring membrane potential and cytoplasmic pH (Decoursey, 2003, 2016). When NOX is activated, H^+ generated from NOX activity induces a sharp decrease in cytoplasmic pH, which in turn suppresses NOX which is optimal at pH 7.5 (Morgan et al., 2005). NOX activity also causes membrane depolarization due to the transferring of electrons across the membrane. As a pH- and voltage-dependent channel, Hv1 is activated by the combined effects of NOX-induced membrane depolarization and intracellular acidosis. Upon activation, Hv1 conducts 10 to 100 protons for every NOX electron transfer, functioning as the most efficient proton channel coupled to the NOX activity (DeCoursey et al., 2003).

The relationship between Hv1 and NOX has been reviewed thoroughly (DeCoursey, 2016). But it is worth noting that although Hv1 indemnifies the NOX activity, depletion of Hv1 only reduces NOX activity but does not eliminate ROS production (DeCoursey, 2016). This means that Hv1 inhibition abrogates functions such as various signaling, while sparing the innate immune response, which could cause susceptibility to infections (Capasso et al., 2011). $Hv1^{-/-}$ mice were able to clear bacterial infections (Ramsey et al., 2009), while after traumatic injuries of the CNS, $Hv1^{-/-}$ mice showed neuroprotective effect compared to WT (Wu et al., 2012; Liu et al., 2015; Li et al., 2020b, c, 2021; Murugan et al., 2020; Ritzel et al., 2021). Deficiency of Hv1 directly alters microglia activation and alleviates secondary damage following SCI.

Hv1-Related Mechanisms in Spinal Cord Injury

Increased expression of Hv1 is noted in several animal models of SCI. In a mouse contusion model, *Hvcn1* mRNA from injured spinal cord tissue was elevated at 3 days after injury and persisted up to 28 days in both sexes; consistently, western blot analysis showed a nearly four-fold increase in Hv1 protein at 3 days post SCI compared with the sham control. To confirm whether the increase of tissue Hv1 is contributed by myeloid cells, $CD11b^+$ cells were sorted from injured spinal cord tissue for qRT-PCR. The results show a significant increase in *Hvcn1* mRNA in isolated $CD11b^+$ cells at 3 days post injury compared to cells from the sham group (Li et al., 2021). In line with this, we found a significant increase in the expression of Hv1 in sorted microglia/macrophages after SCI lasting up to 2 weeks by performing bioinformatics analysis of a recently published RNA sequencing dataset (Noristani et al., 2017). More specifically, the published study used $Cx3cr1^{gfp/+}$ mice to perform laminectomy at thoracic 9 (T9) level to obtain either complete paraplegia or hemi paraplegia (**Figure 1A**). Subsequently, 1 cm length of tissue containing the epicenter of injury was collected at 72 hours, 1 and 2 weeks and $Cx3cr1^+$ cells were isolated for RNA sequencing (Noristani et al., 2017). Analysis of the Noristani et al. (2017) dataset revealed a significant increase in *Hcvn1* mRNA in $Cx3cr1^+$ microglia/macrophages at 1 week and 2 weeks after both hemisection and full transection of the spinal cord (**Figure 1B**). This confirms that local and peripheral immune response in the epicenter of injury has remarkably high expression of Hv1 channels presumably by a combination of resident activated microglia and infiltrated macrophages that contribute to the secondary damage after SCI.

To address the function of Hv1 in SCI, we performed a laminectomy over the dorsal portion of T9 in both WT and $Hv1^{-/-}$ mice. Using a modified NYU impactor, mice were subjected to a 3-g drop with a tip diameter of 0.5-mm flat surface from a height of 6.25 mm, resulting in a moderate contusion injury. Basso mouse scale (BMS) scoring (Basso et al., 2006) for locomotion was performed after the injury and weekly thereafter for up to 8 weeks (**Figure 1D**). We observed significant improvement of motor recovery in the $Hv1^{-/-}$ mice with SCI compared to the WT/SCI mice (**Figure 1E**; Murugan et al., 2020). This robust rescue of behavioral outcomes in $Hv1^{-/-}$ mice compared to WT mice after SCI was also observed independently by two other research groups in similar SCI models (Li et al., 2020b, 2021). Additional readouts of attenuated neuronal damages in the $Hv1^{-/-}$/SCI from these three groups include attenuated neuronal loss, increased white matter sparing, and reduced demyelination. The following paragraph will discuss the current understanding of Hv1-mediated mechanism following SCI. All studies consistently showed the $Hv1^{-/-}$/SCI developed alleviated secondary damages (Li et al., 2020b, c, 2021; Murugan et al., 2020).

Hv1 regulates NOX2 activity and NOX-dependent ROS production after SCI

Several studies have demonstrated that Hv1 channel activity is coupled to NOX-dependent pH regulation, membrane

depolarization, and ROS production. This Hv1-NOX coupling has been shown in a number of immune cells including microglia (Wu, 2014b), neutrophils (El Chemaly et al., 2010), B cells (Capasso et al., 2010), and eosinophils (Zhu et al., 2013). Since there is a potent immune response following SCI, it is no surprise that Hv1-NOX mediated ROS is elevated in injured tissue. Indeed, our analysis of RNA sequencing dataset from Noristani et al. (2017) confirmed a significant increase in *Cybb* gene that encodes the NOX2 protein lasting at 1 week and 2 weeks after SCI (**Figure 1C**).

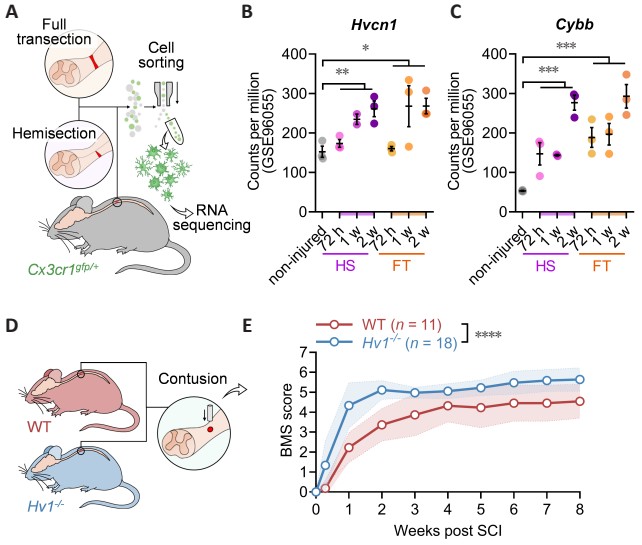

Figure 1 | Hv1 and Nox2 are upregulated in two independent models of spinal cord injury (SCI).
(A) Schematic illustration depicts experimental design of the study by Noristani et al. (2017) showing isolation of Cx3cr1-gfp$^+$ microglia/macrophages from the spinal cord at the epicenter of the injury following either a full transection (FT) or hemi section (HS) for RNA sequencing. (B, C) Plots show counts of Cx3cr1-gfp$^+$ cells with Hcvn1 expression (B) or Cybb expression (C) in non-injured and at 72 hours, 1 and 2 weeks after wither FT or HS injury. The genes Hvcn1 and Cybb encodes for Hv1 channel and NOX2, respectively. *P < 0.05, **P < 0.01 and *** P < 0.001. (D, E) Schematic depicting experiment performed by Murugan et al. (2020) (D) and the results from BMS scoring (E). The total BMS scores in Hv1$^{-/-}$ mice measured at different time points following SCI showed faster locomotor recovery compared to age-matched controls (WT, n = 11; Hv1$^{-/-}$, n = 18) (*P < 0.05, **P < 0.01, ***P < 0.001). BMS: Basso mouse scale; Hv1$^{-/-}$: Hv1 knockout mice; w: week; WT: wild-type.

In line with this, SCI induced upregulation of NOX2 expression was observed at 6–24 hours and remained elevated at 2 weeks in injured spinal cord tissue (Sabirzhanov et al., 2019). Using flow cytometry, microglia being labeled as CD11b$^+$ CD45^{int} display a marked increase of NOX2 activity at 3 days after SCI compared to the sham. The *Hv1*$^{-/-}$/SCI showed significantly attenuated NOX2 activity in both microglia (CD11b$^+$ CD45^{int}) and infiltrating myeloid cells (CD11b$^+$ CD45^{hi}) compared to the WT/SCI. However, it should be noted that NOX2 activity is still robustly elevated in the *Hv1*$^{-/-}$/SCI compared to the sham (Li et al., 2021). This result implies that sodium-proton exchanger and other ion channels may serve as alternative mechanisms to compenstate for NOX2 activation (Wu, 2014b).

Following SCI, the increased NOX2 activity results in excessive ROS production. The production of ROS following SCI is a double-edged sword. Low and mild ROS concentration regulates cell signaling cascades, including cell development, proliferation, stress response, antioxidant defenses and anti-apoptosis. However, high ROS concentration can induce cell signaling disruption, mitochondrial pro-apoptotic signal activation, and lipid/protein/DNA oxidative damage (Tauffenberger and Magistretti, 2021). Scavenging of ROS by phenyl-N-t-butyl nitrone showed improvement of locomotor recovery after SCI (Gwak et al., 2013). Inhibition of NOX2 also showed beneficial outcomes (Khayrullina et al., 2015). Interestingly, a recent study highlighted the importance of ROS in regulation of neurite outgrowth and post-injury axonal regeneration. Axonal active ROS-producing NOX2 originating from macrophage and bone marrow-derived macrophages is required for sensory axonal regeneration (Hervera et al., 2018). Additionally, targeting NOX2, which is expressed widely in various cell types (Diebold et al., 2015) may have broad side effects, especially the apprehension of being susceptible to infections. Taken together, these findings suggest the importance of seeking a target to reduce but not eliminate ROS, and Hv1 fits in well in this context.

Depletion of Hv1 reduces NOX-dependent ROS production. ROS concentration in *Hv1*$^{-/-}$/SCI spinal tissue homogenates is lower than the WT/SCI at early time points. Flow cytometry revealed that both microglia and infiltrating myeloid cells have attenuated ROS production in the *Hv1*$^{-/-}$/SCI compared to the WT/SCI. In line with the other studies, immunofluorescent staining of 8-hydroxyguanosine, a marker of oxidative damage in cellular DNA, showed less co-localization with ionized calcium binding adaptor molecule 1 positive (Iba1$^+$ - a microglia/macrophage marker) cells in the Hv1$^{-/-}$ in the early time points. In agreement with this, ROS production is attenuated in the *Hv1*$^{-/-}$/SCI compared to the sham (Li et al., 2020b, 2021; Murugan et al., 2020; **Figure 2**).

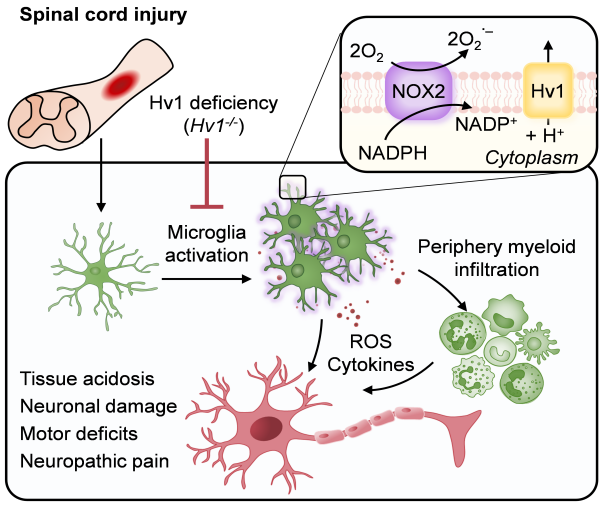

Figure 2 | Microglial Hv1 mechanisms in SCI.
Schematic figure of microglia Hv1-mediated mechanism following SCI. Cooperative function of Hv1 and NOX2 following SCI leads to excessive ROS and pro-inflammatory cytokine production, resulting in increased tissue damages. Hv1$^{-/-}$: Hv1 knockout mice; NOX 2: NADPH oxidase2; ROS: reactive oxygen species; SCI: spinal cord injury.

Moreover, same attenuated oxidative injury phenotypes in *Hv1*$^{-/-}$ was observed in other pathological models including ischemic injury and cuprizone model of multiple sclerosis (Wu et al., 2012, 2014a; Liu et al., 2015; Tian et al., 2016; Chen et al., 2020; Yu et al., 2020; Ritzel et al., 2021), suggesting that Hv1 is a promising target for controlling NOX-dependent ROS production in pathological conditions.

Hv1 regulates microglia/macrophage activation, polarization, and infiltration after SCI

Depletion of Hv1 alleviated microglia/macrophage activation after SCI. From a morphological aspect, microglia/macrophage activation is related to increased cell number, retracted processes, and enlarged soma size. Immunofluorescence staining showed increase in Iba1$^+$ cell number and enlarged soma size in both WT/SCI and *Hv1*$^{-/-}$/SCI. Compared to the WT/SCI, the *Hv1*$^{-/-}$/SCI group had fewer Iba1$^+$ cell numbers and smaller cell soma, indicating that Iba1$^+$ cells are likely to be less activated in the *Hv1*$^{-/-}$/SCI (Murugan et al., 2020). Flow cytometry results showed that SCI-induced increase

in Ly6^{Chi} monocyte and Ly6G^{+} neutrophil was attenuated in *Hv1*$^{-/-}$/SCI group compared to the WT/SCI (Li et al., 2021). However, without proliferated markers such as Ki67 or bromodeoxyuridine (BrdU), it is difficult to determine the contribution from proliferation versus infiltration/migration of microglia/macrophages to the injury site. Taking advantage of *Cx3cr1*$^{creER/+}$:*Rosa26*^{tdTomato} mice and its ability to label microglia while excluding nearly all monocyte-derived macrophages, it was demonstrated that microglia proliferate extensively in the first week post SCI (Bellver-Landete et al., 2019). However, the Hv1 may not be necessary for microglial proliferation as evidenced in a model of photothrombotic ischemic stroke (Tian et al., 2016). In this study, researchers performed BrdU incorporation and found no difference of Iba1^{+} BrdU^{+} cell number between the injured *Hv1*$^{-/-}$ and WT, suggesting Hv1 deficiency may not affect Iba1^{+} proliferation. Taken together, these results suggest that the increased Hv1 might regulate microglial activation independent of proliferation. Further, distinctions in microglia versus macrophage Hv1 is needed to fully appreciate the immune sequelae following SCI.

The microglia/macrophage polarization is a broad terminology, indicating the regulation of distinct patterns of cell programming in response to stimulus (Murray et al., 2014; Qin et al., 2019). Although markers may overlap depending on stimulus, in general, anti-inflammatory microglia/macrophage are considered to express CD206, Arginase 1, chitinase-like 3, downregulating the inflammation responses and repairing tissue damages, whereas pro-inflammatory microglia/macrophage produce cytokines such as interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α), nitric oxide, and ROS (Tang and Le, 2016). Compared to the WT/SCI, in the *Hv1*$^{-/-}$/SCI, the proportion of CD206, Arginase 1, chitinase-like 3 increases, while the proportion of IL-1β, TNF-α, Cxcl10, CD68, Nos2, ROS downregulates, indicating loss of Hv1 shift the microglia/macrophage polarization from pro-inflammatory to anti-inflammatory status (Li et al., 2020b, c, 2021; Murugan et al., 2020). The increased anti-inflammatory proportion of microglia/macrophage in the *Hv1*$^{-/-}$ was also observed in stroke (Tian et al., 2016; Qin et al., 2019) and traumatic brain injury (Ritzel et al., 2021).

Relevantly, the *Hv1*$^{-/-}$/SCI also shows reduction of periphery myeloid infiltration (Li et al., 2021). The reduced infiltration may be due to the decreased pro-inflammatory signals being released from activated microglia. This phenotype is consistently observed in the mice with NOX2 deficiency (*Nox2*$^{-/-}$) after SCI. Less CD11b^{+} CD45^{hi} F4/80^{+} macrophages were detected by flow cytometry in the *Nox2*$^{-/-}$ at 24 hours post injury (Sabirzhanov et al., 2019). Again, the similarities in phenotypes between *Nox2*$^{-/-}$/SCI and *Hv1*$^{-/-}$/SCI indicate the cooperative function of Hv1 and NOX2 in the pathology.

Hv1 contributes to tissue acidosis after SCI

Increased tissue acidosis was reported in many neurological disorders including psychiatric disorders, including schizophrenia, bipolar disorder, and autism spectrum disorder (Hagihara et al., 2018). Early investigations in the 1970s showed elevated lactate levels in the cerebrospinal fluid as a consequence of excessive ROS in a mouse model of contusion injury (Anderson et al., 1976). A recent study systematically characterized the local pH changes after SCI (Li et al., 2021). The extracellular pH recording was performed after saline perfusion and a post-mortem laminectomy exposing the spinal cord using a micro electrode probe. The researchers found that acidosis was most evident at the thoracic injury site which remained elevated at 7 days after injury. Moreover, both ascending cervical and descending lumbar regions show pH decrease indicative of acidosis. In the cervical region, acidosis was noted at 1 day post injury but returned to baseline level by 3 days. In the lumbar region, a persistent decrease of pH continued for 7 days. They then used *Hv1*$^{-/-}$ mice and found a significant reduction in tissue acidosis after SCI compared to WT. Since Hv1 is selectively expressed in microglia and some infiltrated myeloid cells, the current evidence also underscores the contributions of myeloid cells in tissue acidosis. Indeed, as a proton-releasing channel, Hv1 activation is able to increase extracellular protons that can activate acid-sensing ion channels (Zeng et al., 2015). Consistent with the role of Hv1 in tissue acidosis, Li et al. (2021) found both L-lactate and ROS concentration was decreased in the *Hv1*$^{-/-}$/SCI. However, in current understanding, astrocytes but not microglia/macrophage are sources for releasing lactate (Proia et al., 2016). When treating mice with PLX5622 to induce microglia ablation, approximately 40–55% of astrocytes at the lesion epicenter and in adjacent areas were reduced at 7 days post SCI compared to the control group. This suggests that microglia release molecules to trigger astrocyte proliferation and astrocyte scar formation post the injury (Bellver-Landete et al., 2019). Therefore, the reduced lactate in the *Hv1*$^{-/-}$/SCI may be an indirect outcome of reduced microglia-derived signals to astrocytes.

Serendipitously, the researchers also noted a difference in baseline extracellular pH between *Hv1*$^{-/-}$ and WT sham with *Hv1*$^{-/-}$ noting approximately 0.1 pH unit higher than the WT/sham, although there is no lactate nor ROS concentration difference between the sham groups (Li et al., 2021). Our latest understanding of Hv1 channel is that it is supposed to be dormant in the sham, while activated only during membrane depolarization and intracellular acidosis. If we exclude the possibility of experimental error, the baseline difference may indicate unknown Hv1 channel functions in physiological conditions.

Furthermore, although L-lactate contributes to tissue acidosis and its decrease in the *Hv1*$^{-/-}$/SCI relates to a protective phenotype, it should be noted that L-lactate may also play beneficial roles during the regeneration. A recent research indicated that glial reprogramming leads to L-lactate accumulation and L-lactate being transported out of glia cells contributes to axon regeneration. Injection of a mixture of ethyl-L-lactate and sodium L-lactate boosted axon regeneration (Li et al., 2020a). Similar to ROS being a requirement for axonal regeneration (Hervera et al., 2018), researchers should take a dialectical perspective on the roles of L-lactate and tissue acidosis in the subacute phase of SCI.

Hv1 contributes to neuronal damage following SCI

Increased Hv1 expression and activation in SCI is linked to secondary tissue damage including neuronal loss, white matter injury and reduced myelination. These anatomical changes mediated by Hv1 after SCI were in turn found to be linked to behavioral outcomes such as worsened motor deficits. This section briefly describes recent studies that assessed the contribution of Hv1 to these anatomical and behavioral deficits. In these studies, the assessment of (i) neuronal loss and tissue damages were made using histological measures, such as NeuN and cresyl violet staining, (ii) the spared white matter was measured by hematoxylin-eosin and Luxol fast blue staining, (iii) the myelin loss was measured by myelin basic protein (MBP) staining, (iv) the volume of glial scar measured by glial fibrillary acidic protein staining, and (v) the locomotor recovery measured by BMS score.

The attenuated neuronal loss in the *Hv1*$^{-/-}$/SCI was identified to be due to reduced pyroptosis (Li et al., 2020c). Pyroptosis is a type of regulated cell death that depends on the enzymatic activity of pro-inflammatory proteases. Pyroptosis has been observed in myeloid cells, CD4^{+} T cells, epithelial cells, endothelial cells, and neurons. Pro-inflammatory mediators such as proIL-1β, Nlrp3 inflammasome, and caspase-11 are priming signals for pyroptosis. Then the assembly of inflammasome and caspase-1 activation triggers pyroptosis.

One of the hallmarks of pyroptosis is gasdermin D (He et al., 2015; Vande Walle and Lamkanfi, 2016). In the *Hv1*$^{-/-}$/SCI, the reduced pyroptosis was claimed based on the reduced Nlrp3, apoptosis-associated speck-like protein containing a CARD (ASC) responsible for recruiting caspase-1, caspase-1, and the proportions of gasdermin D^{+} neurons (Li et al., 2020c). The key question here is how Hv1 channel is linked with inflammasome activation, possibly through reduced proton or even indirectly through Ca^{2+} elevation (Yi et al., 2021b).

Apart from reduced neuronal loss, depletion of Hv1 also results in attenuated apoptosis of oligodendrocytes and ameliorated myelin loss (Li et al., 2020b). Similar to this, hematoxylin and eosin staining of the lesion area at 7 days after SCI revealed that the *Hv1*$^{-/-}$/SCI has increased spared white matter (Murugan et al., 2020). The prevention of myelin loss in the *Hv1*$^{-/-}$/SCI was prominent near the lesion epicenter and was also observed at distances up to 1500 μm rostral and caudal to the injury epicenter (Li et al., 2020b). Glial fibrillary acidic protein staining also revealed that the volume of lesion epicenter, which is the unstained area enclosed by glial fibrillary acidic protein^{+} cells, is smaller in the *Hv1*$^{-/-}$/SCI compared to the WT/SCI (Li et al., 2021). A recent study showed that damage caused to myelin sheath in a lysophosphatidylcholine model of demyelination was attenuated in *Hv1*$^{-/-}$ mice (Chen et al., 2020). Further, they showed that microglial Hv1 deficiency ameliorated demyelination through inhibition of ROS-mediated autophagy. This suggests that inhibition of ROS-mediated autophagic activation of microglia may be another mechanism for prevention of myelin loss noted in the *Hv1*$^{-/-}$ mice after SCI.

The most interesting aspect in these studies is that the rescue of anatomical features (neuronal loss, white matter damage, myelin loss) in *Hv1*$^{-/-}$mice corresponded with the improved locomotor recovery noted in these mice compared to the WT. Hind limb locomotor function was evaluated by the BMS score, and the areas of stepping frequency, coordination, paw position, trunk stability, and tail position were measured by the BMS subscore. The *Hv1*$^{-/-}$/SCI showed better mobility function recovery compared to the WT/SCI starting as early as the subacute phase, and the significant difference persisted till the end of the 8-week observation period (Murugan et al., 2020; Li et al., 2021; **Figure 2**).

Hv1 in Neuropathic Pain

After SCI, up to 80% of patients develop or experience clinically significant neuropathic pain (Lee et al., 2013). Recent studies have demonstrated the critical role of microglia in chronic pain (Zhuo et al., 2011, 2019; Gu et al., 2016; Peng et al., 2016; Inoue and Tsuda, 2018; Yi et al., 2021a). Although there has not been any direct link between Hv1 and neuropathic pain, NOX2-derived ROS in microglia/macrophage has been reported to contribute to peripheral nerve injury-induced neuropathic pain (Kim et al., 2010). Macrophages are recruited to dorsal root ganglion in response to peripheral nerve injury and increase ROS production via a NOX2-dependent manner. Mice lacking NOX2 (*Nox2*$^{-/-}$) displayed absence of injury-induced upregulation of TNF-α, decreased activating transcription factor 3 induction, and reduced neuropathic pain behavior (Kallenborn-Gerhardt et al., 2014). Given the established link between Hv1-NOX and between NOX2 and the development of neuropathic pain, it is possible that microglial Hv1 might be implicated in the initiation and maintenance of neuropathic pain after SCI and warrants further investigation.

Hv1 in Systemic Inflammation Beyond the Injury Site

SCI-induced systemic inflammatory response syndrome is a life-threatening condition affecting distal organs, including lung, spleen, cardiovascular system, gastrointestinal tract, and urinary system. In the circulation, increased immune cells and pro-inflammatory signals after the injury may result in the immune cell infiltration into secondary organs and prolong inflammatory microenvironment leading to organ dysfunction (Sun et al., 2016). In the blood samples taken at 6, 12, 24, 48, and 72 hours, 1 and 2 weeks post SCI and from corresponding controls, researchers found oxidative activity increased significantly at 12, 24, and 48 hours and 1 week in neutrophils and monocytes (Bao et al., 2009). As discussed in the previous section, the oxidative activity in neutrophils and monocytes is likely through a NOX2-depedent manner. To date, the Hv1 channel has been documented in many immune cell types, including macrophages (Subramanian Vignesh et al., 2013), neutrophils (Okochi et al., 2009; Ramsey et al., 2009; El Chemaly et al., 2010), eosinophils (Zhu et al., 2013), plasmacytoid dendritic cells (Montes-Cobos et al., 2020), B cells (Capasso et al., 2010), and T cells (Sasaki et al., 2013; Asuaje et al., 2018). All these cell types may directly or indirectly contribute to the overall immune response after a traumatic injury. Therefore, although no study reported the role of Hv1 in SCI-induced systemic inflammation, the current understanding of Hv1-NOX2 cooperation may shed light on alleviation of systemic inflammation by targeting Hv1.

Concluding Remarks

Despite the recent surge in reports supporting the detrimental role of microglial Hv1 in SCI, there are still many open questions regarding the mechanisms of Hv1 activation. Future studies are needed to fully appreciate the functions of Hv1 channel in microglial activation, particularly in the context of SCI. Highlighted here are some of the gaps in the field: (1) What are the Hv1-mediated mechanisms other than NOX2 and acidosis? Recent studies show neuronal activity regulates microglia calcium signals (Umpierre et al., 2020; Umpierre and Wu, 2021), although the mechanism is still unclear. Ca^{2+} signaling is known to play a key role regulating microglia activation (Laprell et al., 2021). Activation of Hv1 induces membrane hyperpolarization, which may relate to calcium influx due to the increased driving force for calcium ions. (2) While microglial Hv1 is gaining attention, other immune cells such as neutrophils and macrophages also express Hv1 and are often overlooked. These peripheral immune cells may be important mediators in SCI. Hence, the role of Hv1 in other cell types need to be elucidated in detail, for instance, using single-cell RNA sequencing analysis; (3) Improved research tools, particularly, the development of conditional knockout animals is another approach to investigate cell-specific roles of Hv1 in SCI and related pathological mechanisms; (4) Development of selective small molecule inhibitors and activators are needed for translating the current experimental findings to clinical data. This will be particularly useful in testing whether targeting Hv1 can prevent inflammation, tissue acidosis, secondary injury, and even neuropathic pain after SCI in preclinical models. Overall, this review highlights the significance and novelty of targeting Hv1 in patients with SCI and related neurological disorders.

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