The role of hydrogen sulphide in the control of breathing in hypoxic zebrafish (*Danio rerio*)

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Key points

- Hydrogen sulphide (H_2S), a gaseous neurotransmitter, is involved in oxygen sensing in glomus cells, which are oxygen-sensing cells found in the mammalian carotid body.
- Neuroepithelial cells (NECs) are oxygen-sensing cells of fish and are thought to be phylogenetic precursors of mammalian glomus cells; however, the oxygen-sensing mechanisms of these cells remain largely unknown.
- Both adult and larval zebrafish responded to exogenous H_2S by increasing ventilation in a dose-dependent manner; H_2S increased intracellular $[Ca^{2+}]$ in NECs.
- Inhibiting endogenous H_2S production decreased or abolished the ventilatory response to hypoxia in both adult and larval zebrafish.
- The results demonstrate an important role for H_2S in oxygen sensing in zebrafish.

Abstract The current study investigated the role of hydrogen sulphide (H_2S) in oxygen sensing, intracellular signalling and promotion of ventilatory responses to hypoxia in adult and larval zebrafish (*Danio rerio*). Both larval and adult zebrafish exhibited a dose-dependent increase in ventilation to sodium sulphide (Na_2S), an H_2S donor. In vertebrates, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) are enzymes that catalyse the endogenous production of H_2S. In adult zebrafish, inhibition of both CBS and CSE with aminooxyacetate (AOA) and propargyl glycine (PPG) blunted or abolished the hypoxic hyperventilation, and the addition of Na_2S to the water partially rescued the effects of inhibiting endogenous H_2S production. In zebrafish larvae (4 days post-fertilization), gene knockdown of either CBS or CSE using morpholinos attenuated the hypoxic ventilatory response. Furthermore, the intracellular calcium concentration of isolated neuroepithelial cells (NECs), which are putative oxygen chemoreceptors, increased significantly when these cells were exposed to 50 μM Na_2S, supporting a role for H_2S in Ca^{2+}-evoked neurotransmitter release in these cells. Finally, immunohistochemical labelling showed that NECs dissociated from adult gill contained CBS and CSE, whereas cutaneous NECs in larval zebrafish expressed only CSE. Taken together, these data show that H_2S can be produced in the putative oxygen-sensing cells of zebrafish, the NECs, in which it appears to play a pivotal role in promoting the hypoxic ventilatory response.

Introduction

The capacity to monitor and detect changes in ambient and internal oxygen (O_2) levels is critical to the survival of vertebrates and in particular to that of species which routinely experience fluctuating O_2 levels. Changes in O_2 availability to cells can arise from an inadequate O_2 supply to the body, increased O_2 demand, or changes in the delivery of O_2 within the body. The ability to sustain or maximize O_2 delivery to tissues in the face of alterations in O_2 availability depends on an integrated cascade of cardiorespiratory adjustments that are triggered by O_2-sensing cells or chemoreceptors. Thus, O_2 chemoreceptors increase their activity in response to low O_2 levels (hypoxia) to initiate hyperventilation in an attempt to maintain O_2 delivery to cells. In mammals, the main O_2-sensing organ is the carotid body, found at the bifurcation of the internal and external carotid arteries. This is an ideal location at which to monitor arterial blood O_2 levels because glomus cells receive blood leaving the lungs and can detect hypoxaemia before the blood reaches the brain (Prabhakar, 2006). Despite a wealth of research, the precise nature of the O_2-sensing mechanism(s) of the carotid body glomus cells and other peripheral chemoreceptors remains uncertain, although several theories continue to be debated vigorously (Peers *et al.* 2010; Prabhakar, 2013). For example, some of the prevailing theories of oxygen sensing implicate a change in reactive oxygen species (ROS) production by the mitochondria and/or NADPH oxidase (Dinger *et al.* 2007), activation of AMP-activated protein kinase (AMPK) (Evans *et al.* 2005), and the inhibition of calcium sensitive potassium (BK_{Ca}) channels by haemoxygenase-2 (HO-2) (Prabhakar *et al.* 1995; Williams *et al.* 2004) as steps in the process.

Hydrogen sulphide (H_2S), a gaseous neurotransmitter, is involved in a myriad of physiological functions including epithelial Na^+ transport (Althaus, 2012), metabolism (Zhang *et al.* 2013), cardiorespiratory control (Wang, 2009; Olson *et al.* 2012), catecholamine secretion (Perry *et al.* 2009*a*; Zhu *et al.* 2012) and O_2 chemoreception (Olson, 2008; Peng *et al.* 2010; Telezhkin *et al.* 2010). With respect to the last of these, there is a growing body of evidence implicating H_2S as an O_2 sensor (Olson, 2011) or mediator of hypoxic signalling (Peng *et al.* 2010) within the mammalian carotid body. One proposed mechanism (Olson, 2011; Olson *et al.* 2012) for H_2S-mediated O_2 sensing involves alterations in the balance between the production of H_2S from cysteine by the enzymes cystathionine β-synthase (CBS) and/or cystathionine γ-lyase (CSE) and its oxidative degradation. Under normoxic conditions, H_2S is oxidised in the mitochondria to sulphite (SO_3^{2-}) and sulphate (SO_4^{2-}). However, under hypoxic conditions the mitochondria cannot fully oxidise the H_2S being produced and H_2S accumulates in the cell (Olson, 2011; Olson *et al.* 2012). An alternative mechanism to explain how H_2S mediates O_2 sensing involves haemoxygenase (HO) (Peng *et al.* 2010, 2014; Prabhakar, 2013). In normoxia, HO generates carbon monoxide (CO), which inhibits CSE and thereby lowers H_2S production. Under hypoxic conditions, CO generation by HO is reduced, removing the inhibition to CSE and thereby increasing H_2S production. Several means by which H_2S can influence O_2 sensing have been proposed and include the closure of potassium channels, activation of NADPH oxidase and AMPK, increases in ROS and/or mitochondrial uncoupling (Buckler & Vaughan-Jones, 1998; Dinger *et al.* 2007; Wyatt *et al.* 2007; Olson, 2008; Buckler, 2012). The aim of the current study was to examine in zebrafish (*Danio rerio*) the role of H_2S in O_2 sensing, particularly in the putative cardiorespiratory oxygen chemoreceptors, the neuroepithelial cells (NECs), and its involvement in regulating the magnitude of downstream ventilatory responses to acute hypoxia. The zebrafish is an excellent model organism in which to study O_2 sensing in vertebrates. Firstly, as in other fish, O_2 rather than CO_2 is the principal gas which regulates breathing. Secondly, NECs and carotid body glomus cells are thought to be homologous structures (Milsom & Burleson, 2007). Thirdly, genomic initiatives have yielded a fully sequenced/annotated genome allowing for manipulations such as gene knockdown. Finally, methods for the isolation and primary culture of NECs are well established in zebrafish (Jonz *et al.* 2004).

To test the hypothesis that H_2S is involved in O_2 sensing and promoting the hyperventilatory responses to hypoxia in zebrafish, we first examined the effects of exogenous sodium sulphide (Na_2S) on ventilation in adults and larvae. We predicted that ventilation would increase in a dose-dependent manner and that this response would resemble the hypoxic ventilatory response (HVR). Secondly, we determined the effects of pharmacologically inhibiting in adults, or removing (by splice blocking gene knockdown) in larvae, two of the biosynthetic enzymes of H_2S (CBS and CSE). Based on findings from mammalian studies (Buckler, 2012; Makarenko *et al.* 2012), we predicted that inhibiting or knocking down these enzymes would abolish or blunt the HVR. Thirdly, ratiometric Ca^{2+} imaging was performed to determine the *in vitro* response of isolated NECs to exogenous H_2S. In keeping with a previous report of H_2S-evoked membrane depolarization in zebrafish NECs (Olson *et al.* 2008), we predicted that exogenous H_2S would cause an increase in intracellular $[Ca^{2+}]$ in these cells. In addition, we used immunohistochemistry to localize CBS and CSE to the NECs of adult gill, and CSE to NECs of larval skin.

Methods

Ethical approval

All procedures were conducted in accordance with the guidelines established by the Canadian Council for Animal Care and were approved by the University of British Columbia Animal Care Committee (Protocol A06-1510) and University of Ottawa Animal Care Committee (Protocol BL-226).

Animals

Adult zebrafish, *Danio rerio* (Hamilton 1822), were obtained from commercial suppliers (Noah's Pet Ark or Delta Aquatics, Vancouver, BC, Canada; Big Al's Aquarium Services, Ottawa, ON, Canada) and were maintained on a 12 : 12 h light : dark cycle at 28°C in either dechlorinated City of Vancouver tap water [ionic composition of the water: Na$^+$, 0.08 mM; Cl$^-$, 0.06 mM; Ca^{2+}, 0.03 mM; K$^+$, 0.004 mM; pH 7.0 (Metro Vancouver Water Quality Report, 2011)] or dechloraminated City of Ottawa tap water [ionic composition of the water: Na$^+$, 0.78 mM; Cl$^-$, 0.4 mM; Ca^{2+}, 0.25 mM; K$^+$, 0.025 mM; pH 7.0 (Xxxx)]. Embryos were obtained using standard zebrafish breeding techniques (Westerfield, 2000). Fertilized eggs were placed in Petri dishes under conditions identical to those in which the adults were maintained except that 0.05% methylene blue was added to the holding water.

Series I: adult zebrafish

Ventilatory responses of adult zebrafish to Na$_2$S. The ventilation rate in adult zebrafish was measured non-invasively as described previously (Vulesevic *et al.* 2006). Briefly, adult zebrafish were placed in cylindrical plastic chambers (University of Ottawa). Each chamber was supplied with a continuous water flow ($\sim$1 ml min^{-1}) using gravity. Two electrodes were submerged in the water inside the chamber and mesh was used to prevent the fish from coming into contact with the electrodes. The analog signals from the electrodes, which represented opercular displacements, were amplified (the amplifier was custom-built at the University of Ottawa) and recorded to a computer as digital data using an A/D interfacing system and data acquisition software (AcqKnowledge; BioPac Systems, Inc., Galeta, CA, USA) at a sampling rate of 500 Hz. Although previous studies have used calibration procedures to quantify the magnitude of the linear deflections (in mm) of the opercular movements (Vulesevic & Perry, 2006; Vulesevic *et al.* 2006), the current study focused exclusively on quantifying breathing frequencies because ventilation amplitude in zebrafish is largely unaffected by hypoxia (Vulesevic & Perry, 2006).

Thus, in the current study, each fish was assumed to have a resting breathing amplitude of 0.5 mm and the system was calibrated accordingly. Breathing frequencies were determined by *post hoc* analysis of the AcqKnowledge files.

Adult zebrafish were placed in the breathing recording chambers for 1–3 h prior to the start of experiments. After breathing had been recorded under control conditions, different groups of zebrafish were exposed to one of five concentrations of Na$_2$S (10 μM, 19 μM, 37 μM, 75 μM and 150 μM) for 5 min; no fish was exposed to more than a single level of Na$_2$S. Measurements were taken during the last minute of the recording period. The fish were allowed to recover for 5 min and breathing frequency was quantified during the final minute of recovery.

Effect of H$_2$S synthesis inhibitors on ventilation in adult zebrafish during acute hypoxia. The CBS and CSE inhibitors aminooxyacetate (AOA) (Sigma-Aldrich Canada Co., Oakville, ON, Canada) and propargyl glycine (PPG) (Sigma-Aldrich Canada Co.) were used to further test the role of H$_2$S in the control of breathing in adult zebrafish. Fish were exposed for 16–20 h to a cocktail of the two conventional inhibitors AOA and PPG (0 μM, 100 μM or 250 μM each) prior to the start of experiments. Ventilation was measured as described above during resting conditions (pre-exposure) and the measurements were continued as fish were exposed to hypoxia (40 mmHg) for 5 min and then allowed to recover under normoxic conditions. Measurements were taken over the final minute of each time period (pre-exposure, hypoxia and recovery).

A separate group of zebrafish that had been exposed to a cocktail of AOA and PPG (250 μM) for 16–20 h were exposed to hypoxia (40 mmHg) for 5 min, and then exposed to both hypoxia (40 mmHg) and Na$_2$S (150 μM) for 5 min.

Dissociation, isolation and primary culture of NECs from adult gills. Adult zebrafish were stunned by a sharp blow to the head and killed by decapitation. NECs were isolated as previously described (Jonz *et al.* 2004; Qin *et al.* 2010). All NEC isolation procedures were performed under sterile conditions in a laminar flow hood. Gill baskets were excised and rinsed in 2% penicillin-streptomycin (Sigma-Aldrich Canada Co.) in phosphate-buffered saline (PBS) for 10 min. PBS contained the following: 137 mM NaCl, 15.2 mM Na$_2$HPO$_4$, 2.7 mM KCl, 1.5 mM KH$_2$PO$_4$, at pH 7.8 (Bradford *et al.* 1994; Jonz *et al.* 2004). All eight gill arches were separated and thoroughly cleaned of blood and mucous. Distal filaments rich in NECs were selectively removed and placed in 0.25% trypsin/EDTA (Life Technologies, Invitrogen Canada, Inc., Burlington, ON, Canada) for 1 h at 28°C. Tissue was then subjected to

mechanical dissociation with fine forceps and triturated in a 15 ml centrifuge tube with a Pasteur pipette for 3 min. The trypsin reaction was stopped with the addition of 10% fetal calf serum (FCS) and undigested tissue was allowed to settle to the bottom of the centrifuge tube. The cell suspension was centrifuged (130 g) for 5 min and the pellet triturated in filter-sterilized PBS. The cell suspension was centrifuged once more with PBS and the pellet resuspended in Leibovitz's L-15 medium with L-glutamine (Life Technologies Inc./Gibco, Grand Island, NY, USA) supplemented with 1% penicillin-streptomycin and 2% FCS. Cells were plated in 0.2 ml L-15 on 35 mm glass-bottomed culture dishes (MatTek Corp., Ashland, MA, USA) coated with $0.1 \, mg \, ml^{-1}$ poly-L-lysine (Sigma-Aldrich Canada Co.) and Matri-Gel (Becton, Dickenson & Co., Mississauga, ON, Canada). Cells were supplemented with 2 ml L 16–18 h after isolation, and imaging was carried out 4–5 h thereafter.

Immunohistochemistry of dissociated NECs. Immuno-histochemistry in dissociated cells was performed as previously described (Qin *et al.* 2010). Cells were obtained from two separate dissociations, each performed in 10–12 adult zebrafish. Briefly, 24 h after dissociation, cells that were plated on sterile 35 mm glass-bottomed culture dishes (see above) were fixed with 4% Paraformaldehyde (PFA) in PBS for 30 min at room temperature. The cells were incubated with primary antibodies, mouse anti-5-HT, 1 : 250 (catalogue no. ab16007; Abcam, Inc., Toronto, ON, Canada), and rabbit anti-CSE, 1 : 100, commercial polyclonal antibody raised against human CSE (ARP46067_P050; Aviva Systems Biology, San Diego, CA, USA), the epitope of which shares 100% identity with zebrafish CSE (accession no. AAH67624.1), or rabbit anti-CBS, 1 : 100, a commercial polyclonal antibody raised against human CBS (ARP45746_T100; Aviva Systems Biology), the epitope of which is 92% identical to both zebrafish *cbsa* and *cbsb*. Primary antibodies were diluted in PBS with 0.5% Triton-X overnight at 4°C. The plates were rinsed (3 × 3 min in PBS) and then cells were incubated with the secondary antibody (1 : 200; AlexaFluor 488 anti-rabbit and AlexaFluor 594 anti-mouse; Molecular Probes, Invitrogen Canada, Inc.) in PBS for 1 h at room temperature. The plates were rinsed (3 × 3 min in PBS) and then mounted with 4′6′-diamidino-2-phenylindole (DAPI) (Vectashield; Vector Laboratories, Inc., Burlingame, CA, USA). Cells were visualized using a fluorescent microscope (Observer A1; Carl Zeiss Jena GmbH, Jena, Germany). Control labelling experiments which excluded incubation with primary antibodies demonstrated lack of non-specific labelling of any of the secondary antibodies. Pre-absorption controls were performed by incubating the CSE antibody for 2 h at room temperature in the pre-

sence of CSE blocking peptide (AAP46067; Aviva Systems Biology) at a 1 : 10 antibody to peptide ratio, followed by immunohistochemistry as described above.

Calcium imaging. Calcium imaging was performed as described previously (Zhang *et al.* 2011). The fluorescent indicators Fura-2-LeakRes-AM (Teflabs, Inc., Austin, TX, USA), hereafter referred to as Fura-2, was used to monitor $[Ca^{2+}]_i$. Cells were obtained from two separate cell dissociations. Cells that adhered to the culture substrate were incubated in a solution of 5 μM Fura-2 in normal Ringer solution containing 135 mM NaCl, 5 mM KCl, 2 mM Mg_2Cl, 2 mM Ca_2Cl, 10 mM glucose and 10 mM Hepes (pH 7.8) and 0.1% v/v of 10% w/v Pluoronic F-127 (Invitrogen Canada, Inc.) at 28°C for 1 h. Afterward, cells were washed in indicator-free Ringer solution for an additional 30 min at 28°C. NECs were identified using $4 \, mg \, ml^{-1}$ Neutral Red (NR) (Sigma-Aldrich Canada Co.), a vital marker used to identify neurosecretory cells (Stuart *et al.* 1974; Youngson *et al.* 1993), including NECs (Jonz *et al.* 2004).

Ratiometric imaging was performed using a Nikon Eclipse microscope (Nikon Instruments, Inc., Melville, NY, USA) equipped with a Lamda DG-5 high-speed wavelength changer (Sutter Instrument Co., Novato, CA, USA), a Nikon 40× water-dipping objective lens with a numerical aperture of 0.8 and a digital CCD camera (QImaging Corp., Surrey, BC, Canada). Dual images at 340 nm and 380 nm excitation (510 nm emission) for Ca^{2+} were acquired every 2 s and ratiometric data were obtained using Northern Eclipse software (Empix Imaging, Inc., Mississauga, ON, Canada). Data are represented as raw ratios ($R_{340/380}$) and thus indicate relative changes in $[Ca^{2+}]_i$.

Dishes containing NR/Fura-2 positive cells were fitted with a perfusion chamber insert (Warner Instruments, Inc., Hamden, CT, USA) and mounted on the microscope stage. The chamber was perfused continuously under gravity (3–4 ml min^{-1}) with Ringer solution at room temperature (22–24°C). Multiple perfusion reservoirs (50 ml syringes) were connected to a single gas-impermeable tube that was used to deliver the perfusate. A three-way valve was used to switch between control (Ringer solution bubbled with air) and experimental treatments. Stock solutions of Na_2S were prepared daily and diluted in Ringer solution to achieve a final concentration of 50 $\mu mol \, l^{-1}$. This concentration was chosen as it has been shown previously to produce membrane depolarization in dissociated NECs of zebrafish (Olson *et al.* 2008) and to produce a robust ventilatory response in adult zebrafish (this study). The solution within the reservoir was continuously pumped through the outflow opening of the perfusion chamber via a variable flow mini-pump (Fisher Scientific, Inc.,

Pittsburgh, PA, USA). The volume of Ringer solution within the perfusion chamber never exceeded 400 μl.

Series II: larval zebrafish

Ventilatory responses. Ventilatory responses of zebrafish larvae were obtained as described previously (Jonz & Nurse, 2005; Coccimiglio & Jonz, 2012). Briefly, 4 day post-fertilization (dpf) larvae were anaesthetized with 0.05 mg ml^{-1} Tris buffered MS-222 (Westerfield, 2000) to minimize gross movements. The larvae were placed in a groove in a plastic chamber and were confined to a small area of the well using fine mesh. The dish was perfused with solutions using gravity. The experimental chamber was placed on the water jacket stage of a dissecting microscope (model SZX10; Olympus Canada, Inc., Richmond Hill, ON, Canada), which was maintained at 28°C using a water bath (model RC 6; Lauda-Brinkmann LP, Delran, NJ, USA). Larvae were left undisturbed in the chamber for 20 min before the experiments were started. Hypoxia

was achieved by bubbling a mixture of air and nitrogen to the perfusion chamber using a gas mixer (model GF-3/MP; Cameron Instruments, Inc., Port Aransas, TX, USA). After a pre-exposure period of 3 min, the larvae were exposed for 5 min to either hypoxia (25 mmHg) or Na$_2$S (37 μM, 75 μM or 150 μM made fresh several times per day). The larvae were then allowed to recover for 5 min; breathing rate was determined during the last minute of each of these time periods. Depending on the position and orientation of the larvae, breathing frequency was determined by counting either buccal or opercular movements. For larvae experiencing gene knockdown, the protocol was modified slightly so that the hypoxia and recovery periods were extended to 10 min. In these experiments, breathing rate was measured after 2 min, 4 min, 6 min and 9 min of hypoxia and after 4 min and 9 min of recovery from hypoxia.

**Knockdown of CBS and CSE.** To provide additional evidence that H$_2$S is involved in the control
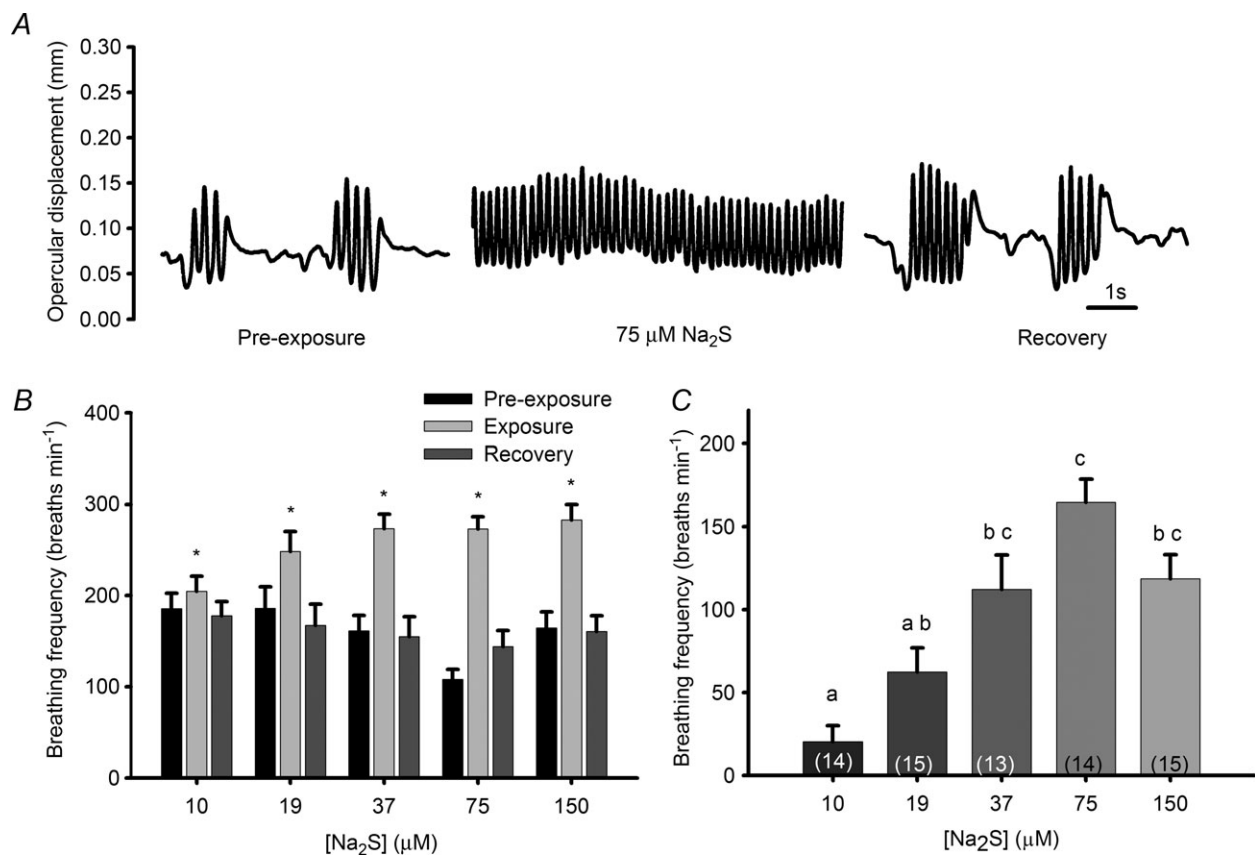
Figure 1. Effects of exogenous hydrogen sulphide (H$_2$S) on ventilation in adult zebrafish (*Danio rerio*)
A, representative ventilation traces from one zebrafish before (pre-exposure), during and after (recovery) exposure to 75 μM sodium sulphide (Na$_2$S), an H$_2$S donor. *B*, average breathing frequency (breaths min^{-1}) of adult zebrafish exposed to various concentrations of Na$_2$S (μM). *C*, changes in breathing frequency of adult zebrafish exposed to increasing concentrations of Na$_2$S (μM). ∗, significant ($P < 0.05$) difference from pre-exposure values. Bars not sharing the same letters are significantly different from one another. Values are means ± s.e.m.; sample sizes are indicated in parentheses.

of breathing, H_2S biosynthetic enzymes were knocked down by injecting fertilized embryos with antisense oligonucleotide morpholinos (Gene Tools LLC, Philomath, OR, USA) targeting CSE (5′-CGCACAAGAGTGAACAGCTCTCTGT-3′) or CBS (5′-TTGTCCTGTGAGAAAAAGCTGCATT-3′). In the zebrafish there are two paralogues of CBS: CBSa and CBSb. Based on its more widespread distribution at 4 dpf (Thisse *et al.* 2001), we chose to focus on CBSb rather than CBSa, which appears to be restricted to the liver and pancreas of larvae at 4–5 dpf (Thisse & Thisse, 2004). The CBSb morpholino was designed to splice out exon 3 (385–491 bp of NM_001014345.2l), and the CSE

morpholino was designed to splice out exon 1 (57–219 bp of NC_007117.5).

The morpholinos, which were designed by GeneTools (http://www.gene-tools.com/), were prepared to a final concentration of 4 ng nl^{-1} in 1× Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂, and 5.0 mM Hepes; pH 7.6) and 0.05% phenol red. Injections were performed using a micro-injector system (model IM 300; Narishige International USA, Inc., New York, NY, USA). Control groups were injected with a standard control morpholino (5′-CCTCTTACCTCAGTTACAATTTATA-3′) from GeneTools prepared in the same way as the CSE and
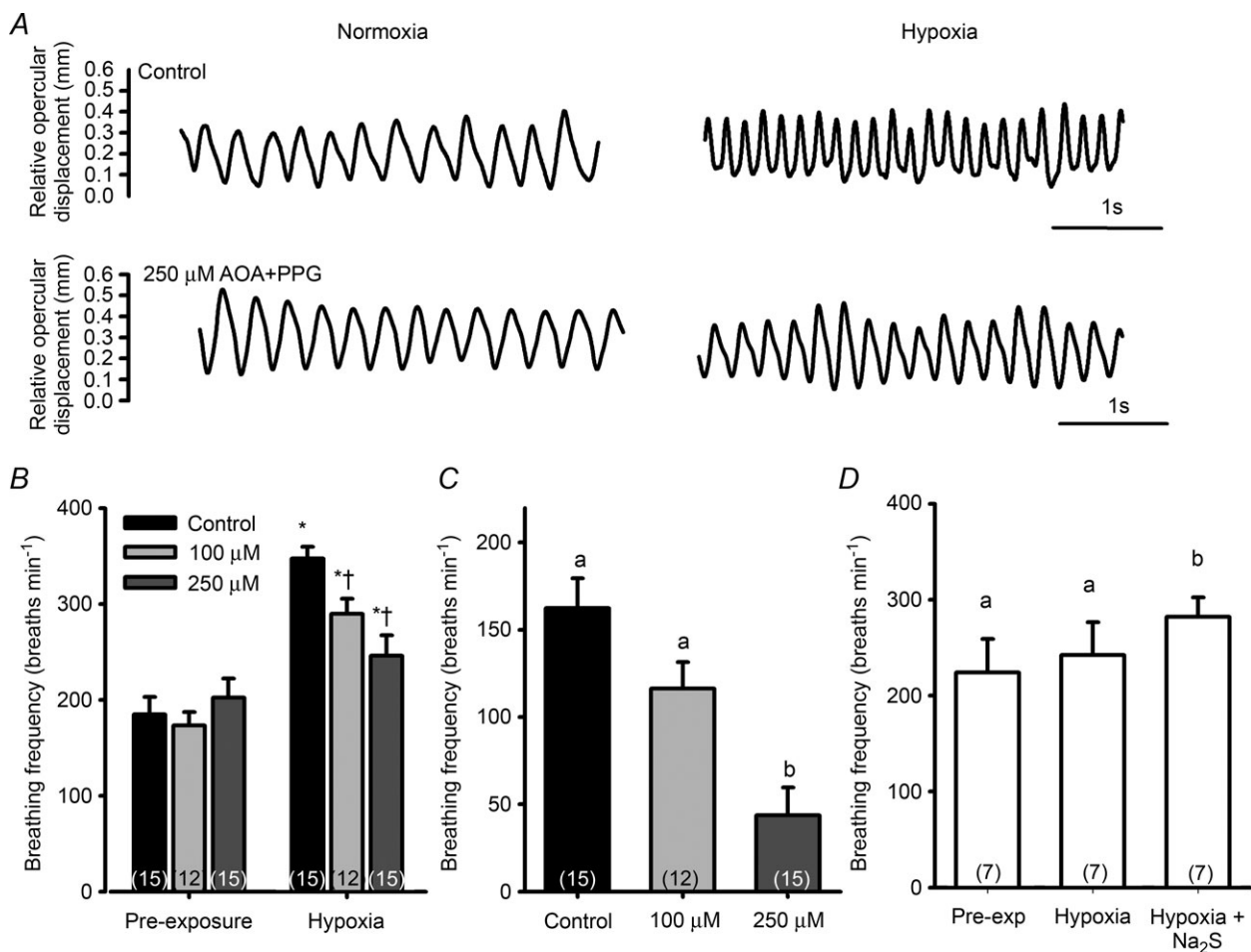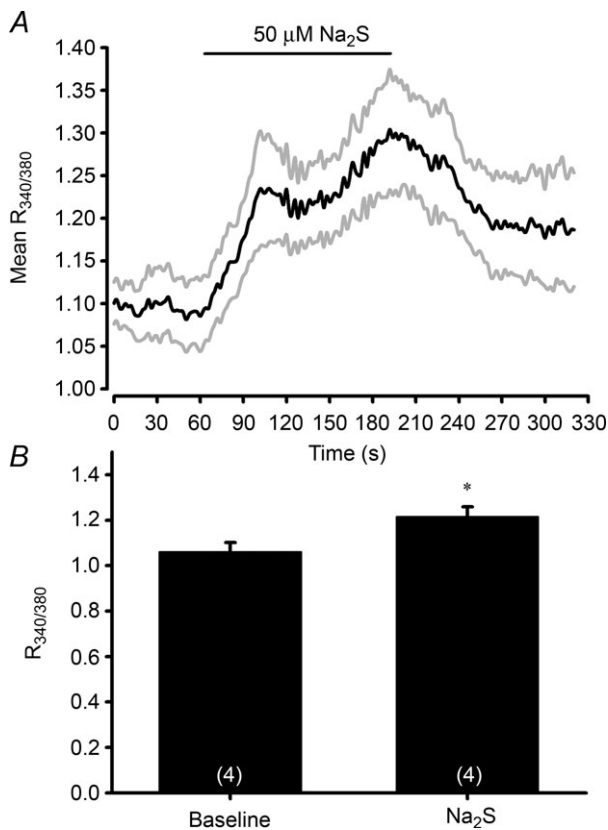

Figure 2. Effects of simultaneously inhibiting *both* cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS) on ventilatory responses of adult zebrafish (*Danio rerio*) to acute hypoxia
A, representative ventilation traces from one zebrafish during exposure to normoxia and hypoxia (40 mmHg) before (control) and after exposure to 250 μM of both aminooxyacetate (AOA) and propargyl glycine (PPG). *B*, average ventilatory responses (breaths min^{-1}) of adult zebrafish exposed to hypoxia after treatment with 100 μM or 250 μM of both AOA and PPG. *C*, average changes in breathing frequency of fish exposed to hypoxia (40 mmHg) under control conditions or after exposure to either 100 μM or 250 μM of both AOA and PPG. *D*, fish pretreated with 250 μM AOA and PPG exhibited no hypoxic ventilatory response. However, adding sodium sulphide (Na₂S) [a hydrogen sulphide (H₂S) donor] to the water partially rescued (restored) the response. *, significant (*P* < 0.05) difference from pre-exposure values; †, significant difference from control. Bars not sharing the same letters are significantly different from one another. Values are means ± S.E.M.; sample sizes are indicated in parentheses.

CBS morpholinos. The morpholino was injected at a dose of 4 ng embryo^{-1} at the 1–2 cell stages. No significant mortality or deformities were observed up to 4 dpf. Morpholinos were conjugated with fluorescein isothiocyanate (FITC) and 1 day after injection, embryos were screened using a microscope (model SMZ1500; Nikon Instruments, Inc.) for the presence of widely distributed carboxyfluorescein. Embryos that were FITC-positive were raised to 4 dpf in dechloraminated University of Ottawa tap water (see above) supplemented with 0.05% methylene blue.

Immunohistochemistry of larvae. Larvae were killed by an overdose of MS-222 and placed in 4% paraformaldehyde prepared in PBS and left overnight at 4°C. Larvae were rinsed with PBS and placed in a permeabilizing solution containing 2% Triton-X in PBS overnight at 4°C. The larvae were then blocked using 10% normal donkey serum (NDS) in PBS for 1 h and incubated in primary antibodies (goat anti-5-HT, 1 : 500, Immuno-Star and rabbit anti-CSE, 1 : 250, or rabbit anti-CBS, 1 : 250; see above) diluted in PBS containing 3% NDS overnight at room temperature. Next, the larvae were rinsed in PBS and incubated with the secondary antibodies (1 : 500, AlexaFluor 488 and 1 : 1000, AlexaFluor 568; Molecular Probes, Invitrogen Canada, Inc.) diluted in PBS containing 3% NDS for 2 h at room temperature. Following another rinse in PBS, the larvae were mounted in water onto glass slides. The larvae were observed and images were captured using a confocal microscope (Fluoview FV10i; Olympus Corp., Tokyo, Japan) equipped with a solid state laser emitting at 405 nm, 473 nm and 559 nm. Z-stacks of 9–19 optical sections taken 1.0 μm apart were captured using the 60× objective of this microscope.

Immunohistochemistry and knockdown validation experiments. In addition to pre-absorption controls (see above), the effectiveness of the CSE antibody and the CSE knockdown were confirmed by Western blotting using the same polyclonal antibody used for immunohistochemistry (see above). Total protein was extracted from morpholino- and sham-injected larvae using Tris buffer (10 mM Tris-HCl with 2% Triton X-100; pH adjusted to 7.4) supplemented with protease inhibitor tablet (Complete Mini; Roche, Mississauga, ON, Canada). Ten larvae were pooled to prepare one sample ($n = 1$). Extracted samples were loaded onto a 10% SDS-PAGE, size-fractionated at 200 V and transferred onto a polyvinylidene difluoride (PVDF) membrane [Bio-Rad Laboratories (Canada) Ltd, Mississauga, ON, Canada]. After transfer, membranes were blocked with 5% BSA in 0.2% Tween 20 in Tris-buffered saline (TBST) for 2 h at room temperature. Membranes were incubated overnight with gentle shaking with anti-CSE antibody (1 : 750 in 2% BSA in TBST) at 4°C. After that, membranes were washed (3 × 5 min) with TBST and incubated with horseradish peroxidase-conjugated secondary antibody against rabbit IgG (Life Technologies, Invitrogen Canada, Inc.) (1 : 15,000 in 2% BSA in TBST) for 2 h at room temperature. The membranes were then washed (4 × 10 min) and the immunoreactive bands were detected using enhanced chemi-luminescence (Millipore Corp., Billerica, MA, USA) with a ChemiDoc system [Bio-Rad Laboratories (Canada) Ltd]. Afterwards, the membrane was re-probed with β-actin antibody (1 : 4000; Sigma-Aldrich Canada Co.) after stripping with Re-Blot Plus solution (Millipore Corp.).

The effectiveness of the CBS knockdown was confirmed by RT-PCR using a Bio-Rad S1000 Thermal Cycler. Whole-body RNA extraction was performed in 100 embryos per treatment using Trizol (Life

Figure 3. Effects of sodium sulphide (Na$_2$S) on intracellular calcium levels (as estimated by Fura-2 340/380 nm excitation ratios) in dissociated neuroepithelial cells (NECs) from gills of adult zebrafish (*Danio rerio*)
A, mean normalized trace of all dissociated NECs responding to 50 μM Na$_2$S. An increase in the R$_{340/380}$ represents a rise in intracellular Ca^{2+}. The grey lines represent continuous standard errors. *B*, average response of dissociated NECs to 50 μM hydrogen sulphide (H$_2$S). *, significant ($P < 0.05$) difference from the pre-exposure value. Values are means ± s.e.m.; numbers are indicated in parentheses.

Technologies, Invitrogen Canada, Inc.) according to the manufacturer's specifications and cDNA was prepared by treating 1 μg of extracted RNA with DNase (Life Technologies, Invitrogen Canada, Inc.) and RevertAid M-MNuLV reverse transcriptase (Fermentas International, Inc., Burlington, ON, Canada) according to the manufacturers' instructions. Four sets of primers were used: set 1: forward 5′-ATAGACATGCTGGTCGCAGG-3′ and reverse 5′-CCTCCGGATCCACTCCAATG-3′; set 2: forward 5′-TCAGAATCAGTTGGCACTGG-3′ and reverse 5′-AATCATACCCGATGCCTTCA-3′; set 3: forward 5′-TCAGAATCAGTTGGCACTGG-3′ and reverse 5′-ATCATACCCGATGCCTTCAA-3′, and set 4: forward 5′-AACCATGCCAGCAGTACCAT-3′ and reverse 5′-AATCATACCCGATGCCTTCA-3′. Additionally, 18 S primers (forward 5′-GGCGGCGTTATTCCCATGACC-3′ and reverse 5′-GGTGGTGCCCTTCCGTCAATTC-3′) were used as controls. The PCR conditions were: 94°C for 4 min; 21 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min, and a final step of 72°C for 5 min.

Statistics

Data are routinely presented as the mean $\pm$ 1 S.E.M. All statistical analyses were performed using SigmaPlot Version 10.0 (Systat Software, Inc., San Jose, CA, USA). A significance level of 0.05 was used throughout. One-way repeated measures (RM) ANOVA was used to determine the effects of individual concentrations of Na_2S, followed by a Holm–Sidak *post hoc* test. A one-way ANOVA was performed to compare the magnitude of responses to the various concentrations of Na_2S, followed by a *post hoc* test on ranks (Dunn's test). To evaluate the responses to hypoxia with and without exposure to AOA/PPG cocktails, a two-way RM ANOVA was used to test for differences between treatments (control, 100 μmol l^{-1} and 250 μmol l^{-1}) and between normoxia and hypoxia within a treatment. This analysis was followed by a Holm–Sidak *post hoc* multiple comparison test. To test for differences in the ventilatory responses of morphant larvae, a two-way RM ANOVA was used, followed by a Holm–Sidak *post hoc* comparison. A two-way RM ANOVA was used to test for differences within and among different concentrations of Na_2S in larvae and was followed by a Holm–Sidak *post hoc* comparison. A paired *t* test was used to determine if Na_2S produced a significant increase in $[Ca^{2+}]_i$ in isolated NECs.

Results

Series I: adult zebrafish

Ventilatory responses of adult zebrafish to Na_2S. Many adult zebrafish exhibited episodic breathing under control conditions, but switched to continuous breathing when exposed to Na_2S or hypoxia (Fig. 1*A*). Adult zebrafish significantly increased gill breathing frequency by 11%, 36%, 69%, 152% and 72% compared with pre-exposure conditions when exposed to 10 μM, 19 μM, 37 μM, 75 μM and 150 μM Na_2S, respectively (Fig. 1*B* and *C*).

Effect of inhibiting endogenous H_2S production on ventilation. Zebrafish exposed to a cocktail of AOA and
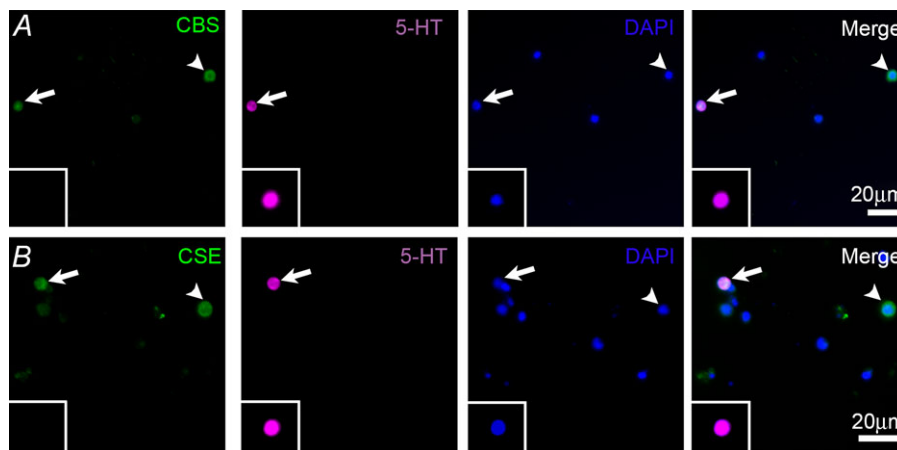
Figure 4. Immunohistochemical labelling of dissociated neuroepithelial cells (NECs) from gills of adult zebrafish (*Danio rerio*)
*A*, NECs were labelled using antibodies against cystathionine β-synthase (CBS) and serotonin (5-HT). *B*, NECs were labelled using antibodies against cystathionine γ-lyase (CSE) and 5-HT. In both panels cell nuclei were labelled with DAPI. The merged image is an overlay of all three channels. Arrows indicate cells labelled with either CBS or CSE and 5-HT. The inset shows CSE pre-absorption controls showing a 5-HT positive NEC without any CSE labelling. Arrowheads show cells labelled with either CBS or CSE, but not 5-HT. Minor adjustments to the brightness and contrast of the whole image were made using Adobe Photoshop Version 7.0.1.

PPG exhibited a dose-dependent blunting of the hypoxic ventilatory response ($P < 0.05$) (Fig. 2A–C). When fish treated with 250 μM of each of AOA and PPG were exposed to hypoxia, the addition of Na$_2$S partially rescued the hypoxic ventilatory response ($P < 0.05$) (Fig. 2D).

Calcium imaging. Four out of six cells tested responded to 50 μM Na$_2$S with an increase in $[Ca^{2+}]_i$; this rate of response (66%) was similar to response rates (60%; based on membrane potential measurements) reported previously for zebrafish NECs exposed to acute hypoxia (Jonz & Nurse, 2003). On average, the responsive NECs exhibited a small (22%) but significant ($P < 0.05$) increase in $[Ca^{2+}]_i$ (Fig. 3) when exposed to 50 μM Na$_2$S.

Immunohistochemistry of dissociated NECs. Triple labelling of dissociated cells labelled with markers for serotonin (5-HT), CBS and cell nuclei (DAPI) revealed that NECs, identified via 5-HT-immunoreactivity (5-HT-IR) expressed CBS (Fig. 4A, arrows). In addition, other unidentified cells in the culture (non 5-HT-IR cells) were positive for CBS-IR but not all cells (based on DAPI staining) expressed CBS (Fig. 4A, arrowheads). Triple labelling of dissociated cells labelled with markers for 5-HT, CSE and cell nuclei revealed that all dissociated NECs that were 5-HT-IR contained CSE (Fig. 4B, arrows); other cells in culture were only CSE-IR (Fig. 4B, arrowheads), and others were negative for both 5-HT and CSE. CSE peptide pre-absorption resulted in the absence of CSE labelling of 5-HT positive cells (Fig. 4B, insets).
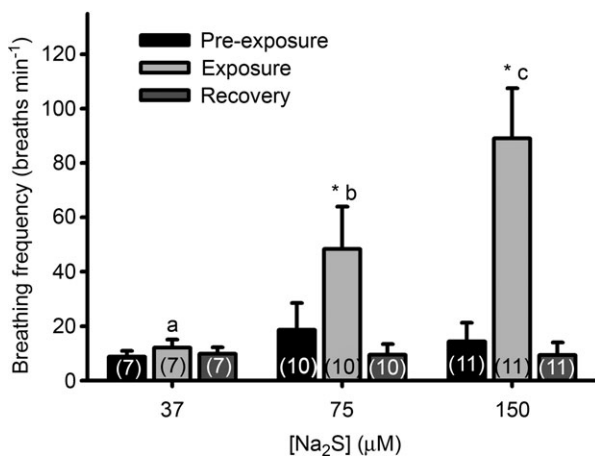
Figure 5. Ventilatory responses (breaths min⁻¹) of zebrafish larvae (4 days post-fertilization) exposed to various concentrations of sodium sulphide (Na₂S)
∗, significant ($P < 0.05$) difference from pre-exposure value. Bars not sharing the same letters are significantly different from one another. Values are means ± S.E.M.; sample sizes are indicated in parentheses.

Series II: larval zebrafish

Ventilatory responses of zebrafish larvae to Na₂S. Ventilatory frequency was low (14.4 ± 6.8 breaths min⁻¹) in larval zebrafish exposed to control conditions (aerated water), but increased by 37%, 160% and 520% when exposed to 37 μM, 75 μM and 150 μM Na$_2$S, respectively (Fig. 5). These changes were statistically significant for the two highest concentrations ($P < 0.05$) and differed significantly from one another, indicating a dose-dependent response ($P < 0.05$).

Effect of CBS/CSE knockdown on ventilatory responses to hypoxia. Double knockdown of CBS and CSE was lethal; the embryos did not survive past 24 h of development. Larvae experiencing CBS or CSE knockdown developed normally to 4 dpf and appeared similar to sham-injected larvae. Sham-injected larvae exhibited a robust increase in ventilation frequency in response to hypoxia ($P < 0.001$) (Fig. 6A and B). Larvae experiencing CSE (Fig. 6A) or CBS (Fig. 6B) knockdown failed to exhibit a statistically significant hypoxic ventilatory response. At all measurement periods during hypoxia, ventilation frequency in the CSE/CBS morphants was significantly lower than in the sham-injected larvae ($P < 0.05$) (Fig. 6A and B). CSE knockdown was confirmed by Western blotting (Fig. 6C), which showed a 98% reduction in CSE protein expression level (data not shown). CBS knockdown was confirmed using RT-PCR (Fig. 6D).

Whole-mount immunohistochemistry. In all immunohistochemical procedures, omission of the primary antibody eliminated positive labelling. The efficiency of the CSE antibody was confirmed by Western blotting (Fig. 6C). Double labelling of dissociated cells using antibodies against 5-HT and CSE revealed that some 5-HT-IR cells also labelled with CSE (Fig. 7). However, some cells were 5-HT-IR but did not contain CSE, but very few CSE-IR cells did not label for 5-HT. No positive labelling was detected in NECs with the CBS antibody (data not shown).

Discussion

In zebrafish, NECs in culture experience membrane depolarization in response to hypoxia (Jonz *et al.* 2004) and hypercapnia (Qin *et al.* 2010). Further, NECs are believed to be phylogenetic precursors to the carotid body glomus cells with which they share several distinct characteristics (Milsom & Burleson, 2007) including K⁺ conductance, which is inhibited by lowered O$_2$ and increased CO$_2$. In addition, they are innervated and contain synaptic vesicles and neurotransmitters. These findings, when combined with the results of

pharmacological treatments used to characterize chemoreceptors in mammals, have led to the theory that NECs are the putative O_2 and CO_2 chemoreceptors in fish (Jonz *et al.* 2004; Qin *et al.* 2010). Both NECs and glomus cells are in close proximity to blood vessels carrying oxygenated blood and thus are ideally located for sensing changes in blood O_2 tension. Although several mechanisms have been proposed to explain O_2 sensing in mammalian glomus cells (López-Barneo *et al.* 2001; Chandel, 2010; Peers *et al.* 2010; Weir & Archer, 2010), the mechanisms by which NECs detect changes in O_2 remain largely unknown.

To determine if exogenous H_2S is capable of stimulating breathing *in vivo* in zebrafish, larvae and adults were exposed to Na_2S in order to generate H_2S. Although previous studies have used similar approaches to increase exogenous H_2S, it is nevertheless challenging to calculate

the exact amount of H_2S produced by these chemical treatments and therefore the exact dose to which cells, or in this case fish, are being exposed (Olson, 2013*a*). Moreover, H_2S is highly volatile and thus levels of H_2S in solution after the addition of Na_2S are certain to decrease over time. Despite these issues, both zebrafish adults and larvae showed a dose-dependent increase in breathing frequency in response to H_2S produced via dissociation of Na_2S (Figs 1 and 5). Similar increases in ventilation to exogenous H_2S used at similar concentrations were reported previously in mice (Li *et al.* 2010), rats (Peng *et al.* 2010) and trout (Olson *et al.* 2008; Li *et al.* 2010; Peng *et al.* 2010). Unlike in trout (Olson *et al.* 2008), in zebrafish, breathing frequency rather than breathing amplitude increased in response to Na_2S. The differing modes of hyperventilation are likely to reflect species differences because trout
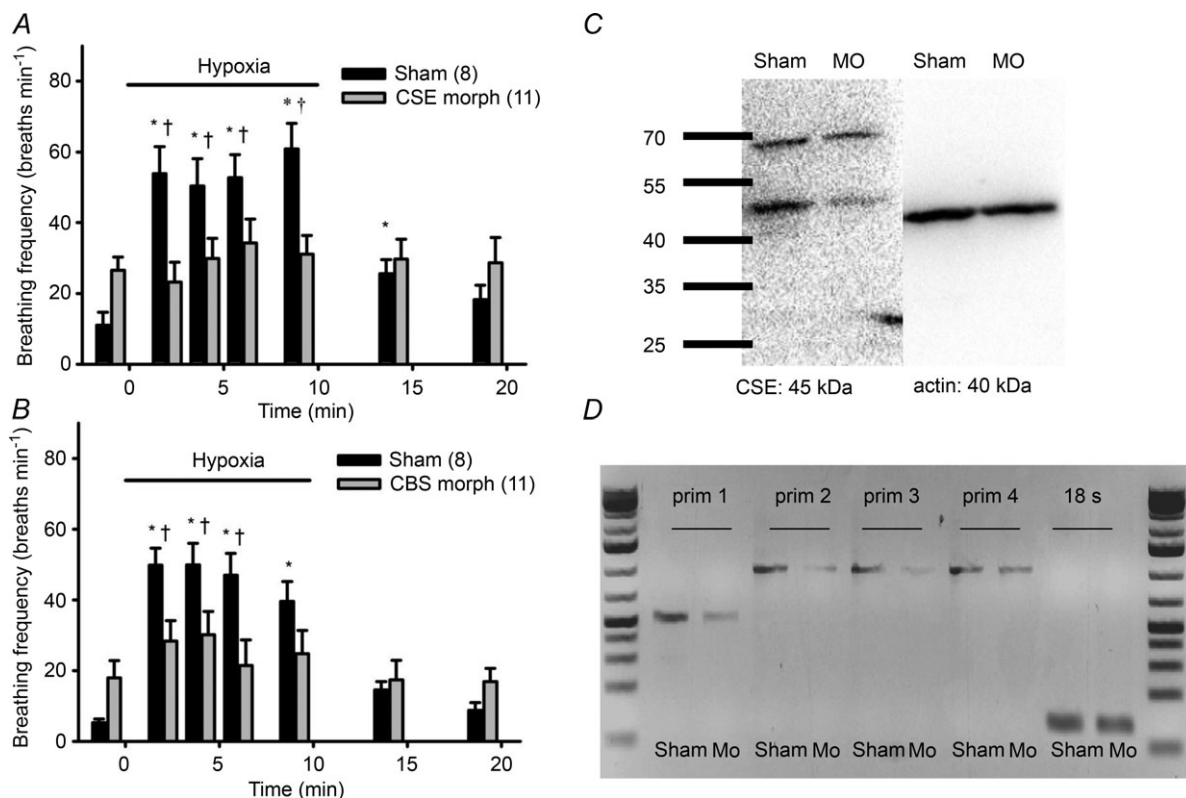
Figure 6. Effects of knockdown on ventilatory responses in larval zebrafish (*Danio rerio*) hypoxia
The hypoxic (25 mmHg) ventilatory response of zebrafish larvae (4 days post-fertilization) in which expression of either cystathionine γ-lyase (CSE) (*A*) or cystathionine β-synthase (CBS) (*B*) was knocked down using morpholinos. *C*, representative Western blot of protein derived from morpholino- (MO) and sham-injected zebrafish larvae labelled with the CSE antibody confirming specificity of this antibody. *D*, confirmation of CBS knockdown using RT-PCR in both CBS- (Mo) and sham morpholino-injected larvae. Four sets of primers were used: set 1 (prim 1): forward 5′-ATAGACATGCTGGTCGCAGG-3′ and reverse 5′-CCTCCGGATCCACTCCAATG-3′; set 2 (prim 2): forward 5′-TCAGAATCAGTTGGCACTGG-3′ and reverse 5′-AATCATACCCGATGCCTTCA-3′; set 3 (prim 3): forward 5′-TCAGAATCAGTTGGCACTGG-3′ and reverse 5′-ATCATACCCGATGCCTTCAA-3′; set 4 (prim 4): 5′-AACCATGCCAGCAGTACCAT-3′ and reverse 5′-AATCATACCCGATGCCTTCA-3′. Additionally, 18 S primers (forward 5′-GGCGGCCGTTATTCCCATGACC-3′ and reverse 5′-GGTGGTGCCCTTCCGTCAATTC-3′) were used. *, significant difference ($P < 0.05$) from pre-exposure (normoxia) value; †, significant difference ($P < 0.05$) from CBS or CSE morphants. Mo, CBS or CSE morphant. Values are means ± S.E.M.; sample sizes are indicated in parentheses.

respond to hypoxia by predominantly increasing breathing amplitude, whereas zebrafish typically show a much larger increase in frequency (Perry *et al.* 2009*b*). Overall, H$_2$S produces an increase in ventilation similar to the HVR in both trout and zebrafish, indicating that exogenous H$_2$S produces effects similar to hypoxia in fish.

To determine if H$_2$S was necessary to initiate the HVR in zebrafish, H$_2$S biosynthesis was impaired. In adult zebrafish, inhibiting CBS and CSE with a cocktail of AOA and PPG blunted or abolished the HVR and exogenous H$_2$S produced from Na$_2$S rescued, at least in part, the response (Fig. 2*A–D*). Rats and mice treated with PPG showed a blunted or no carotid body response to hypoxia and a blunted or no HVR (Peng *et al.* 2010; Makarenko *et al.* 2012), indicating that CSE plays an important role in the HVR in mammals. Similarly, in a separate study, mice treated with AOA, a CBS inhibitor, also showed a blunted HVR and impaired carotid body sensory response (Li *et al.* 2010). In trout, the addition of a cocktail of both CBS and CSE inhibited H$_2$S production in homogenized gill filaments, but AOA and PPG did not blunt the HVR or hypoxic bradycardia (Olson *et al.* 2008). It is possible that the discrepancy between the effects of enzyme inhibition on ventilation in these two studies is related to methodological differences. Trout were exposed to a lower dose (5 mg l^{-1}; 46 μM and 44 μM of AOA and PPG, respectively) of the AOA/PPG cocktail for a shorter period of time (30 min), whereas in the current study zebrafish were exposed to higher doses (100–250 μM) of the AOA/PPG cocktail for longer periods of time (16–20 h). Our results suggest that longer incubation periods might be necessary to allow for uptake of these inhibitors from the water and thus inhibition of the enzymes.

In zebrafish larvae, double knockdown of CBS and CSE was lethal past 24 h, confirming the importance of H$_2$S in the development of these animals. The efficacy of CSE knockdown was confirmed by Western blotting (Fig. 6*C*) with a 98% reduction in CSE protein expression level (quantification data not shown), whereas the knockdown of the CBS antibody was confirmed by RT-PCR (Fig. 6*D*). Knocking down either CBS or CSE abolished the HVR, indicating that H$_2$S is necessary for the HVR of larvae (Fig. 6*A* and *B*). Similarly, CSE knockout mice had reduced carotid body sensitivity, a blunted HVR, and reduced H$_2$S generation (Peng *et al.* 2010; Makarenko *et al.* 2012). Interestingly, in zebrafish larvae only CSE was localized to NECs; however, larvae experiencing CBS knockdown clearly exhibited a blunted HVR. As CBS is predominantly found in the central nervous system in other vertebrates (Olson, 2008), it is possible that the HVR may have been inhibited in the CNS of these animals, perhaps at the sensory integration sites of the medulla or the central rhythm generators. Indeed, the idea that CSE and CBS may generate H$_2$S at two different sites involved in the regulation of breathing is consistent with our results showing that knockdown or pharmacological inhibition of a *single* isoform is able to abolish the HVR. At present, we do not have a way of labelling the brain of zebrafish larvae with these antibodies because antibody penetration of this tissue is poor in whole-mount preparations. In the current study, we showed that inhibiting H$_2$S production blunts or abolishes the HVR in adults and larvae, indicating that H$_2$S is necessary and sufficient to produce the HVR in zebrafish.

In fish, NECs are innervated by afferent nerves from cranial nerves IX and X (de Graaf, 1990; Sundin & Nilsson, 2002), which project to the general visceral nucleus, an area thought to be homologous to the *nucleus tractus solitarius* (NTS) in mammals (Sundin *et al.* 2003). In the present study, dissociated NECs showed an increase in [Ca^{2+}]$_i$ in response to 50 μM Na$_2$S. Although absolute intracellular Ca^{2+} levels were not measured, the increase in the 340/380 nm excitation ratio was markedly lower
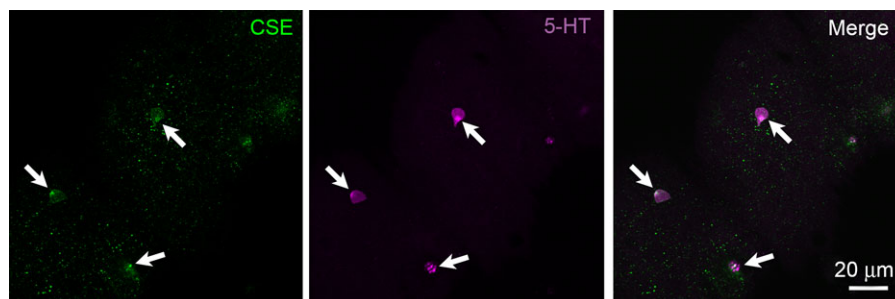
Figure 7. Immunohistochemical labelling of neuroepithelial cells (NECs) of developing zebrafish (*Danio rerio*)
Whole mounts of 4 days post-fertilization larvae were labelled with antibodies against cystathionine γ-lyase (CSE) and serotonin (5-HT). The image shows a section of the body, posterior to the yolk sac projection; the anterior part of the larvae is to the top right and the posterior towards the bottom left. Arrows indicate cells that double-label with CSE and 5-HT. Minor adjustments to the brightness and contrast of the whole image were made using Adobe Photoshop Version 7.0.1.

than in zebrafish NECs exposed to 5% CO_2 (S. Abdallah, M. Jonz and S. F. Perry; unpublished observations, 2013). The changes in 340/380 nm excitation ratios reported here for cells exposed to Na_2S were similar to the increases in 340/380 nm excitation ratios reported for dissociated NECs of goldfish (*Carassius auratus*) exposed to hypoxia (Zachar, 2013). Moreover, in a previous study, dissociated NECs of zebrafish depolarized ($\sim$10 mV) in response to 50 μM Na_2S and this depolarization was similar in magnitude to that observed in response to hypoxia (Olson *et al.* 2008). Taken together, these results show that H_2S can depolarize NECs and lead to an increase in Ca^{2+}, steps necessary for the release of neurotransmitters onto afferent nerve fibres and thus a reflex ventilatory response to hypoxia.

Immunohistochemistry was used to determine if the putative O_2-sensing cells of zebrafish, the NECs, have the capacity to produce H_2S. Similarly to the carotid body glomus cells of mammals (Li *et al.* 2010; Telezhkin *et al.* 2010; Fitzgerald *et al.* 2011; Makarenko *et al.* 2012), dissociated NECs of adult zebrafish expressed both CBS and CSE. These results are consistent with CBS and CSE mRNA expression in trout gills (Olson *et al.* 2008) and further demonstrate that the biosynthetic enzymes of H_2S are present not only in the gills but specifically in NECs. Interestingly, zebrafish larvae appeared to express only CSE, which is predominantly expressed in peripheral tissues, whereas CBS is predominantly expressed in the CNS in mammals (Olson, 2008). In zebrafish larvae, the HVR develops before NECs form in the gills and innervated NECs on the skin are thought to be responsible for initiating the HVR (Coccimiglio & Jonz, 2012) prior to maturation of gill NECs. Skin NECs are similar to gill NECs, but may represent a different population of cells with similar characteristics. Our results suggest that CBS either does not become expressed in NECs until later in development or that skin NECs never express CBS. Additionally, we cannot exclude the possibility that CBS, although present in skin NECs, was undetectable by immunohistochemistry (as in the adult gill) on whole-mount preparations. Although the scope of this issue is challenging, future studies should attempt to localize CBS mRNA in larval NECs by *in situ* hybridization or to dissociate NECs from zebrafish larvae in a further attempt to localize CBS in these cells by immunohistochemistry.

There are two proposed mechanisms underlying H_2S in O_2 sensing based on observations that H_2S concentration increases in tissues exposed to hypoxia. In one scenario, H_2S production is dependent on O_2 availability (Olson, 2013*b*). If O_2 levels are adequate, H_2S is oxidised in the nearby mitochondria (Olson, 2013*b*). When O_2 levels in the cell drop, H_2S accumulates as the rates of mitochondrial oxidation of H_2S fall and vice versa (Olson *et al.* 2008). An alternative mechanism involves

the production of CO by HO in normoxia and the inhibition of CSE by CO, and, therefore, an inhibition of H_2S production (Prabhakar, 2013; Peng *et al.* 2014). In hypoxia, production of CO decreases, releasing the inhibition on CSE and thereby resulting in an increase in H_2S production. In mammals, H_2S is thought to act as a signalling molecule by inhibiting K^+ channels, leading to an increase in $[Ca^{2+}]_i$ (Li *et al.* 2010; Telezhkin *et al.* 2010; Buckler, 2012). Although further research is necessary to confirm a similar mode of action of H_2S on K^+ channels in fish, our data clearly revealed an increase in $[Ca^{2+}]_i$ in response to exogenous H_2S, presumably arising from membrane depolarization (Olson *et al.* 2008).

Conclusion

In the current study, adult and larval zebrafish exhibited a dose-dependent increase in breathing frequency when exposed to Na_2S. Furthermore, adult zebrafish displayed a blunted HVR when pre-exposed to AOA and PPG, inhibitors of the biosynthetic enzymes of H_2S; the normal hyperventilatory response was partially restored by the addition of Na_2S. In larvae, knockdown of either CBS or CSE inhibited the HVR. Immunohistochemistry of NECs revealed the presence of both CBS and CSE in adults, whereas NECs of larvae appeared to express only CSE. Finally, in Ca^{2+} imaging experiments, dissociated NECs significantly increased $[Ca^{2+}]_i$ in the presence of Na_2S. Combined, these results indicate that H_2S in NECs is involved in oxygen sensing and that it plays an important role in the HVR.

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Additional information

Competing interests

None declared.

Author contributions

S.F.P., C.S.P., Y.K., S.J.A., J.P. and W.K.M. designed and conceptualized the experiments. S.F.P., C.S.P., S.J.A., J.P., R.W.M.K., H.M.Y. and Y.K. carried out the research. C.S.P, S.J.A., J.P., R.W.M.K., H.M.Y. and S.F.P analysed the data; S.F.P. and C.S.P. wrote the paper, and all authors contributed feedback to various drafts of the manuscript.

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