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SYMPOSIUM REVIEW

# Regulation of the T-type $Ca^{2+}$ channel Cav3.2 by hydrogen sulfide: emerging controversies concerning the role of $H_2S$ in nociception

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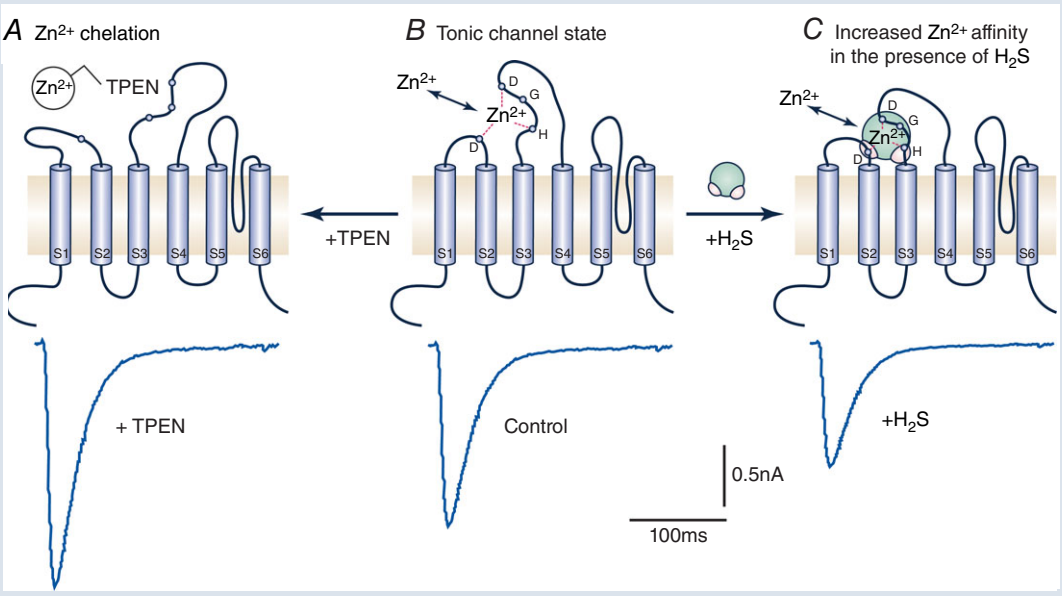
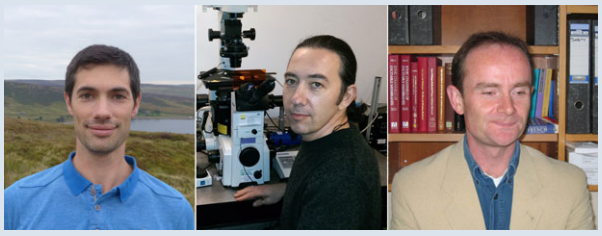



**Abstract** Ion channels represent a large and growing family of target proteins regulated by gasotransmitters such as nitric oxide, carbon monoxide and, as described more recently, hydrogen sulfide. Indeed, many of the biological actions of these gases can be accounted for by their ability



**Jacobo Elies** studied BSc Pharmacy and completed his PhD at the Department of Pharmacology of the University of Santiago de Compostela, Spain. In 2009 he joined Professor Chris Peers as a postdoctoral researcher. Since then he has been investigating the regulation of ion channels by gasotransmitters (CO and $H_2S$) in cardiovascular disease. **Nikita Gamper** obtained his PhD in Physiology at the Sechenov Institute, St Petersburg, Russia. After postdoctoral work in Tübingen (Germany) and the University of Texas at San Antonio (USA) he joined the University of Leeds where he studies molecular and cellular mechanisms of nociception. His group investigates regulation of ion channels and G protein coupled receptors that control or influence excitability of peripheral 'pain' neurons. **Chris Peers** obtained his BSc in Physiology and his PhD in Pharmacology at the University of London. He entered the world of oxygen sensing as a postdoctoral researcher in Piers Nye's laboratory in Oxford. He then moved to the University of Leeds. His interests in the effects of hypoxia have extended to incorporate effects of gasotransmitters on ion channels and how this impacts on the cardiovascular and neurodegenerative diseases.

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to modulate ion channel activity. Here, we report recent evidence that $H_2S$ is a modulator of low voltage-activated T-type $Ca^{2+}$ channels, and discriminates between the different subtypes of T-type $Ca^{2+}$ channel in that it selectively modulates Cav3.2, whilst Cav3.1 and Cav3.3 are unaffected. At high concentrations, $H_2S$ augments Cav3.2 currents, an observation which has led to the suggestion that $H_2S$ exerts its pro-nociceptive effects via this channel, since Cav3.2 plays a central role in sensory nerve excitability. However, at more physiological concentrations, $H_2S$ is seen to inhibit Cav3.2. This inhibitory action requires the presence of the redox-sensitive, extracellular region of the channel which is responsible for tonic metal ion binding and which particularly distinguishes this channel isoform from Cav3.1 and 3.3. Further studies indicate that $H_2S$ may act in a novel manner to alter channel activity by potentiating the zinc sensitivity/affinity of this binding site. This review discusses the different reports of $H_2S$ modulation of T-type $Ca^{2+}$ channels, and how such varying effects may impact on nociception given the role of this channel in sensory activity. This subject remains controversial, and future studies are required before the impact of T-type $Ca^{2+}$ channel modulation by $H_2S$ might be exploited as a novel approach to pain management.

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**Abstract figure legend** Schematic diagram showing the linear structure of the transmembrane domain I of the Cav3.2 T-type $Ca^{2+}$ channel $\alpha$ subunit. In the 'tonic' channel state (middle), zinc partially occupies a binding site formed by interacting residues (indicated by white dots) present in IS1–IS2 and IS3–IS4 linkers, including H191. Under these conditions, zinc causes tonic inhibition of evoked currents (as illustrated by example current shown below). Chelation of zinc by TPEN (left) augments current amplitudes via relief of tonic zinc inhibition. In the presence of $H_2S$, zinc affinity appears augmented, leading to increased zinc binding and a reduction in current amplitude.

**Abbreviations** CBS, cystathionine $\beta$ synthase; CSE, cystathionine $\gamma$ lyase; DRG neuron, dorsal root ganglion neuron; HEK293 cells, human embryonic kidney cells; $K_{ATP}$, ATP-sensitive $K^+$ channel; 3-MST, 3-mercaptopyruvate sulfurtransferase; TPEN, *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethane-1,2-diamine.

## Introduction

Ion channel activity is central to a vast and diverse array of cellular functions in both excitable and non-excitable cell types. For example, ion channel activity controls gene expression, apoptosis, proliferation, contractility, fluid transport, exocytosis, excitability and action potential propagation. It is therefore unsurprising that the number of genes encoding ion channel proteins (either the pore-forming proteins or auxiliary subunits) runs into the hundreds. This diversity is further increased by splice variation and also the fact that subunits of different channel types can form functional channels by combining with other (albeit related) subunits to form heteromeric complexes. Of the many functions regulated by ion channels one of the most important is the control of $[Ca^{2+}]_i$ as this ion is a ubiquitous intracellular signalling molecule that mediates many of the ways in which channels influence the above-mentioned fundamental cellular functions (Berridge *et al.* 2003; Clapham, 2007). Here, we discuss recently discovered new modes of regulation of one specific class of $Ca^{2+}$ channel, the voltage-gated T-type $Ca^{2+}$ channel.

Tailoring ion channel activity to serve specific and often co-ordinated roles requires not only the availability of multiple channel types, but also that they can be dynamically regulated. This can occur by a plethora of means, for example by coupling to specific G proteins and via numerous post-translational modifications, including phosphorylation, ubiquitination, sumoylation, nitrosylation, sulfhydration and *S*-acylation (Rajan *et al.* 2005; Gonzalez *et al.* 2009; Mustafa *et al.* 2011; Lipscombe *et al.* 2013; Shipston, 2014). Such modifications can regulate ion channel trafficking and membrane insertion, as well as ongoing activity. In recent years, it has become apparent that ion channels are also modulated by endogenously generated, biologically active gases, often termed gasotransmitters, such as nitric oxide, carbon monoxide and hydrogen sulfide (NO, CO and $H_2S$, respectively). The roles of these gases, especially NO, in diverse physiological and pathological settings have in many instances been long-established, but the concept that they represent a new class of ion channel regulators is currently emerging (Wilkinson & Kemp, 2011; Peers *et al.* 2012, 2014). In this article, we focus on $H_2S$ specifically as a modulator of voltage-gated T-type $Ca^{2+}$ channels.

## T-type $Ca^{2+}$ channels

When first described at the single channel level, this class of voltage-gated $Ca^{2+}$ channel was named 'T-type' since members had a relatively *t*iny conductance and gave rise to *t*ransient currents (Nowycky *et al.* 1985). These channels are also distinguished from other voltage-gated $Ca^{2+}$ channels by their rapid activation, slow deactivation and very negative activation thresholds, as low as −60 mV or even below (Carbone & Lux, 1984; Perez-Reyes, 2003; Iftinca & Zamponi, 2009). Example currents are shown in Fig. 1*A*, and a typical current–voltage relationship is presented in Fig. 1*B*. Owing to this low threshold for activation, they also may display a significant window current (i.e. display tonic activity; Fig. 1*C*, arrowed) at potentials close to the resting membrane potential (RMP), and so can provide a sustained route for $Ca^{2+}$ entry into resting cells (exemplified in Fig. 1*D*) and also contribute to setting the RMP (Perez-Reyes, 2003).Three genes, *CACNA1G, CACNA1H and CACNA1I* give rise to pore-forming $\alpha$-subunits of T-type $Ca^{2+}$ channels, which are nowadays referred to as Cav3.1–3.3, respectively (Catterall *et al.* 2005), with Cav3.3 showing relatively slower activation and inactivation kinetics than Cav3.1 or Cav3.2 (Fig. 1*A*). Heterologous expression of individual Cav3 $\alpha$-subunits gives rise to currents which

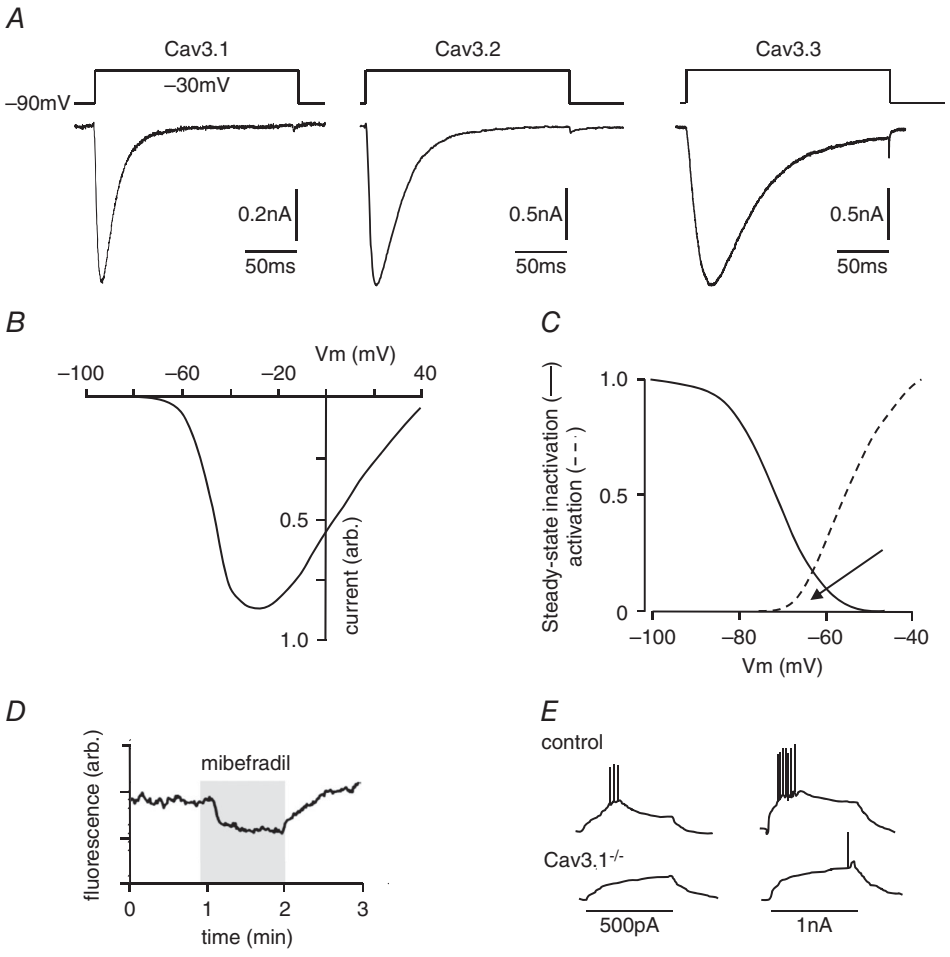


**Figure 1. Properties of T-type $Ca^{2+}$ channels**
*A*, example currents evoked by step depolarizations applied (according to the protocol shown above each trace) to HEK293 cells expressing each of the 3 classes of T-type $Ca^{2+}$ channel, as indicated. Note the relatively slow activation and inactivation of Cav3.3 as compared with Cav3.1 and Cav3.2. *B*, schematic current–voltage relationship typical of T-type $Ca^{2+}$ channels; note the low threshold for activation. *C*, superimposition of steady-state inactivation plot (continuous line) and activation profile (dashed line) typical of T-type $Ca^{2+}$ channels. Note the pronounced window current (region of overlap, arrowed). *D*, fluorimetric recording of $[Ca^{2+}]_i$ in a Fura-2-loaded HEK293 cell expressing Cav3.2. For the period indicated by the shaded area, the T-type channel blocker mibefradil (1 $\mu$M) was applied, causing a reduction in basal $[Ca^{2+}]_i$ (N.T. Hettiarachchi and C Peers, unpublished observations). *E*, schematic diagram of membrane depolarizations evoked by current injections in thalamic neurones. Note the evoked burst activity is almost fully suppressed in cells lacking the T-type channel Cav3.1 (see Kim *et al.* 2001).

closely resemble native T-type $Ca^{2+}$ currents, suggesting that auxiliary subunits are not required for assembly of functional $Ca^{2+}$ channels, although other channel properties and trafficking may indeed be affected by the auxiliary subunits (Dolphin *et al.* 1999).

T-type $Ca^{2+}$ channels are widely distributed, but their physiological functions have sometimes proved difficult to resolve. This is certainly true in vascular smooth muscle cells (VSMCs), where Cav3.1 and Cav3.2 are expressed. Numerous attempts have failed to record 'classic' T-type $Ca^{2+}$ channel activity in VSMCs (reviewed in Kuo *et al.* 2011, 2014). Recent studies suggest this is because VSMCs primarily express T-type $Ca^{2+}$ channel splice variants which do not activate at the low voltages normally associated with T-type $Ca^{2+}$ channels. Instead, they activate over more depolarized voltage ranges as are observed for L-type $Ca^{2+}$ channels. Splice variation accounting for these differences occurs in Cav3.1 (and similarly in Cav3.2) primarily around exons 25 and 26, corresponding to the intracellular linker region between domains III and IV (Chemin *et al.* 2001; Latour *et al.* 2004; Emerick *et al.* 2006). Four variants have been identified termed 25a, 25b, 25bc and 25ac, with variant 25bc predominating in both the juvenile and mature systemic vasculature. These variants all contribute to vasomotor tone (Kuo *et al.* 2011, 2014), and may also have a pacemaker role, controlling slow $Ca^{2+}$ waves and contractile oscillations (Cribbs, 2006).

Despite continued uncertainty around the physiological roles of T-type $Ca^{2+}$ channels in the vasculature, it appears that in several vascular diseases the influence of T-type $Ca^{2+}$ channels, particularly Cav3.1, becomes more prominent. Their increased expression and activity appears to be instrumental in pathological vascular remodelling in both the systemic and pulmonary circulation, providing a route for $Ca^{2+}$ entry which is required for proliferation (Tzeng *et al.* 2012; Chevalier *et al.* 2014). Interestingly, numerous cancers also rely on the expression of T-type $Ca^{2+}$ channels for proliferation, and hence tumour growth (Dziegielewska *et al.* 2014). These findings suggest that they represent a promising therapeutic target in the treatment of various cancers and cardiovascular diseases.

In the nervous system, T-type $Ca^{2+}$ channels serve better defined roles. Thus, for example, in thalamic and corticothalamic neurones T-type $Ca^{2+}$ channels are responsible for pacemaker activity and low threshold spikes (Huguenard & Prince, 1994), as illustrated in Fig. 1*E*. They contribute to 'rebound' bursts of spikes following a hyperpolarizing postsynaptic potential. Indeed, evidence suggests that all three isoforms of T-type $Ca^{2+}$ channels can contribute to this excitability (Kim *et al.* 2001; Joksovic *et al.* 2006; Lee *et al.* 2014). In the peripheral nervous system, T-type $Ca^{2+}$ channels are prominent in somatosensory fibres including small, capsaicin-sensitive (presumed nociceptive) dorsal root ganglion (DRG) neurons (Jevtovic-Todorovic & Todorovic, 2006; Nelson *et al.* 2006; Rose *et al.* 2013) as well as in two distinct populations of low-threshold mechanoreceptors (LTMRs), Aδ- and C-LTMRs, innervating skin hair follicles (Francois *et al.* 2015). Cav3.2 is the dominant form in DRG and may even be the exclusive form in some mechanosensitive neurons (Shin *et al.* 2003). Cav3.2 channels can control burst firing in DRG neurons (White *et al.* 1989) and so strongly influence excitability (Nelson *et al.* 2005, 2007*a*), implying they are of central importance to nociception since stimulus intensity correlates with burst frequency. Indeed, the role of T-type $Ca^{2+}$ channels in pain is well recognized and has been covered in depth in several recent reviews (Bourinet *et al.* 2014; Todorovic & Jevtovic-Todorovic, 2014; Francois *et al.* 2014; Waxman & Zamponi, 2014; Zamponi *et al.* 2015). Thus, conditional genetic deletion (Francois *et al.* 2015), or downregulation of Cav3.2 in DRG using intrathecal injection of antisense oligonucleotides (Bourinet *et al.* 2005; Messinger *et al.* 2009) produced strong anti-nociceptive effects in rodent pain models of neuropathic and inflammatory pain. Conversely, T-type $Ca^{2+}$ currents are often increased in pathological conditions associated with chronic pain, such as diabetic neuropathy (Jagodic *et al.* 2007; Messinger *et al.* 2009), peripheral nerve injury or inflammation (Jagodic *et al.* 2008; Marger *et al.* 2011; Garcia-Caballero *et al.* 2014). Both the enhancement of channel trafficking (Orestes *et al.* 2013; Weiss *et al.* 2013) and enhanced deubiquitination (Garcia-Caballero *et al.* 2014) were reported as underlying mechanisms for the latter phenomenon.

It is not entirely clear how exactly T-type $Ca^{2+}$ channels participate in the nociceptive transmission. Cav3.2 channel expression has been detected in different compartments of afferent fibres, including peripheral nociceptive terminals and axons of skin afferents (Rose *et al.* 2013; Francois *et al.* 2015), nodes of Ranvier of Aδ fibres (Francois *et al.* 2015), as well as in the presynaptic terminals of nociceptive fibres in the spinal cord (Jacus *et al.* 2012; however, cf. Francois *et al.* 2015). Therefore, multiple mechanisms (or their combinations) are conceivable, including setting the threshold for action potential generation (at the nerve terminals) and propagation (at the nodes of thinly myelinated fibres), supporting burst firing, or indeed promoting synaptic activity at the central terminals of afferent fibres. Finally, since T-type $Ca^{2+}$ channels are expressed in skin terminals of low threshold mechanoreceptors, including D-hair cells (Coste *et al.* 2007; Francois *et al.* 2015), a more direct role of T-type $Ca^{2+}$ channels in mechanotransduction also cannot be excluded.

In accordance with strong evidence for the physiological role of T-type $Ca^{2+}$ channels in pain, recent data clearly demonstrated that pharmacological inhibition of T-type

$Ca^{2+}$ channel activity produces strong anti-nociceptive effects in various rodent pain models (Todorovic *et al.* 2001, 2002, 2004; Latham *et al.* 2009). Together with N-type $Ca^{2+}$ channels, T-type $Ca^{2+}$ channels are clinically validated drug targets for pain (Bourinet *et al.* 2014). Accordingly, intense search is currently underway for novel pharmacological tools targeting T-type $Ca^{2+}$ channel activity. These need to be more selective than widely used blockers such as mibefradil which, despite its ability to inhibit T-type $Ca^{2+}$ channels, is not highly selective (Bezprozvanny & Tsien, 1995). This new generation of more selective T-type inhibitors (e.g. TTA-A2, TTA-P2, KYS-05090S) shows promising analgesic properties in animal models of pain (Choe *et al.* 2011; Francois *et al.* 2013). Another novel, selective and orally bioavailable T-type $Ca^{2+}$ channel blocker, Z944, which showed analgesic activity in animal models of inflammatory and neuropathic pain, is currently being tested in clinical trials as a first-in-class novel oral analgesic (Lee, 2014). Moreover, Zamponi's lab has recently developed small molecule modulators that prevent deubiquitination of the Cav3.2 channels by the deubiquitinase USP5, which in turn can prevent channel upregulation in chronic pain conditions (Gadotti *et al.* 2015).

## A redox- and $Zn^{2+}$-sensitive 'module' within the Cav3.2 subunit

Studies in both native DRG neurons and recombinant expression systems have revealed a regulatory site within Cav3.2 which confers high sensitivity to redox agents, distinguishing it functionally from both Cav3.1 and Cav3.3. Thus, for example, T-type $Ca^{2+}$ currents recorded in native nociceptive neurones and recombinant Cav3.2 currents are enhanced by reducing agents (dithiothreitol (DTT) or L-cysteine) and inhibited by the oxidizing agent 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) (Nelson *et al.* 2007*a*). Sensitivity to these agents was exploited in order to demonstrate the importance of Cav3.2 in nociception: hindpaw injections of DTT or L-cysteine induces thermal and mechanical hyperalgesia (Todorovic *et al.* 2001), and such effects are prevented with the $Ca^{2+}$ channel inhibitor mibefradil; furthermore, analgesic effects are observed with DTNB (Todorovic *et al.* 2001). Such findings were confirmed in a subpopulation of nociceptive neurons expressing high levels of Cav3 channels (Nelson *et al.* 2005). An important break-through in the field was achieved by the joint work of Perez-Reyes' and Lee's groups which allowed the characterization of specific Cav3.2 residues involved in the metal-induced inhibition of the $Ca^{2+}$ channel. The redox-sensitive module is located extracellularly, involving interaction of the extracellular IS1–IS2 linker region with the IS3–IS4 linker region (Kang *et al.* 2010), the latter region containing a histidine residue (H191) which is key to conferring high redox sensitivity to this channel. Intriguingly, the same H191 residue that is involved in modulation of T-type $Ca^{2+}$ channel activity by redox mechanisms also mediates T-type $Ca^{2+}$ channel inhibition by low (submicromolar) concentrations of extracellular $Zn^{2+}$ (Kang *et al.* 2006, 2010). The full $Zn^{2+}$ binding site also includes residues that precede H191, namely D189 and G190, as well as the negatively charged residues at the outer portion of the IS2 segment (Kang *et al.* 2010). It is, as yet, unclear how exactly the redox- and $Zn^{2+}$-mediated modulatory pathways converge at the same site but one hypothesis suggests that H191 may represent a general binding site for transition metals (e.g. iron, copper, zinc) and can be subject to oxidation via a metal-catalysed oxidation (MCO) reaction (Stadtman, 2001; Nelson *et al.* 2007*a*). It is conceivable therefore that either binding of metal (e.g. $Zn^{2+}$) or $Zn^{2+}$-independent MCO of H191 may result in a similar inhibition of channel activity. An alternative hypothesis is that oxidative modification (possibly but not necessarily at H191) can increase the effect of metal binding either by increasing the affinity at the binding site or by enhancing coupling efficiency between the metal binding and channel inhibition. In such a scenario, oxidative modification could result in sensitization of the channel to $Zn^{2+}$ (and potentially other transition metals) making the channel sensitive to ambient concentrations in biological fluids. Total $Zn^{2+}$ concentration in human plasma is reported to be within the range of 5–20 $\mu$M (Moran *et al.* 2012). Although it is likely that the free $[Zn^{2+}]$ is considerably lower, it is still quite plausible that the extracellular free $[Zn^{2+}]$ can reach the nanomolar range at which changes in $Zn^{2+}$ affinity/sensitivity of Cav3.2 subunits may result in noticeable changes in channel activity. While the exact mechanism of regulation of Cav3.2 activity via the H191-containing regulatory site remains to be elucidated, it is clear that redox modulation is involved in the action on the T-type $Ca^{2+}$ channels of some pharmacological agents and physiological signalling cascades including nitrous oxide (Orestes *et al.* 2011), CO and thioredoxin (Boycott *et al.* 2013), as well as $GABA_B$ receptors (Huang *et al.* 2015).

## $H_2S$ as a gasotransmitter

$H_2S$ is regarded as the third 'gasotransmitter' since it is an endogenous, enzymatically generated, biologically active gas. Emerging evidence indicates that it has widespread physiological and pathophysiological importance throughout the body (Li *et al.* 2011). Unsurprisingly, therefore, it has received increasing interest as a potential therapeutic target over the past decade or so, as was the case previously for both NO and CO (Moore *et al.* 2003; Leffler *et al.* 2006; Szabo, 2007). $H_2S$ is generated primarily by the action of two widely

distributed enzymes, cystathionine $\gamma$ lyase (CSE) and cystathionine $\beta$ synthetase (CBS; Fig. 2). Both synthesize the gas from L-cysteine. More recently, the mitochondrially located 3-mercaptopyruvate sulfurtransferase (3-MST) has also been found to generate $H_2S$ in both the brain and vasculature (Fig. 2). 3-MST generates $H_2S$ from 3-mercaptopyruvate which is itself generated by cysteine aminotransferase (CAT; Kimura, 2010). Red blood cells can generate $H_2S$ non-enzymatically from inorganic polysulfides, a finding which has prompted the idea that $H_2S$ may mediate the beneficial vascular effects of dietary garlic (Benavides *et al.* 2007). $H_2S$ is also known to be liberated in a redox- or pH-sensitive manner from sulfur 'stores', i.e. sulfur bound to proteins in mitochondria or the cytosol (Kimura, 2010). Differential distribution of $H_2S$-generating enzymes results in a predomination of CBS in the central nervous system, along with 3-MST (Leffler *et al.* 2006), whereas CSE is dominant peripherally, generating the majority of $H_2S$ elsewhere in the body, including the vasculature. More recently, $H_2S$ has been shown to be generated from D-cysteine (Fig. 2), particularly in the cerebellum and kidney, via a novel pathway involving 3-MST and D-amino acid oxidase (Shibuya *et al.* 2013).

## $H_2S$ as a regulator of ion channels

Ion channels (specifically NMDA receptors; Abe & Kimura, 1996) were amongst the first family of cellular proteins recognized as being molecular targets of $H_2S$. Since then, reports of ion channel regulation by $H_2S$ have grown rapidly (Peers *et al.* 2012; Kuksis & Ferguson, 2015; Zhang *et al.* 2015), as indicated by Fig. 2. It is clear that modulation by $H_2S$ is not confined to any particular class of ion channels (such as voltage- or ligand-gated channels) or to channels which are selective for specific ions (e.g. $K^+$, $Na^+$, $Ca^{2+}$ or $Cl^-$) (Zhang *et al.* 2015). $H_2S$ is also known to interfere with a number of intracellular signalling pathways (Wang, 2012), and it is via these pathways that $H_2S$, in some cases, modifies ion channel activity. In other cases, direct post-translational modification accounts for its effects. The best studied of these is the vascular smooth muscle ATP-sensitive $K^+$ channel ($K_{ATP}$ channel), which becomes more active when sulfhydrated (i.e. when –SH groups within cysteine residues are altered to –SSH groups). This direct process (also known as persulfidation; see Paul & Snyder, 2015) contributes to vasorelaxation and thereby protects against hypertension (Mustafa *et al.* 2011; Paul & Snyder, 2012).

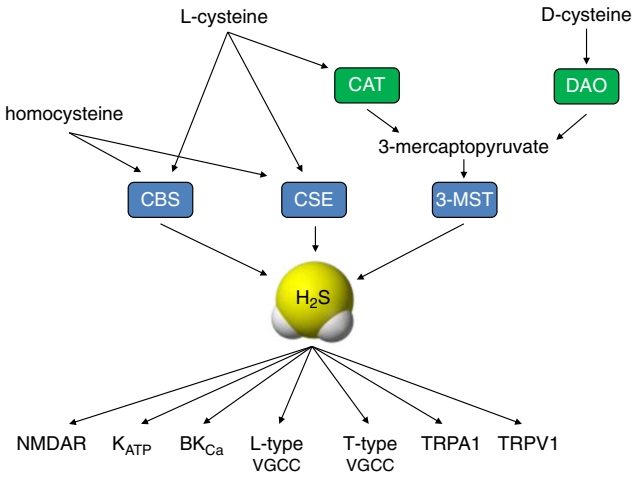


**Figure 2. Hydrogen sulfide synthesis and effector ion channels**
Schematic diagram illustrating the synthesis of $H_2S$ from homocysteine, L- and D-cysteine via enzymes shown in boxes (CBS, cystathionine $\beta$ synthase; CSE, cystathionine $\gamma$ lyase; 3-MST, 3-mercaptopyruvate sulfurtransferase; CAT, cysteine aminotransferase; DAO, D-amino acid oxidase). Some of the target ion channels modulated by $H_2S$ are also indicated (NMDAR, *N*-methyl- D-aspartate receptor; $K_{ATP}$, ATP-sensitive $K^+$ channel; $BK_{Ca}$, large conductance $K^+$ channel; VGCC, voltage-gated $Ca^{2+}$ channel; TRP, transient receptor potential channel).

The list of ion channels regulated by $H_2S$ is long and continues to grow. However, some areas of contention have arisen along the way. Thus, for example, inhibition by $H_2S$ of the high-conductance, $Ca^{2+}$-sensitive $K^+$ channel has been described in detail (Telezhkin *et al.* 2010), yet others report activation of this channel by $H_2S$ (Sitdikova *et al.* 2010; Jackson-Weaver *et al.* 2013). Similarly, inhibition of L-type $Ca^{2+}$ channels in cardiac myocytes (Sun *et al.* 2008; Avanzato *et al.* 2014) contrasts with the observed augmentation of L-type $Ca^{2+}$ channel activity reported in astrocytes (Nagai *et al.* 2004). Such discrepancies may have simple explanations, but these need to be identified if $H_2S$ modulation of ion channels is to be exploited therapeutically. The remainder of this review is focused on the known pro-algesic effects of $H_2S$ and considers the recent, conflicting data concerning the role of T-type $Ca^{2+}$ channels in this process.

## $H_2S$ as a regulator of Cav3.2

Our group has recently reported an inhibitory effect of micromolar concentrations of $H_2S$ on recombinant and native T-type $Ca^{2+}$ channels in sensory neurons (Elies *et al.* 2014). Among Cav3 subunits, this effect was selective to Cav3.2 while Cav3.1 and Cav3.3 were insensitive to $H_2S$. In agreement with the predominant expression of Cav3.2 subunits in DRG neurons, $H_2S$ also strongly inhibited endogenous low-voltage activated (LVA) currents in these neurons. Interestingly, the same extracellular histidine residue, H191, which is necessary for Cav3.2 modulation by $Zn^{2+}$ and redox agents, was also found critical for the effect of $H_2S$. Thus, the H191Q mutation (where histidine has been substituted by glutamine) in Cav3.2 abolished channel sensitivity to $H_2S$ and the analogous reciprocal mutation in Cav3.1 (Q172H) conferred sensitivity to

$H_2S$ on this subunit (Elies *et al.* 2014). It is not entirely clear exactly how redox/$Zn^{2+}$ binding of Cav3.2 is implicated in this $H_2S$-mediated effect but one theory is that $H_2S$ may increase channel sensitivity to extracellular $Zn^{2+}$. Indeed, chelation of ambient $Zn^{2+}$ with TPEN (*N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethane-1,2-diamine) fully reversed the $H_2S$-mediated T-type $Ca^{2+}$ channel inhibition. Moreover, pretreatment with TPEN abolished $H_2S$-mediated inhibition when $H_2S$ was applied in the continued presence of TPEN. However, subsequent washing out of TPEN in the presence of $H_2S$ allowed the inhibition to commence (Elies *et al.* 2014). Thus, the action of $H_2S$ on Cav3.2 seems to depend on ambient $Zn^{2+}$, assumed to be present in extracellular solutions in trace amounts. Indeed, according to our atomic absorption spectroscopy measurements, total $Zn^{2+}$ levels can reach micromolar levels in nominally zinc-free laboratory solutions (D. Huang & N. Gamper, unpublished observations); likewise, micromolar concentrations of $Zn^{2+}$ in plasma are also reported (Moran *et al.* 2012). While levels of free $Zn^{2+}$ are likely to be much lower as compared to the total zinc, it is still likely that this metal is present in the extracellular milieu at levels sufficient to affect channel activity. We hypothesize that the action of $H_2S$ results in the potentiation of channel inhibition by such ambient $Zn^{2+}$, via a mechanism which may share commonalities with the redox modulation of Cav3.2 (see Abstract figure).

Although our data strongly suggest that $H_2S$ inhibits Cav3.2 and the endogenous T-type $Ca^{2+}$ currents in DRG neurons, there is an alternative theory suggesting that $H_2S$ augments T-type $Ca^{2+}$ currents, possibly by chelating $Zn^{2+}$, and that this augmentation underlies the pro-algesic actions of this gasotransmitter (Kawabata *et al.* 2007; Maeda *et al.* 2009; Takahashi *et al.* 2010; Matsunami *et al.* 2011). Thus, injection of the $H_2S$ donor NaHS into the rat hindpaw produced hyperalgesia which was abolished by the oxidizing agent DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)) and by the pharmacological inhibition of T-type $Ca^{2+}$ channels (Kawabata *et al.* 2007). Inhibition of endogenous $H_2S$ production also produced an anti-algesic effect. This and other studies by the same group led them to conclude that $H_2S$ could activate or 'sensitize' Cav3.2 channels in order to account for their pro-algesic effects (Kawabata *et al.* 2007; Takahashi *et al.* 2010). Furthermore, $H_2S$-induced colonic pain could be mimicked by chelation of $Zn^{2+}$ (Matsunami *et al.* 2011). Yet, most of the evidence suggesting that the pro-algesic effects of $H_2S$ are mediated by T-type $Ca^{2+}$ channel augmentation is somewhat circumstantial and direct electrophysiological evidence is sparse and insubstantial. For example, pre-incubation of NG108-15 (mouse neuroblastoma and rat glioma hybridoma) cells with 1.5 mM NaHS resulted in ∼20% augmentation of endogenous T-type $Ca^{2+}$ current while at 0.5 mM the effect was not significant (Kawabata *et al.* 2007). Another study demonstrated that pre-incubation of human embryonic kidney (HEK) 293 cells exogenously expressing Cav3.2 channels with the CSE inhibitor propargylglycine resulted in a reduction of the current, which was interpreted as tonic augmentation of recombinant Cav3.2 by endogenous $H_2S$ (Sekiguchi *et al.* 2014). Interestingly, only in the presence of this inhibitor were currents augmented by NaHS. These experiments are consistent with potential augmentation of T-type $Ca^{2+}$ currents by $H_2S$ but the following has to be taken into account: (i) only very high, millimolar concentrations of NaHS were efficacious; (ii) due to the experimental protocol used (pre-incubation) these experiments did not directly assess the acute effect of $H_2S$ on T-type $Ca^{2+}$ channel activity.

In our hands, increasing the NaHS concentration to 3 mM did augment recombinant Cav3.2 currents, while even higher concentrations were needed to significantly augment native T-type $Ca^{2+}$ channel currents in DRG neurons (Elies *et al.* 2014). These experiments can, to some degree, reconcile conflicting experimental evidence from different laboratories and suggest a dual effect of $H_2S$ on T-type $Ca^{2+}$ channels: H191-mediated inhibition at low (micromolar) concentrations and potentiation (due to an unknown mechanism) at high (several millimolar) concentrations. It is important to point out, however, that the presence of $H_2S$ at millimolar concentrations in mammalian tissues is highly unlikely. Initial reports estimated plasma $H_2S$ levels within the range of 20–100 $\mu$M (Li & Moore, 2008), but even these values are now regarded as overestimations (Li *et al.* 2011). Therefore, physiologically relevant concentrations of $H_2S$ are unlikely to be sufficiently high to produce T-type $Ca^{2+}$ channel augmentation. The expected effect of endogenous $H_2S$ is, therefore, inhibition of T-type $Ca^{2+}$ channel currents, at least in cells and tissues that express significant amounts of Cav3.2 (such as sensory neurons). Therefore, the hypothesis that physiological levels of $H_2S$ can trigger pro-algesic effects via T-type $Ca^{2+}$ channel activation appears questionable at present. Furthermore, in our view, it is also unlikely that $H_2S$ can act as a $Zn^{2+}$ chelator (as was suggested by Matsunami *et al.* 2011) as (i) the chemical properties of $H_2S$ do not suggest metal-chelating properties (e.g. the ability to form polydentate complexes); (ii) acute application of $H_2S$ leads to T-type $Ca^{2+}$ channel current inhibition, whereas $Zn^{2+}$ chelation with TPEN causes current augmentation (Nelson *et al.* 2007*b*; Elies *et al.* 2014); (iii) application of TPEN completely reverses $H_2S$-induced T-type $Ca^{2+}$ channel inhibition; (iv) pre-application of TPEN renders $H_2S$ unable to inhibit T-type $Ca^{2+}$ channels.

At present, whilst it is hard to envisage how the painful/hyperalgesic effects of $H_2S$ (especially those of endogenously produced $H_2S$) can be mediated by its effect on T-type $Ca^{2+}$ channels, other possible

targets may account for this action. Thus, $H_2S$ was suggested to produce some of its pro-algesic effects via transient receptor potential channel TRPA1 activation (Andersson *et al.* 2012; Hsu *et al.* 2013). Additionally, $H_2S$ was suggested to inhibit voltage-gated $K^+$ channels in trigeminal sensory neurons (most likely Kv1.1 and Kv1.4), thus producing depolarizing and excitatory effects (Feng *et al.* 2013).

## Conclusions and perspectives

Tonic $H_2S$ production clearly impacts a variety of both physiological and pathological processes, and probably does so in part via direct and indirect modulation of ion channel activity. The numerous and diverse pathways that can be modified by $H_2S$ suggest that interventional control of this gasotransmitter could be therapeutically beneficial (e.g. in the cardiovascular system and peripheral sensory neurones). However, conflicts have arisen and must be resolved. We believe that at least some issues could be rectified if the experimental conditions through which we study the effects of $H_2S$ are standardized (e.g. preparation of stock solutions of $H_2S$ donors, flow rates of solutions containing donors, exposure time and obligatory use of agar bridges for reference electrodes).

Twenty years after the discovery of $H_2S$ as an endogenously bioactive molecule (Abe & Kimura, 1996), one of the main challenges within the field remains the measurement of absolute concentrations of $H_2S$, in real time, both intracellularly and in extracellular compartments. For instance, development of appropriate intracellular fluorescence probes will expand not only the knowledge regarding intracellular concentrations of $H_2S$ in space and time, but it will also help to correlate experimental data obtained from exogenously administered $H_2S$ with experimental data obtained from endogenously generated $H_2S$. Additionally, future research regarding the physiological effects of $H_2S$ must bear in mind the crosstalk signalling with other gasotransmitters (NO and CO). Only then can we obtain more comprehensive and uniformly acceptable data on which to build in order to design modulators of $H_2S$ production and signalling for translational benefit.

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## Additional information

### Competing interests

None declared.

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