



REVIEW

# Neuroprotective Effects of Molecular Hydrogen: A Critical Review

Wei Chen<sup>1,2</sup> · Han-Ting Zhang<sup>3</sup> · Shu-Cun Qin<sup>1,2</sup>

Received: 19 February 2020 / Accepted: 31 May 2020  
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**Abstract** Molecular hydrogen (H<sub>2</sub>) is a physiologically inert gas. However, during the last 10 years, increasing evidence has revealed its biological functions under pathological conditions. More specifically, H<sub>2</sub> has protective effects against a variety of diseases, particularly nervous system disorders, which include ischemia/reperfusion injury, traumatic injury, subarachnoid hemorrhage, neuropathic pain, neurodegenerative diseases, cognitive dysfunction induced by surgery and anesthesia, anxiety, and depression. In addition, H<sub>2</sub> plays protective roles mainly through anti-oxidation, anti-inflammation, anti-apoptosis, the regulation of autophagy, and preservation of mitochondrial function and the blood-brain barrier. Further, H<sub>2</sub> is easy to use and has neuroprotective effects with no major side-effects, indicating that H<sub>2</sub> administration is a potential therapeutic strategy in clinical settings. Here we summarize the H<sub>2</sub> donors and their pharmacokinetics. Meanwhile, we review the effectiveness and safety of H<sub>2</sub> in the treatment of various nervous system diseases

based on preclinical and clinical studies, leading to the conclusion that H<sub>2</sub> can be a simple and effective clinical therapy for CNS diseases such as ischemia-reperfusion brain injury, Parkinson's disease, and diseases characterized by cognitive dysfunction. The potential mechanisms involved in the neuroprotective effect of H<sub>2</sub> are also analyzed.

**Keywords** Molecular hydrogen (H<sub>2</sub>) · Neurological disease · Neuroprotection · Anti-oxidation · Anti-inflammation · Anti-apoptosis

## Introduction

Molecular hydrogen (H<sub>2</sub>) was first discovered by the chemist Henry Cavendish in 1766. It is a colorless, odorless, and physiologically inert gas. The biological functions of H<sub>2</sub> were gradually confirmed by scientists in the late 20th century. In 1975, Dole [1] discovered that hyperbaric H<sub>2</sub> (2.5% O<sub>2</sub> and 97.5% H<sub>2</sub>) causes regression of squamous cell carcinoma in hairless albino mice. He hypothesized that the effect of H<sub>2</sub> might be attributed to its ability to scavenge the most damaging oxidant hydroxyl radical ( $\cdot\text{OH}$ ). In 2001, Gharib [2] found that 0.7 MPa H<sub>2</sub> has an anti-inflammatory effect on the chronic liver inflammation associated with schistosomiasis. Several years later, in 2007, Shigeo Ohta demonstrated that H<sub>2</sub> exhibits protective effects as an antioxidant on brain tissues under oxidative stress by selectively scavenging the cytotoxic free radical  $\cdot\text{OH}$  and peroxynitrite (ONOO<sup>-</sup>) [3]. Since then, these findings have led to a number of studies that have explored the potential protective effects of H<sub>2</sub> against a variety of diseases and the related molecular mechanisms.

✉ Han-Ting Zhang  
hzhang@hsc.wvu.edu

✉ Shu-Cun Qin  
13583815481@163.com

<sup>1</sup> Taishan Institute for Hydrogen Biomedicine, Shandong First Medical University and Shandong Academy of Medical Sciences, Tai'an 271000, China

<sup>2</sup> Key Laboratory of Atherosclerosis in Universities of Shandong and Institute of Atherosclerosis, Shandong First Medical University and Shandong Academy of Medical Sciences, Tai'an 271000, China

<sup>3</sup> Departments of Neuroscience and Behavioral Medicine and Psychiatry, Rockefeller Neurosciences Institute, West Virginia University Health Sciences Center, Morgantown, WV 26506, USA

Diseases caused by abnormalities in the structure and function of the nervous system are usually most devastating disorders with a high incidence of disability and mortality worldwide. The recovery of neural structure and function from either acute injury or chronic neurodegeneration remains challenging, as most neurological diseases do not have effective approaches to cure and are poorly responsive to traditional interventions such as medications, physical therapies, neuro-rehabilitation, and preventative measures. In contrast, accumulating evidence has shown protective effects of H<sub>2</sub> against various neurological diseases, including ischemia/reperfusion injury [4–6], traumatic damage [7, 8], subarachnoid hemorrhage (SAH) [9, 10], neuropathic pain [11, 12], Alzheimer's disease [13, 14], Parkinson's disease [15, 16], mood disorders [17, 18], glioblastoma [19], and cerebral infarction [20]. To date, more than 60 clinical trials on the use of H<sub>2</sub> in many diseases involving multiple systems have been conducted; at least ten of the trials were on diseases in the nervous system, including acute cerebral ischemia [4], acute cerebral infarction [20], post-cardiac arrest syndrome [21, 22], Parkinson's disease [23, 24], and mood disorders [18].

As a neuroprotective gas, H<sub>2</sub> has a variety of advantages. First, it can cross the blood-brain barrier (BBB), penetrate biomembranes, and diffuse into the cytosol and organelles [3]. In addition, it has no documented major side-effects [4]. Importantly, repeated administration of H<sub>2</sub> does not cause tolerance [25]. Further, various easy and convenient approaches to its administration are available [26, 27]. Finally, H<sub>2</sub> has protective effects against multiple diseases, including peripheral and central nervous system (CNS) diseases [26, 28]. For instance, in Japan, 2% H<sub>2</sub> inhalation has been approved for clinical emergency treatment for cardiac arrest. Thus, H<sub>2</sub> is a novel and potential therapeutic strategy for the prevention and treatment of a variety of diseases, including neurological diseases. The advantages of H<sub>2</sub> have further promoted the development of the H<sub>2</sub> healthcare industry.

In this review, we aim to summarize the current knowledge about the neuroprotective effects of H<sub>2</sub> against various diseases in the nervous system (Fig. 1) and the possible mechanisms involved (Table 1).

## H<sub>2</sub> Donors

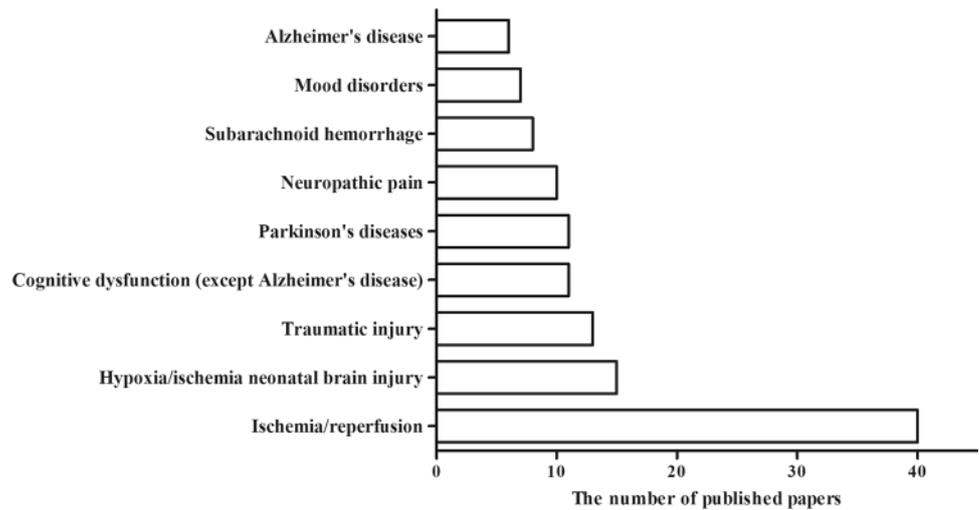
Currently, there are multiple routes of H<sub>2</sub> administration, including inhalation of H<sub>2</sub> gas, drinking H<sub>2</sub>-rich water (HRW), injection of H<sub>2</sub>-rich saline (HRS), H<sub>2</sub>-water bathing, intake of a solid H<sub>2</sub> carrier (coral calcium hydride), and H<sub>2</sub>-producing precursors (such as lactulose

and L-arabinose) [27, 65–67], as well as functional micro/nanomaterials for targeted H<sub>2</sub> delivery [68].

In clinical applications, the common routes of H<sub>2</sub> administration mainly include inhalation of H<sub>2</sub> gas, drinking HRW, injection of HRS, and H<sub>2</sub>-water bathing. Inhalation H<sub>2</sub> is the simplest and most direct route, and the most commonly used concentrations are 1%–4% (safe concentration). Compared with inhaling H<sub>2</sub> gas, drinking HRW is safer and more convenient. HRS is usually administered by intravenous or intraperitoneal injection. Hydrogen bathing is often used for the treatment of skin diseases [69]. When H<sub>2</sub> is given by drinking HRW, 59% of the ingested H<sub>2</sub> is released in the breath, ~ 0.1% is released from the body surface, and ~ 40% is consumed in the body [70]. After consumption of 500 mL of HRW within 1 min in volunteers, the concentration of breath H<sub>2</sub> reaches a peak level of ~ 36 ppm at 10 min and then gradually drops to the baseline level of ~ 7.0 ppm after 60 min [70]. Ono *et al.* found that the H<sub>2</sub> concentration in both the arterial and venous blood rapidly increases and reaches a plateau level (10 μmol/L to 20 μmol/L) in 20 min after the initiation of 3% and 4% H<sub>2</sub>. When H<sub>2</sub> inhalation is discontinued, the H<sub>2</sub> concentration in arterial blood decreases to < 10% of the plateau level in ~ 6 min, but in ~ 18 min in venous blood [4]. During 30 min of intravenous HRS (0.8 mmol/L) infusion, the concentration of H<sub>2</sub> in both the arterial and venous blood rapidly increases to a maximum (< 1.8 ppm) at ~ 15 min and rapidly decreases with the cessation of HRS infusion [71]. Generally speaking, inhalation leads to a higher blood H<sub>2</sub> concentration than intravenous infusion [71]. Animal experiments have also shown that inhalation induces higher H<sub>2</sub> concentrations in the brain than the other routes of H<sub>2</sub> administration (oral, intraperitoneal, intravenous) [72]. These results suggest that inhalation is the preferred route in H<sub>2</sub> therapy for CNS disease.

Although inhalation, oral ingestion, or injection of H<sub>2</sub> effectively alleviates diseases in the nervous system, as demonstrated by different research groups [6, 20, 73], there is a lack of comparisons in terms of biological effects between different hydrogen intervention methods in a specific disease. As the H<sub>2</sub> concentrations in tissues and organs significantly differ according to which intervention is selected [72, 74], its administration through different routes may have varied effects in the same damaged tissues. Moreover, only a few studies have focused on dose- and time-dependent effects and tolerance to H<sub>2</sub> in basic and clinical studies. To choose the most effective H<sub>2</sub> therapy method for each disease, it is therefore important to further understand the pharmacokinetics and therapeutic effects of different types of H<sub>2</sub> donors. In mouse models of Alzheimer's disease, intracerebral injection of Pd hydride (PdH) nanoparticles reduces the over-generation of

**Fig. 1** The numbers of published papers (clinical trials and animal experiments) in the PubMed database on the use of H<sub>2</sub> in various neurological diseases from 2007 to 2020.



amyloid beta (A $\beta$ ) in the brain [14], whereas the intake of HRW does not show a similar effect [13]. H<sub>2</sub> has low solubility, and its concentrations in the brain of mice are significantly lower (< 30 ppb/g) *via* traditional administration (intake of HRW, injection of HRS, or inhalation of 4% H<sub>2</sub>) [72]. The PdH nanoparticle, which is a high-payload H<sub>2</sub> storage material, sustainably releases ~ 6  $\mu$ mol/L H<sub>2</sub> within 60 h [14, 75]. This phenomenon has indicated that A $\beta$  clearance may be correlated with the concentration and duration of H<sub>2</sub> in the brain. Namely, a high H<sub>2</sub> concentration in the target tissue has a better performance than a low concentration. To maximize the therapeutic action of H<sub>2</sub>, the developing effective storage and targeted delivery by H<sub>2</sub> donors may be one of the future research directions.

## Protective Effects of H<sub>2</sub> Against Nervous System Diseases

### Ischemia/Reperfusion Injury

Ischemia/reperfusion injury is a condition characterized by tissue damage caused by an ischemic or anoxic period, followed by the re-establishment of blood supply to the tissue. Ischemia/reperfusion injury in the CNS is associated with disorders such as stroke, brain trauma, cerebral infarction, and cardiac arrest.

The restoration of circulation after deprivation of an adequate supply of O<sub>2</sub> in the blood induces a burst of reactive oxygen species (ROS), which then triggers inflammatory responses and oxidative damage. The production of ROS may directly destroy cell membranes by inducing lipid peroxidation, and thus antioxidant agents are considered as a therapeutic option. First, H<sub>2</sub> has been considered as an antioxidant that can buffer the destructive

effect of oxidative stress in the brain after focal ischemia/reperfusion by selectively reducing cytotoxic oxygen radicals [3]. This is the basis on which *in vivo* studies have been conducted to validate the protective role of H<sub>2</sub> in ischemia/reperfusion injury in the CNS. In animal models of cerebral ischemia/reperfusion, inhalation of 66.7% H<sub>2</sub> significantly increases the activity of SOD and GSH-Px, reduces the level of malondialdehyde (MDA), reduces infarct volume, alleviates brain edema and hemorrhage, and improves neurobehavioral deficits [5, 76, 77]. Brain ischemia/reperfusion injury is a common secondary effect of cardiac arrest which is responsible for mortality and morbidity after cardiopulmonary resuscitation. In experimental cardiac arrest/resuscitation, H<sub>2</sub> intervention also significantly diminishes neurologic injury and improves the survival rate and neurological outcome in animals [78, 79]. H<sub>2</sub> inhalation or injection after cardiac arrest effectively controls neuronal death and microglial activation in the hippocampus and decreases the serum S100 $\beta$  protein level [80–82]. In addition, H<sub>2</sub> inhalation alone or in combination with therapeutic hypothermia has been demonstrated to be superior to hypothermia alone [38, 80, 81, 83].

Clinical ischemia/reperfusion does not often occur in stroke and cerebral infarction, whereas patients who receive thrombolysis treatment or interventional thrombectomy are more likely to develop the most feared brain reperfusion injury. Importantly, the safety and efficacy of H<sub>2</sub> has been confirmed in clinical trials for cerebral ischemia, cerebral infarction, and cardiac arrest. In patients with acute cerebral ischemia, inhalation of 3% H<sub>2</sub> for 30 min or intravenous H<sub>2</sub> administration can deliver sufficient H<sub>2</sub> in the blood without compromising safety [4, 84]. A randomized controlled clinical study of patients with acute cerebral infarction showed that inhalation of 3% H<sub>2</sub> gas for 1 h twice a day for 7 days improved O<sub>2</sub> saturation without causing adverse effects. Patients who receive H<sub>2</sub> inhalation

**Table 1** Neuroprotective effects of H<sub>2</sub> against neurological diseases and related mechanisms.

Disease type	Object	Hydrogen administration	Mechanism	References
Ischemia/reperfusion injury in the CNS	Rat, mouse, rabbit, pig, human	Inhalation, intraperitoneal injection, oral, intravenous injection	Preservation of BBB; preservation of mitochondrial function; inhibition of endoplasmic reticulum stress; anti-oxidation; anti-inflammatory; anti-apoptosis (PI3K/Akt/GSK3 $\beta$ signaling pathway, Cyt c/caspase-3 pathway); microRNA regulation	[5, 6, 20, 29–36]
Hypoxia/ischemia neonatal brain injury	Rat, mouse, pig	Inhalation, intraperitoneal injection	Alleviation of oxidative stress (MAPK/HO-1/PGC-1 $\alpha$ pathway); inhibition of endoplasmic reticulum stress; promoting autophagy; regulating microglial polarization; improving neurovascular dysfunction	[37–41]
Traumatic injury in the CNS	Rat, mouse	Inhalation, intraperitoneal injection, subarachnoid perfusion	Preservation of BBB; attenuation of neuronal apoptosis; preservation of mitochondrial function; anti-oxidation; regulation of oxidative stress-related genes; suppression of astrocyte activation	[7, 8, 42–45]
Subarachnoid hemorrhage	Rat, rabbit,	Inhalation, intraperitoneal injection	Preservation of BBB; amelioration of cerebral vasospasm; anti-apoptosis (Akt/GSK3 $\beta$ signaling pathway); inhibition of oxidative stress; inhibition of NF- $\kappa$ B activation and NLRP3 inflammasome formation	[9, 10, 46–49]
Neuropathic pain				
Hyperalgesia	Rat	Intraperitoneal injection	Anti-inflammatory; inhibition of GSK-3 $\beta$ activity and NMDA receptor membrane trafficking; inhibition of oxidative stress (removing ONOO <sup>-</sup> and blocking MnSOD nitration)	[50–52]
Nerve injury	Rat, mouse	Intrathecal infusion, oral, intraperitoneal injection	Suppression of oxidative stress; inhibition of spinal astrocytes and microglia activation; inhibition of inflammation by activating HO-1/CO signaling; down-regulation of p38MAPK and BDNF expression; activation of autophagy <i>via</i> HIF-1 $\alpha$ pathways	[11, 12, 25, 53, 54]
Parkinson's disease	Rat, mouse, human	Inhalation, oral	Inhibition of oxidative stress; prevention of the dopaminergic cell loss; activation gastric ghrelin system	[15, 16, 23, 55–58]
Alzheimer's disease	Rat, mouse	Oral, intraperitoneal injection, intracerebral injection	Recovering mitochondrial dysfunction; enhancing the anti-oxidative system by stimulating AMPK and up-regulating Sirt1-FoxO3a axis; suppression of inflammatory response by inhibiting JNK, NF- $\kappa$ B, and NLRP3 activation; activation of E <sub>2</sub> -ER $\beta$ -BDNF signaling pathways	[13, 14, 31, 59–61]
Mood disorders	Mouse, human	Inhalation, oral, intraperitoneal injection	Suppression of oxidative stress, anti-apoptosis and anti-inflammation; regulation of hypothalamus-pituitary-adrenal axis activity	[17, 18, 62–64]

therapy have a significantly decreased infarction site, and experience better improvement in the neurological status and the ability to execute activities of daily living relative to controls [20]. Furthermore, intravenous H<sub>2</sub> administration in combination with edaravone has more evident and significant favorable effects than edaravone administration alone [85]. In a human study of patients with post-cardiac arrest syndrome, inhalation of a low concentration of H<sub>2</sub> for 18 h had a favorable cerebral performance category score after 90 days, with no adverse events reported [21]. To further evaluate the efficacy and safety of H<sub>2</sub> inhalation, a larger, phase II clinical trial is being conducted in patients with post-cardiac arrest syndrome in Japan [22]. Although no side-effects of H<sub>2</sub> have been found in animal studies, the potential adverse effects should be investigated further, as

diarrhea has been reported in a small number of patients after receiving H<sub>2</sub> therapy [84].

### Hypoxic/Ischemic Neonatal Brain Injury

Hypoxic/ischemic brain injury is a leading cause of death and disability during the perinatal period, and an effective treatment is not available. However, recent studies using rodent models of neonatal hypoxia/ischemia have shown that H<sub>2</sub> can protect neonatal brains from injury by hypoxia/reoxygenation. Several studies have demonstrated that intraperitoneal injection of HRS significantly suppresses autophagy and neuro-inflammation, promotes M2 microglia polarization, rescues synaptic loss, and then restores behavioral deficits in a neonatal mouse model of hypoxia/

ischemia [39, 40]. Similar results have been obtained from hypoxia/ischemia models in neonatal rats and piglets [37, 38]. In a rat model of neonatal hypoxic-ischemic encephalopathy, H<sub>2</sub> inhalation has been shown to reduce the infarct size, neuronal loss, and astrocyte activation in the cortex and hippocampal CA3 region [37]. Meanwhile, the early behavioral reflexes of neonatal rats significantly improve after inhalation of H<sub>2</sub> [86]. A study by Htun *et al.* shows that H<sub>2</sub> ventilation combined with mild hypothermia improves the neurological score and walking function in a 5-day neonatal hypoxia/ischemia piglet model [38]. H<sub>2</sub> has not only short-term neuroprotective effects but also long-term neurological and neurobehavioral effects. Ten weeks after the hypoxic/ischemic insult in a neonatal rat model, early administration of H<sub>2</sub> also improves learning and memory [37]. The neurovascular unit is necessary for maintaining the fragile homeostasis of the brain. ROS produced in the early reoxygenation period severely decreases cerebrovascular reactivity and induces dysfunction of the neurovascular unit. H<sub>2</sub> preserves cerebrovascular reactivity and alleviates the development and persistence of delayed neurovascular dysfunction caused by hypoxic stress in newborn piglets [41, 87]. These findings taken together indicate that the use of H<sub>2</sub> may be considered a therapeutic approach to neonatal brain injury after asphyxia.

### Traumatic Injury

The incidence of traumatic injury in the brain and spinal cord is constantly increasing in modern society. Although progress has been made in prophylactic and therapeutic treatment of traumatic injury, recovery of neural function remains a huge challenge. In animals, trauma in the brain and spinal cord causes hemorrhage, edema, cell death, inflammatory cell infiltration, and increases BBB permeability and neurological deficits; however, H<sub>2</sub> treatment significantly improves injuries and promotes the recovery of nerve function [7, 8, 42, 88, 89].

H<sub>2</sub> or HRS treatment decreases the expression of caspases-3, caspase-9, and Bax, increases the expression of Bcl-2, and significantly attenuates neuronal apoptosis after mechanical injury [8, 42]. H<sub>2</sub> treatment also decreases oxidative products, such as MDA, 8-iso-prostaglandin F<sub>2</sub> $\alpha$ , 8-hydroxydeoxyguanosine (8-OHdG), and carbonyl protein, increases endogenous antioxidant enzymatic activity (SOD, CAT, and GPx), suppresses the levels of MPO, NOX2, and NOX4, and elevates Sir2, PrxIII, Trx2, and CGRP in contused brain and spinal cord [42, 45, 90, 91]. Moreover, Dohi *et al.* have demonstrated that treatment with HRW reverses the expression of genes involved in oxidative stress, carbohydrate metabolism, and neuroinflammation after traumatic brain injury [43]. Furthermore,

HRS treatment controls the inflammatory process in the brain tissues of traumatic brain injury-challenged rats by decreasing the levels of pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , and HMGB-1) and the number of inflammatory cells (Iba1) and inflammatory metabolites (Cho) [88, 89]. In addition, H<sub>2</sub> molecules may even suppress reactive astrogliosis, which is correlated with oxidative injury in the spinal cord [44]. Notably, in the contused spinal cord of rats, HRS attenuates the local release of pro-inflammatory cytokines and the production of specific markers (STAT3, p-STAT3, and GFAP) expressed by astrocytes, as well as suppressing astrogliosis [44].

Survivors of traumatic brain injury often present with cognitive impairment, including impaired learning and memory. These deficits can be reversed by HRS treatment, as indicated by improved cognitive performance in the Morris water maze after mild traumatic brain injury in the presence of HRS [91]. HRS may ameliorate cognitive deficits after trauma by maintaining synaptic plasticity. In rats with traumatic brain injury, HRS significantly elevates the levels of brain-derived neurotrophic factor (BDNF), calcium/calmodulin-dependent protein kinase II, synapsin I, and cyclic AMP-response element binding (CREB) protein in the hippocampus [91]. These molecules are involved in the mediation of synaptic plasticity and cognition. Generally, H<sub>2</sub> not only alleviates traumatic brain injury *via* its common anti-oxidative, anti-inflammatory, and anti-apoptotic effects, but also attenuates traumatic brain injury-induced cognition disorders by improving neuronal synaptic plasticity.

### Subarachnoid Hemorrhage

Subarachnoid hemorrhage is a devastating cerebrovascular event with high morbidity and mortality, and a poor prognosis. Oxidative stress is a key factor involved in the pathogenesis of early brain injury after SAH. Therefore, an antioxidant therapy that involves scavenging free radicals is effective in its treatment. H<sub>2</sub> is a promising therapeutic method for patients with early-stage SAH. Several studies in animals have shown that HRS treatment remarkably attenuates the early brain injury 24 h after SAH [9, 10]. Similarly, inhalation of 1.3%–2.9% H<sub>2</sub> for 2 h after intracerebral hemorrhage (ICH) in rats attenuates brain edema, maintains the integrity of the BBB, reduces apoptosis and neuroinflammation, and improves neurological function, with decreased production of MDA, nitrotyrosine, and 8-OHG in the brain [46, 92]. However, H<sub>2</sub> has a neuroprotective effect 24 h after SAH (acute phase), but not after 72 h (delayed phase) [46]. Interestingly, Manaenko *et al.* have found that 2% H<sub>2</sub> inhalation for 1 h significantly decreases brain water content and improves neurological outcomes, whereas H<sub>2</sub> inhalation for 2 h does

not have any effects 24 h (acute phase) after ICH [93]. Moreover, 72 h after ICH, H<sub>2</sub> inhalation is more likely, but not significantly, to improve neurological deficits. In contrast, HRS treatment attenuates the increased levels of MDA, caspase-12, and caspase-3 and substantially alleviates the brain injury and brain edema 72 h after SAH in rabbits [49]. Based on these reports, H<sub>2</sub> may have a dose- and time-dependent effect on SAH. In addition, H<sub>2</sub> can ameliorate cerebral vasospasm, a common complication in patients with SAH. In rats, HRS attenuates neurological functional deficits and morphological vasospasm of the basilar artery after SAH [47].

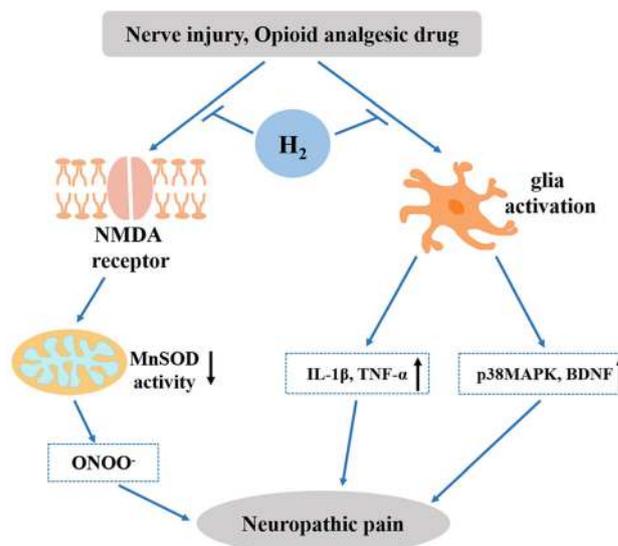
### Neuropathic Pain

Neuropathic pain is a type of pain attributed to damage or disease affecting the somatosensory nervous system; it significantly affects quality of life. Neuropathic pain is troublesome and extremely challenging to treat. Importantly, H<sub>2</sub> treatment may be a therapeutic approach to alleviating neuropathic pain under various pathological conditions, including opioid-induced hyperalgesia, spinal cord injury, and post-herpetic neuralgia.

**Postoperative hyperalgesia** Remifentanyl, a potent, short-acting, synthetic opioid analgesic, is used as an adjunct to an anesthetic during surgery to relieve pain. Hyperalgesia is a side-effect after the administration of intraoperative analgesia with remifentanyl. During the development of opioid-induced hyperalgesia, membrane trafficking of the NMDA receptor NR1 and NR2B subunits is increased in the spinal cord, and this trafficking is mediated by the activation of glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) [51]. Zhang *et al.* have found that remifentanyl infusions induce rapid and prolonged mechanical and thermal hyperalgesia and facilitate NR1 membrane trafficking and GSK-3 $\beta$  activation in the dorsal root ganglion (DRG) [51]. More importantly, HRS treatment partially attenuates remifentanyl-induced hyperalgesia without affecting the baseline nociceptive threshold, decreases the expression of inflammatory mediators (TNF- $\alpha$ , IL-1 $\beta$ , and IL-6), and suppresses NR1 membrane trafficking through the inhibition of GSK-3 $\beta$  activity in the DRG in a dose-dependent manner [51]. In a rat model of incisional postoperative pain, the production of ONOO<sup>-</sup> in the spinal cord increases after administration of remifentanyl. ONOO<sup>-</sup> activates divalent metal transporter 1 without iron-responsive element [DMT1(-)IRE] and induces abnormal iron accumulation, leading to the development of hyperalgesia [50]. Meanwhile, intraperitoneal delivery of HRS can remove ONOO<sup>-</sup> from the spinal cord, protect against remifentanyl-induced postoperative hyperalgesia, and attenuate DMT1(-)IRE activation and iron accumulation [50]. In addition, pretreatment with HRS successfully attenuates

the postoperative mechanical and thermal hyperalgesia induced by remifentanyl in incisional pain in rats, interdicts NR2B expression and membrane trafficking from the intracellular pool to the surface pool, as well as blocking MnSOD nitration in the dorsal horn [52]. These findings indicate that HRS exhibits anti-hyperalgesic effects possibly *via* the inhibition of oxidative stress and the GSK-3 $\beta$ -NMDA-MnSOD pathway (Fig. 2).

**Neuropathic pain after nerve injury** Results of a study on neuropathic pain induced by L5 spinal nerve ligation (L5 SNL) in a rat model show that intrathecal infusion of HRS relieves L5 SNL-induced mechanical allodynia and thermal hyperalgesia and provides a relatively long-lasting pre-emptive effect [25]. In chronic constriction-induced injury in another neuropathic pain rat model, intrathecal or intraperitoneal injection of HRS also significantly elevates the mechanical withdrawal threshold and thermal withdrawal latency of neuropathic pain [11, 53]. In a partial sciatic nerve ligation mouse model, the intake of H<sub>2</sub> water also significantly alleviates mechanical allodynia and thermal hyperalgesia [12]. In addition to oxidative stress, pain is also triggered by inflammation and the activation of immune cells and glial cells in the DRG [94]. The analgesic effect of H<sub>2</sub> might also be associated with the suppression of inflammation and oxidative stress. The administration of H<sub>2</sub> inhibits the activation of spinal astrocytes and microglia, reverses the overexpression of pro-inflammatory cytokines (IL-1 $\beta$ , TNF- $\alpha$ , and HMGB), and decreases the levels of tyrosine-nitrated MnSOD, MDA, protein carbonyl, 8-hydroxyguanosine (8-OHG), 8-OHdG, HNE, and MPO (Fig. 2) [12, 25, 53, 54]. In a rat model with sciatic nerve trunk ligation, HRS treatment



**Fig. 2** Protective effects of H<sub>2</sub> against neuropathic pain and its related molecular mechanisms.

alleviates pain, decreases pro-inflammatory cytokine levels, and increases HO-1 protein expression and activity in the DRG and spinal cord. Meanwhile, the effects of H<sub>2</sub> are reversed by the HO-1 inhibitor SnPP-IX and further enhanced by hemin and CORM-2. Therefore, Chen *et al.* have indicated that HO-1/CO signaling is involved in the analgesic and anti-inflammatory effects of H<sub>2</sub> in neuropathic pain [54].

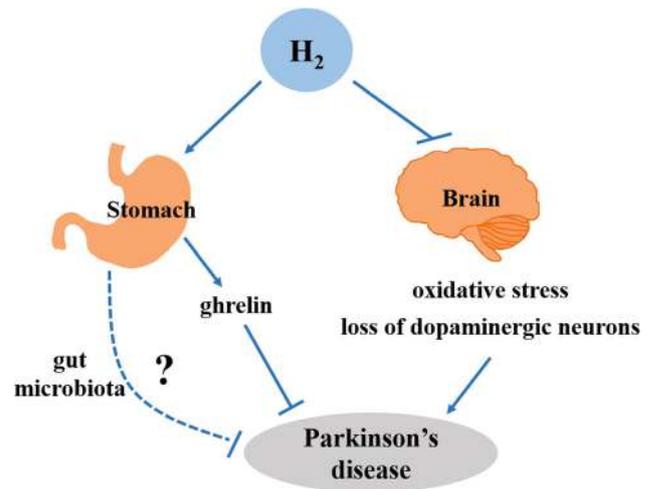
### Parkinson's Disease

Parkinson's disease is the second most common neurodegenerative disorder, mainly affecting the motor system. It is characterized by progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta. Thus far, numerous animal experiments and a few human trials have shown that H<sub>2</sub> has an attenuating effect on the development of Parkinson's disease.

A randomized double-blind placebo-controlled trial on 18 patients has found that patients drinking HRW for 48 weeks showed improvement in their total Unified Parkinson's Disease Rating Scale (UPDRS) scores, whereas patients drinking placebo water had worse UPDRS scores. The preliminary results indicate that drinking HRW is safe and well-tolerated. Moreover, it has significant effects on Parkinson's disease [23]. However, in another clinical trial with 20 Parkinson's disease patients, inhalation of 1.2%–1.4% H<sub>2</sub> for 10 min twice a day for 4 weeks had no beneficial effect [95]. Disappointingly, drinking HRW for 72 weeks also failed to improve the total UPDRS scores in 178 patients with Parkinson's disease in a randomized, double-blind, multicenter trial [96]. Although there were no significant differences in the changes of the score between the HRW drinking group and the placebo group in this trial, it demonstrated the safety of drinking HRW once again [96]. These negative results may be related to the stage of Parkinson's disease, the phase of the UPDRS score, the H<sub>2</sub> concentration in HRW, and the duration of H<sub>2</sub> inhalation.

In 6-hydroxydopamine or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) rodent models of Parkinson's disease, the administration of H<sub>2</sub> is effective in inhibiting the development and progression of the disease [16, 55–57]. Further analysis has shown that the therapeutic effects of H<sub>2</sub> in Parkinson's disease models may be correlated with the prevention of dopaminergic neuron loss in the substantia nigra, as well as a decrease in the production of 4-HNE in the SN dopaminergic neurons and accumulation of 8-oxoguanine in the striatum (Fig. 3) [16, 55, 57].

H<sub>2</sub> is one of the main intestinal gases, produced and utilized by gut microbiota. Gut microbiota are closely related to Parkinson's disease [97]. Thus, the relationship between brain and gut during H<sub>2</sub> treatment has become a



**Fig. 3** Neuroprotective effects of H<sub>2</sub> against Parkinson's disease.

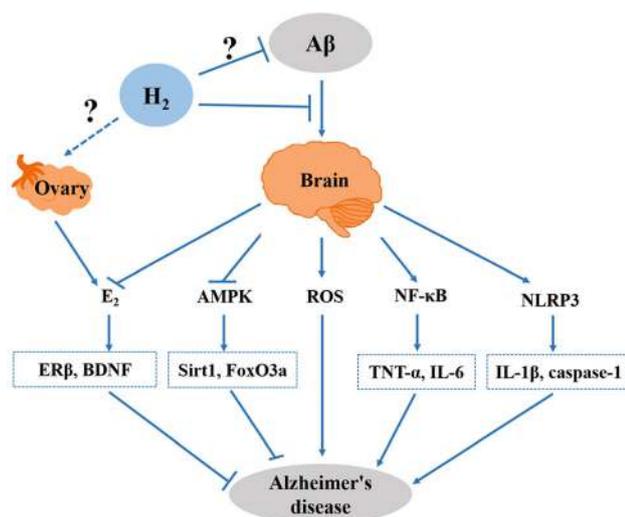
topic of interest. Matsumoto *et al.* reported that the oral intake of HRW significantly enhances ghrelin gene expression in the stomach and the ghrelin level in the plasma of mice. More importantly, activation of the  $\beta$ 1-adrenergic receptor is required for H<sub>2</sub> to induce an increase in ghrelin in plasma [58]. Thus, studies have shown that drinking HRW may ameliorate the pathological process of Parkinson's disease *via* activation of the gastric ghrelin system (Fig. 3) [58]. To further validate the contribution of ghrelin in the mouse model of H<sub>2</sub>-treated Parkinson's disease, ghrelin-knockout (KO) mice have been used. Yoshii *et al.* have found that the administration of HRW significantly decreases the loss of dopaminergic cells in ghrelin-KO mice with MPTP insult [15]. Furthermore, the administration of D-Lys<sup>3</sup>GHRP-6 is not effective in inhibiting the neuroprotective effect of H<sub>2</sub> in ghrelin-KO mice with Parkinson's disease, unlike in wild-type mice [15]. Therefore, they believed that ghrelin is not the only factor associated with the H<sub>2</sub>-induced neuroprotective effect in Parkinson's disease (Fig. 3) [15], and more studies are needed to assess the relationship between the brain and stomach during H<sub>2</sub> treatment. Mikako *et al.* have found that consumption of the H<sub>2</sub>-producing precursor lactulose (substrate for microbial fermentation) increases the H<sub>2</sub> concentration in breath and marginally ameliorates the motor deficits in a rat model of Parkinson's disease [56]. It seems that gut microbiota mediate gut-brain communication by microbial metabolites, including H<sub>2</sub> [98], or H<sub>2</sub> may modulate the gut microbiota dysbiosis in Parkinson's disease (Fig. 3).

### Cognitive Dysfunction (Alzheimer's Disease)

Alzheimer's disease is the most common neurodegenerative disorder worldwide and is the most prevalent cause of

dementia in elderly individuals. Women are more commonly affected than men. H<sub>2</sub> treatment may be a feasible option for patients with this disease, as it has been reported to improve the cognitive impairment in animal models of the disease. The accumulation of A $\beta$  deposits is widely believed to be the fundamental cause of Alzheimer's disease. Intracerebral injection of A $\beta$ 1-42 in male rats increases the level of oxidative stress in brain tissue, indicating enhanced levels of MDA and 8-OHdG [59, 60]. In a transgenic mouse model of Alzheimer's disease, the administration of H<sub>2</sub> has protective effects [13, 14]. The intake of HRW significantly reduces the level of MDA and improves the activity of T-SOD and GSH in APP/PS1 mice [13]. In mice with 3  $\times$  Tg Alzheimer's disease, the intracerebral injection of PdH nanoparticles (a high-payload H<sub>2</sub> carrier) effectively scavenges  $\cdot$ OH, reduces A $\beta$  generation and aggregation, ameliorates mitochondrial dysfunction, reverses the synaptic deficits, and inhibits neuronal death in the brain [14]. *In vitro*, H<sub>2</sub> treatment enhances the anti-oxidative system in human neuroblastoma SK-N-MC cells under A $\beta$ -stimulated oxidative stress by stimulating AMPK and up-regulating the downstream Sirt1-FoxO3a axis, which prevents mitochondrial dysfunction and the production of ROS, thereby ultimately maintaining cell survival (Fig. 4) [61].

H<sub>2</sub> improves cognitive impairment in rodent models of Alzheimer's disease not only by the reduction of oxidative stress but also by the suppression of the inflammatory response in the brain. The administration of H<sub>2</sub> effectively prevents excessive neuroinflammation in A $\beta$ 1-42-challenged mice, with the suppression of astrocyte activation and inhibition of pro-inflammatory factors (IL-1 $\beta$ , IL-6, and TNF- $\alpha$ ) in the brain [59, 60]. In APP/PS1 mice, the



**Fig. 4** Molecular mechanisms of H<sub>2</sub> in ameliorating A $\beta$ -induced Alzheimer's disease.

intake of H<sub>2</sub> water significantly reduces the mRNA levels of IL-6 and TNF- $\alpha$  in the brain (Fig. 4) [13], indicating an anti-inflammatory effect of H<sub>2</sub>.

Interestingly, H<sub>2</sub> has a sex-specific cognitive benefit in APP/PS1 mice without altering A $\beta$  clearance or APP processing. Oral HRW is effective in improving spatial learning deficits and memory impairment and estrogen (E<sub>2</sub>) levels in both the brain and serum and in increasing ER $\beta$ , BDNF, and TrkB expression in the brains of female, but not male, APP/PS1 mice. This is consistent with the recent studies showing that female 3  $\times$  Tg-AD mice display more prominent amyloid plaques, neurofibrillary tangles, neuroinflammation, and spatial cognitive deficits than male 3  $\times$  Tg-AD mice [99]. These data indicate that the mechanism underlying the beneficial effect of H<sub>2</sub> is most likely through the E<sub>2</sub>-ER $\beta$ -BDNF signaling pathway (Fig. 4) [13]. Using a mouse model of premature ovarian failure induced by zona pellucida 3, He *et al.* found that HRW improves the serum anti-Müllerian hormone levels and reduces ovarian granulosa cell apoptosis, thereby exerting a protective effect on ovarian function [100]. In addition, the ovaries primarily produce E<sub>2</sub>. Thus, we speculated that H<sub>2</sub> may alleviate cognitive impairment by improving ovarian function in female mice with Alzheimer's disease (Fig. 4). However, the precise mechanism by which H<sub>2</sub> prevents the decline of E<sub>2</sub> is unknown and needs further investigation. The overexpression of BDNF, TrkB, and synaptic proteins (postsynaptic density 95, synapsin I, and synaptophysin) [13, 14] has been reported in mouse models of Alzheimer's disease after H<sub>2</sub> treatment, which indicates that H<sub>2</sub> may have a neuroprotective effect by restoring neuronal plasticity. In APP/PS1 mice, the researchers also found that oral HRW did not decrease the concentration of A $\beta$ 42 or APP nor increase the protein expression of NEP and IDE in the cerebral cortex [13]. These phenomena indicate that H<sub>2</sub> does not have an effect on A $\beta$  clearance and APP processing in APP/PS1 mice; in other words, H<sub>2</sub> can improve the cognitive deficiency but may not inhibit or reverse the progression of the disease.

In addition to ameliorating the cognitive dysfunction in Alzheimer's disease, H<sub>2</sub> also attenuates the cognitive impairment induced by operation, isoflurane anesthesia, vascular dementia, hypoxia, radiation, stress, status epilepticus, and aging [39, 78, 101–108], which are not discussed here.

## Mood Disorders

Mood disorders (such as anxiety and depression) are common but serious mental health diseases worldwide, which have negative effects on a person's mood, daily life, work, and social communication. Most types of available anti-anxiety drugs and antidepressants are

generally safe and effective. However, > 50% of patients do not achieve complete remission after drug therapy. In addition, these drugs have side-effects and warnings regarding their use.

In recent years, animal experiments have revealed that H<sub>2</sub> has an anti-anxiety and anti-depression effect. The inhalation of 67% H<sub>2</sub> or 4 mL oral HRW per day significantly prevents depressive and anxiety-like behaviors in mice undergoing chronic mild stress (CMS) [17, 62], a rodent model of depression [109]. On the other hand, H<sub>2</sub> treatment successfully suppresses the increase in IL-1 $\beta$ , caspase-1, and ROS in the hippocampus and prefrontal cortex of depressed mice [62]. By contrast, the administration of H<sub>2</sub> also decreases the serum levels of corticosterone (CORT), ACTH, IL-6, and TNF- $\alpha$  in CMS-challenged mice [17]. Moreover, H<sub>2</sub> may have a long-lasting effect on stress resilience in mice, since H<sub>2</sub> inhalation in adolescents significantly increases resilience to acute stress in early adulthood [17]. Anxiety is a negative psychological consequence of opioid withdrawal, which can be attenuated by the administration of H<sub>2</sub>. In a morphine-dependent mouse model of naloxone-precipitated withdrawal, Wen *et al.* found that the administration of HRS not only significantly reduces body weight loss, jumping behavior, and wet-dog shakes but also suppresses anxiety-like behaviors in mice after naloxone-precipitated withdrawal or a spontaneous withdrawal period [63]. HRS treatment even reverses the hyperactivity of the hypothalamus-pituitary-adrenal axis induced by morphine withdrawal and inhibits the increase of CORT and cortisol levels in the plasma. In an mouse model of autism, the pre- and post-administration of HRW significantly improves the valproic acid-induced anxiety-like behaviors and reduces the serum IL-6 and TNF- $\alpha$  levels in mouse offspring [64].

To further validate the unique role of H<sub>2</sub> in emotional regulation, a double-blinded, placebo-controlled study of 31 adult volunteers aged between 20 and 49 years was carried out. Mizuno *et al.* found that the consumption of HRW for 4 weeks significantly decreases (post *versus* pretreatment) scores for K6 (mood and anxiety), the Chalder Fatigue Scale (severity of fatigue), and the Pittsburgh Sleep Quality Index (general sleepiness and daytime sleepiness scores) [18]. In addition, the changes in the post/pretreatment ratios for K6 score and low-frequency component power (sympathetic nerve activity test) were significantly lower in the drinking HRW group than in the placebo water group. This finding indicates that the administration of HRW may offer an effective method to reinforce the quality of life and maintain good health by improving mood, anxiety, and autonomic nerve function in daily life.

## Mechanisms of the H<sub>2</sub>-Induced Neuroprotective Effect

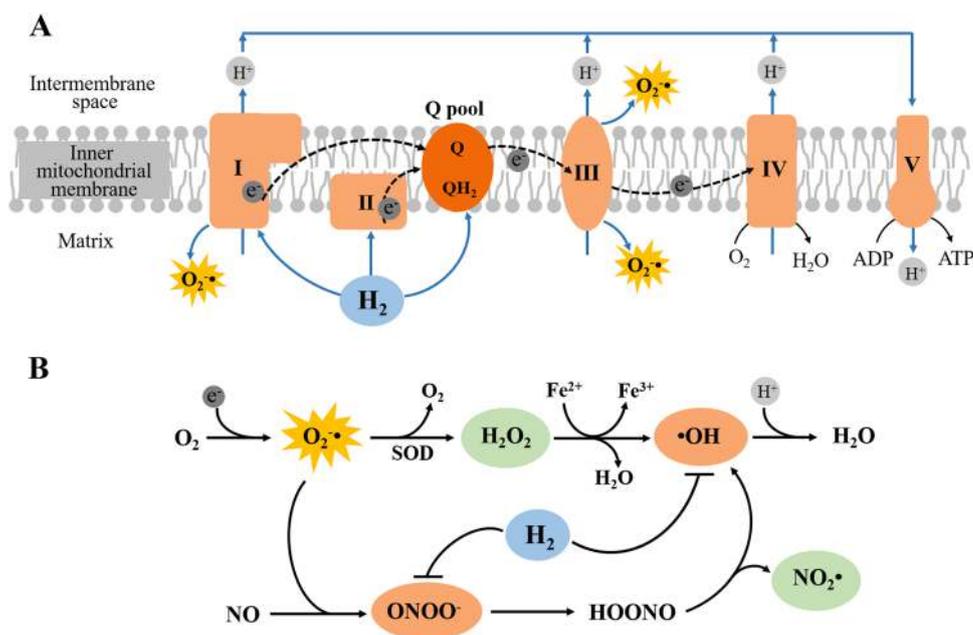
Several potential mechanisms may be involved in the neuroprotective effect of H<sub>2</sub> against neurological diseases: anti-oxidation, anti-inflammation, anti-apoptosis, regulation of autophagy, preservation of mitochondrial function, and preservation of BBB integrity.

### Anti-oxidation Effect of H<sub>2</sub>

Many diseases are linked to oxidative stress damage which comes from an imbalance between ROS production and scavenging. The excessive production of peroxides and free radicals can damage cellular components, including proteins, lipids, and DNA. H<sub>2</sub> is widely recognized as an antioxidant by directly neutralizing cytotoxic  $\cdot\text{OH}$  and ONOO<sup>-</sup> (Fig. 5B), but does not affect other ROS, such as superoxide anion (O<sub>2</sub><sup>-</sup> $\cdot$ ), nitric oxide (NO $\cdot$ ), and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), which play physiological roles as signaling molecules [3]. Currently, some researchers propose that H<sub>2</sub> may have an antioxidant effect by working upstream of the generation of ROS. ROS mainly originate at the electron transport chain during the process of oxidative phosphorylation (Fig. 5). Meanwhile, mitochondrial complex I has close homology and an evolutionary relationship with [NiFe]-hydrogenases. Therefore Toru Ishibashi proposed that H<sub>2</sub> may function as the rectifier of the mitochondrial electron flow to convert ubiquinone to ubiquinol, thereby suppressing excessive electron leakage and ROS generation [110]. A new study by Ishibashi found that H<sub>2</sub> suppresses superoxide generation in complex I and reduces the mitochondrial membrane potential ( $\Delta\Psi\text{m}$ ) [111]. Meanwhile, Gvozdjáčková *et al.* also reported that HRW consumption increases the levels of mitochondrial ATP production by complex I and complex II substrates and increases the levels of mitochondrial oxidized coenzyme Q (ubiquinone) in heart tissue of rats [112]. The latest results from Professor Ma's research team demonstrated that the evolving activity of H<sub>2</sub> in eukaryotic mitochondria is closely related to complex I and the activity occurs around the fully oxidized ubiquinone binding site [113]. Moreover, they have also found that H<sub>2</sub> significantly enhances the activity of mitochondrial complex I under hypoxic conditions [114]. These results thus reinforce the hypothesis that H<sub>2</sub> may control ROS generation at the source by functioning as a rectifier of mitochondrial electron flow in the quinone chamber (Fig. 5A).

Apart from its properties as a free radical scavenger and rectifier of the mitochondrial respiratory chain, H<sub>2</sub> can also enhance the body's resistance to excessive oxidative stress

**Fig. 5** Hypotheses of the anti-oxidative mechanisms of H<sub>2</sub>. **A** The novel hypothesis that H<sub>2</sub> suppresses ROS generation by functioning as a rectifier for mitochondrial electron flow in the ubiquinone (Q) pool. **B** The conventional “scavenger theory” that H<sub>2</sub> directly neutralizes ·OH and ONOO<sup>-</sup> produced by the mitochondrial respiratory chain. Mitochondrial complexes I to V are marked as I, II, III, IV, and V; QH<sub>2</sub>, ubiquinol; HOONO, peroxynitrous acid; NO<sub>2</sub><sup>•</sup>, nitrogen dioxide.



by improving the levels of antioxidant enzymes and modulating the expression of redox-related genes. H<sub>2</sub> treatment increases the activity of endogenous antioxidant enzymes in the brain and spinal cord, such as SOD, CAT, and GPx [29, 47, 115–117]. Moreover, H<sub>2</sub> can regulate the expression of antioxidant genes. *Via* RNA sequencing and RT-PCR analysis, Chen *et al.* have found that the expression of oxidative stress-related genes, including Cox8b, Cox6a2, Cox7a1, Hspb7, and Atp2a1, are significantly downregulated following H<sub>2</sub> treatment in mice with spinal cord injury [42]. In a model of hypoxia/reoxygenation injury, administration of H<sub>2</sub> significantly promotes the expression of HO-1 and nNOS [36, 115]. Strong expression of HO-1 has also been reported after HRS treatment in a rat model of sciatic nerve trunk ligation [54]. Nrf2 is a critical factor for the endogenous antioxidant system, which plays important neuroprotective roles in various neurological diseases by regulating the production of numerous cytoprotective proteins [118]. Yuan *et al.* have reported that the administration of HRS increases Nrf2 expression and promotes the translocation of Nrf2 from the cytoplasm to the nucleus and increases the expression of downstream factors, such as HO-1 and NQO1 [7]. Meanwhile, enhanced expression of p-p38 MAPK, HO-1, and Nrf2 by H<sub>2</sub> treatment have been found in more experiments *in vitro* and *in vivo* [37, 119]. Therefore, H<sub>2</sub> treatment might induce adaptive responses against oxidative stress in the nervous system by evoking the Nrf2 antioxidant defense system.

### Anti-inflammatory Effect of H<sub>2</sub>

Extensive studies have provided strong evidence for the anti-inflammatory effect of H<sub>2</sub> in various diseases in the nervous system. H<sub>2</sub> treatment significantly suppresses microglia activation and the secretion of pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and HMGB-1) in animal models of various neurological diseases [5, 26, 120, 121]. *In vitro*, pretreatment with H<sub>2</sub>-rich medium significantly inhibits the hypertrophy and proliferation of astrocytes, mitigates the expression of GFAP, and weakens the increased secretion of pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, and TNF- $\alpha$ ) in primary astrocytes after H<sub>2</sub>O<sub>2</sub>-induced injury [44]. H<sub>2</sub> not only effectively inhibits the expression of pro-inflammatory factors, but also enhances the immunosuppressive cytokines IL-10, TGF- $\beta$ , and YM-1 in the brain [39, 88]. The M1/M2 polarization of microglia is an important participant in neuroinflammation [122]. Ning *et al.* have reported that H<sub>2</sub> treatment markedly inhibits the proportion of M1 microglia, but has no influence on M2 microglia *in vitro* [123]. Chu *et al.* have noted the phenomenon that the number of M1 microglia is significantly reduced and the number of M2 microglia is significantly increased in the cortex after HRS treatment of mice with hypoxic/ischemic insult [39]. In addition, fewer amoeboid/round microglia and more intermediate microglia are obtained in mice given a high concentration of H<sub>2</sub> [5]. It seems that H<sub>2</sub> may suppress neuroinflammation by inhibiting microglial activation and regulating microglial polarization.

The incorrect regulation of NF- $\kappa$ B has been correlated with neuroinflammation. Some studies have indicated that H<sub>2</sub> may play an anti-inflammatory role by inhibiting NF- $\kappa$ B activation. In rodents with hypoxia/ischemia, HRS treatment significantly promotes AMPK activation and inhibits NF- $\kappa$ B activation accompanied by miR-21 and miR-210 downregulation in the brain [6, 39]. In a rat model of subarachnoid hemorrhage, HRS treatment decreases the protein levels of p-I $\kappa$ B $\alpha$  and nuclear p65, and increases the level of cytosolic p65 [10, 48]. In a rat model of Alzheimer's disease, the intraventricular injection of HRS inhibits JNK and NF- $\kappa$ B activation in the hippocampus [60]. Moreover, the intake of HRW significantly attenuates activation of the NLRP3 inflammasome and decreases the expression of its downstream signaling molecules (cleaved caspase-1 and IL-1 $\beta$ ) in the brain of female APP/PS1 mice [13]. HRS treatment after subarachnoid hemorrhage also decreases the protein expression of NLRP3, ASC, caspase-1, and IL-1 $\beta$ , and cleaves caspase-3 in the cerebral cortex of rats [10]. These results indicate that, in addition to modulating NF- $\kappa$ B pathways, H<sub>2</sub> may also attenuate the inflammatory response *via* suppressing formation of the NLRP3 inflammasome.

Oxidative stress can induce cell damage and promote inflammation in CNS diseases. Excessive ROS have been shown to stimulate the expression of transcription factors such as NF- $\kappa$ B, and promote the secretion of IL-1 $\beta$  by activating the NLRP3 inflammasome [124, 125]. In fact, the anti-inflammatory effect of H<sub>2</sub> is usually paralleled by the antioxidant effect [26, 126]. Therefore, the anti-inflammatory action of H<sub>2</sub> may also be due to the mechanism of gene expression changes caused by ROS.

### Anti-apoptotic Effect of H<sub>2</sub>

In addition to the anti-oxidative and anti-inflammatory effects of H<sub>2</sub>, it also plays an anti-apoptotic role against nerve damage. H<sub>2</sub> suppresses the expression of Bax, caspase-3, and caspase-12, promotes the expression of Bcl-2 and Bcl-xL, and increases the Bcl-2/Bax ratio in the brain [9, 37, 42, 48, 101, 103, 107]. Moreover, HRS treatment significantly attenuates the loss of motor neurons, inhibits the release of mitochondrial cytochrome c (Cyt c), and inhibits the activation of downstream caspase-9 and caspase-3 [127]. Further research has shown that H<sub>2</sub> treatment significantly decreases the expression levels of Akt, GSK3 $\beta$ , p-Akt, and p-GSK3 $\beta$  in brain tissue and cerebral microvascular endothelial cells (CMECs) after hypoxia/reoxygenation [33]. Other studies have obtained similar results showing that HRS treatment enhances the phosphorylation of Akt and GSK3 $\beta$  after subarachnoid hemorrhage. Furthermore, these beneficial effects of H<sub>2</sub> are abolished by Ly294002, a selective inhibitor of the PI3K pathway

[9, 33]. These data suggest that H<sub>2</sub> may protect the brain against apoptosis by the Akt/GSK3 $\beta$  signaling pathway.

### Regulation of Autophagy

Recently, some studies have revealed that H<sub>2</sub> may have a neuroprotective effect by regulating the signaling pathways of autophagy. In a rat model of neuropathic pain, Wang *et al.* found that HRS treatment attenuates hyperalgesia and activates autophagy [11]. Moreover, the intraperitoneal injection of HRS significantly upregulates Beclin-I, HIF-1 $\alpha$ , and BNIP3 mRNA and protein expression, downregulates p62 mRNA and protein expression, and increases the number of autophagosomes and autolysosomes in the spinal cord [11]. In a rat model of post-herpetic neuralgia, the intraperitoneal injection of HRS relieves neuralgia and activates autophagy by upregulating the expression of LC3, Beclin-I, and p62 [128]. In addition, in neonatal hypoxic/ischemic brain injury, Bai *et al.* reported that the administration of HRS elevates the LC3II/LC3I ratio, increases Beclin-1 protein expression, and decreases the levels of phosphorylated mTOR, Stat3, and ERK in the lesioned cortex [40]. Studies have shown that H<sub>2</sub> can not only promote autophagy but also inhibit it. In a rat model of vascular dementia, the intake of HRW decreases the number of autophagosomes, which is accompanied by the downregulation of FoxO1 and Atg7 expression levels, attenuation of the LC3-II/I ratio, and upregulation of p62 level [101].

### Preservation of Mitochondrial Function

The production of ROS primarily occurs in the mitochondria and it is also commonly affected. Researchers have indicated that HRS can attenuate neuronal injury, possibly by preserving mitochondrial function. Cui *et al.* found that HRS decreases the degree of mitochondrial swelling and ultrastructural disruption, maintains the integrality of the mitochondrial membrane, and preserves the loss of  $\Delta\Psi$ m and Cyt c release in the hippocampus of rats with ischemia/reperfusion injury [35]. In mouse models of amyotrophic lateral sclerosis, HRS attenuates the release of mitochondrial Cyt c, restores the activity of complexes I and IV, suppresses the formation of ROS in mitochondria, and enhances the production of mitochondrial ATP [127]. Mechanical injury damages the structure and function of the mitochondria, leading to mitochondrial permeability transition pore (mPTP) opening and ATP loss in neurons; however, the effects of the damage are reversed by H<sub>2</sub> treatment [42, 43]. In human neuroblastoma SH-SY5Y cells, H<sub>2</sub> pretreatment enhances mitochondrial activity,

indicating an increase of  $\Delta\Psi_m$ , cellular ATP concentration, and  $O_2$  consumption rate [119]. The mPTP is one of the direct sites of ROS [129]; thus, in this study, the neuroprotection by  $H_2$  may be associated with the inhibition of mPTP opening. Previous research indicates that the activation of mitochondrial ATP-sensitive  $K^+$  (mitoK<sub>ATP</sub>) channels protect neurons against the injury and death caused by ischemia/reperfusion [130]. Zhou *et al.* speculated that HRS also activates mitoK<sub>ATP</sub> channels, as the beneficial effects of HRS on ischemia/reperfusion in the spinal cord are partially reversed by 5-hydroxydecanoate, a selective mitoK<sub>ATP</sub> channel antagonist [116]. Meanwhile, the relationship between  $H_2$  and the mPTP or mitoK<sub>ATP</sub> channels need further validation.

### Preservation of the BBB

The BBB, a highly selective and organized structure, is composed of endothelial cells, pericytes, astrocytes, neurons, and extracellular matrix. It is also known as the neurovascular unit and is considered the gatekeeper of the CNS [131]. Disruption of the BBB primarily results from ischemia/reperfusion, spontaneous hypertensive stroke, and mechanical trauma-induced nervous system injury, and  $H_2$  has been found to attenuate BBB dysfunction [73, 82, 88, 90]. Takeuchi *et al.* found that oral intake of HRW reduces the number of 8-OHdG-positive cells and vessels with extravasated albumin in the hippocampus of spontaneously hypertensive stroke-prone rats [73]. Matrix metalloproteinases (MMPs) are important factors in BBB disruption. In animals with traumatic brain injury, the intake of HRW might have protective effects against edema and BBB disruption by suppressing the decrease in AQP-4 and MMP-2 levels and by inhibiting the increase in HIF-1 and MMP-9 levels after cortical impact [43]. In addition, brain-derived CMECs play an important role in the BBB, and  $H_2$  treatment inhibits CMEC apoptosis after hypoxia/reoxygenation [33].

### Conclusions and Perspectives

$H_2$  has been reported to have a variety of biological properties including anti-oxidative, anti-inflammatory and anti-apoptotic effects, as well as its protection of mitochondria and the BBB. Oxidative stress modulates the expression of a wide range of genes that influence many biological responses, such as apoptosis, autophagy, and the inflammatory response; thus, the anti-oxidative action of  $H_2$  may be its most basic property. At present, there are two hypotheses for the mechanisms behind the anti-oxidative action of  $H_2$ . The generally accepted hypothesis is that  $H_2$

directly reacts with  $\cdot OH$  and  $ONOO^-$ , namely the conventional “scavenger theory”. The other considers that  $H_2$  suppresses ROS generation by functioning as a rectifier for mitochondrial electron flow. Although several recently-published papers provide some preliminary evidence for the new hypothesis, more compelling evidence is needed.

The neuroprotective effects of  $H_2$  have been demonstrated by growing evidence from animal experiments, meanwhile its efficacy has also been reported in many clinical trials, especially in cerebral ischemia, post-cardiac arrest syndrome, and Parkinson’s disease. Although the protective effect of  $H_2$  has rapidly developed from many fundamental studies to preliminary clinical application studies in recent years, more work on the clinical applications is needed. Numerous studies have shown that  $H_2$  has no toxic effects, but adverse events including diarrhea, heartburn, and headache have been reported in individual cases [84, 132]. In addition, Wang *et al.* found that  $H_2$  supplementation substantially increases the activity of hydrogenase in *Helicobacter pylori*. More importantly, higher hydrogenase activity and hydrogen metabolism in *H. pylori* may induce gastric cancer by promoting the translocation of the carcinogenic factor CagA into host cells [133]. This indicates that  $H_2$  may enhance the pathogenicity of pathogens and facilitate the development of diseases in some special cases. Thus, the indications and potential side-effects of  $H_2$  cannot be ignored in clinical use.

In some clinic trials with Parkinson’s disease,  $H_2$  administration did not have any beneficial effect, which means that the protective effect of  $H_2$  is somehow limited in certain pathological conditions. As previously noted, the distribution of  $H_2$  in tissue and organs varies with different administration methods and thus influences its biomedical effects. It appears that the limited effects of  $H_2$  may be related to the concentration, duration, and route of administration of  $H_2$ , apart from the stage of disease. Therefore, further studies are required to investigate the pharmacokinetics and the dose-effect relationship of  $H_2$  and then develop more effective  $H_2$  delivery methods.

Overall, despite limited side-effects and negative results reported in individual studies,  $H_2$  may be a promising new treatment modality for numerous nervous system diseases.

**Acknowledgements** This review was supported by the National Natural Science Foundation of China (81770855 and 81773717), the Taishan Scholarship from the Shandong Province Government (ts201511057), and the High-Level Talent Training Program of Taishan Medical University (2018GCC08). We are grateful to Nikoli Peacher of West Virginia University for assistance in editing and proofreading.

**Conflict of interest** The authors declare that they have no conflict of interest.

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