Cholinergic signaling is essential for hydrogen sulfide responses in *Caenorhabditis elegans*

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Abstract

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Hydrogen sulfide, H_2S, is one of three endogenously produced gaseous molecules that can act in cellular signaling. In the brain, H_2S has proposed roles in modulating long-term potentiation, nociception, and neuroprotection. To further understand the impact of H_2S on neurons, we conducted a candidate screen in *Caenorhabditis elegans* of neuronal factors to identify those involved in the normal response to H_2S. Our studies show that cholinergic signaling is essential to survive exposure to H_2S. We found animals with mutations in genes required for the biosynthesis and trafficking of acetylcholine died when exposed to H_2S in conditions where wild-type animals survive. Our data further indicate that the levamisole-sensitive acetylcholine receptor subunit, *unc-29*, is involved in H_2S signaling. Unexpectedly, we discovered that the requirement for *unc-29* in H_2S is abrogated by entry into dauer. Together, our results suggest there are multiple neuronal responses to H_2S that vary based on physiological context. Both H_2S and cholinergic signaling have been implicated in neurodegenerative diseases, including Parkinson ’s disease. Our data suggest that understanding the interactions between cholinergic signaling and H_2S could lead to new therapeutic strategies for these devastating diseases.

Introduction

Acetylcholine (ACh) was the first neurotransmitter to be discovered (Loewi, 1921). ACh is the major excitatory neurotransmitter at the neuromuscular junction (NMJ) where it causes muscle contraction in most organisms (del Castillo, 1963). In the brain, ACh has diverse effects on neuromodulation, synaptic plasticity, and neuronal excitation (Piccioto, 2012). Disturbances of cholinergic signaling contribute to a variety of neurological disorders, including autism spectrum disorder, Alzheimer's Disease, and Down's Syndrome (Deutsch, 2010; Francis, 1999; Fodale, 2006).

H_2S is produced in the brain by cystathionine beta synthase and 3-mercaptopyruvate sulfurtransferase (Shibuya, 2009; Kimura, 2002). H_2S influences insulin release and nociception (Kimura, 2010) and acts as a neuromodulator, facilitating hippocampal long-term potentiation (Abe, 1996; Kimura, 2002). In frogs and mice, H_2S increases quantal ACh release at the NMJ (Gerasimova 2013, 2015). Endogenously produced H_2S enhances carotid body responses to hypoxia in mice (Peng, 2010). Normal carotid body responses to hypoxia lead to the release of ACh and ATP, however in cats exogenous H_2S decreases the release of ACh from isolated carotid bodies without hypoxic treatment (Fitzgerald, 2011). It is especially critical to gain insight into the complex and sometimes contradictory interplay between ACh and H_2S signaling, because both are implicated in Parkinson's Disease in rodent models (Aosaki, 2010; Ryan, 2001; Janson, 1988; Gorell, 1999; Hu, 2010).

In the nematode *C. elegans*, exogenous H_2S increases lifespan and protects against hypoxic stress, including protein misfolding (Miller, 2011; Fawcett, 2015). Here we show that cholinergic signaling through the levamisole-sensitive nicotinic receptor is an essential aspect of the physiological response to H_2S in *C. elegans*. We further demonstrate that transient entry into the alternative developmental stage dauer eliminates the requirement for cholinergic signaling in H_2S. We propose that H_2S acts through distinct pathways in fed and dauer worms, and that the response in fed animals requires cholinergic signaling.

These data demonstrate an important physiological interaction between cholinergic signaling and alternative developmental progression, and highlight a critical novel role for ACh in the H_2S response. Understanding the role of ACh in H_2S could lead to novel strategies for the treatment of neurological diseases.

Materials and methods

Strains

Wild-type *C. elegans* (N2 Bristol) and mutant strains were grown at room temperature (25 degrees C) on nematode growth medium (NGM) plates seeded

with *Escherichia coli* OP50 food, as previously described (Brenner, 1974). Stocks were obtained from the *C. elegans* genetic stock center (see supplemental table 1).

Atmospheric Chambers

Construction of atmospheric chambers has been described previously (e.g. Nystul, 2004; Fawcett, 2012). In short, atmospheres were made by combining compressed H_2S stock mixtures with room air at a controlled rate to produce the desired final gas concentration of 50 parts per million (ppm). All compressed gas mixtures were obtained from Airgas (Radnor, PA) and certified standard to within 2% of the indicated concentration.

Crosses

To obtain males, 5-10 fourth-stage larvae (L4) animals of strain 1 were heat shocked at 37 degrees for one hour. Post-heat shock, hermaphrodites produce males with higher frequency (He, 2011). 7-10 males were then picked to small plates with 3-4 hermaphrodites of strain 2. Once strain 2 hermaphrodites become gravid (egg-containing), they were removed to individual small plates. Plates with roughly 50% males several days later were considered successfully mated, and 3 or 4 hermaphrodite offspring (heterozygous for strains 1 and 2—F1) were picked to individual plates and allowed to self-fertilize to create progeny. Then, roughly 20 second generation (F2) offspring were picked to individual plates and allowed to lay eggs. Roughly 1/16 of these F2s are double mutants given standard Mendelian genetic inheritance. After laying eggs, adults were genotyped for mutations in gene 1 and gene 2, and plates whose parent was a double mutant were kept. Several offspring from these plates were re-genotyped to confirm that the resulting strain contains two mutations.

Survival Assays

Ten to 25 L4 animals per strain were transferred to clean plates with food. Plates were put in atmospheric chambers as described above and left for 15-24 hours. Plates were removed and left to sit at room temperature for at least half an hour. Death was assayed as failure to move when tapped at the head or tail after this recovery period.

mRNA Quantification

Quantitative polymerase chain reactions (qPCRs) have been described elsewhere (e.g. Budde, 2010). Briefly, a mixed population of *C. elegans* was exposed to air or 50 ppm H_2S for 1 hour. 1 mL of TRIzol (Invitrogen) was added to 0.1 mL of packed worms, and worms were frozen in liquid nitrogen. RNA was extracted as described in the TRIzol manual, and cDNA was synthesized using a ThermoScript RT-PCR system (Thermo Fisher Scientific) according to manufacturer's instructions. qPCR was performed in an Realplex MasterCycler (Eppendorf) by using Platinum SYBR Green qPCR Supermix (Invitrogen) in 0.03-mL reactions. Standard curves were

generated from cDNA, and data were analyzed as per Pfaffl (2001). cDNA levels were normalized to the geometric mean of *hil-1, tba-1,* and *irs-2* cDNA, which do not change expression in H_2S (Miller, 2007). *gen-2* cDNA provided a genomic DNA control and cDNA expressed at a lower level than *gen-2* was excluded from analysis.

Paralysis Assay

Ten animals per condition were put into H_2S for the designated length of time (10, 20, 30, 60, or 120 minutes). After that time had elapsed, plates were removed and animals were immediately poked on the head and tail. Paralysis was scored as a failure to move in response to gentle taps, and was recorded as a fraction out of ten. Unless otherwise specified, paralysis assays were carried out on day one adults; L4 animals were picked the day before the assay was performed.

Dauer isolation

Dauers were isolated from naturally starved plates by applying 1% SDS for 10 minutes. Dauer animals survive immersion in this liquid while non-dauers do not (Hu, 2007). Dauers were then picked immediately to plates with or without food. Foodless plates were inoculated with CARB to prevent growth of bacteria. For the dauer recovery assay, worms were left on food for 24 hours at 25 degrees C, at which point they were in the L4 stage.

Results

Neuronal expression of *hif-1* is sufficient for H_2S responses.

C. elegans requires *hif-1* to survive in H_2S (Budde, 2010). *hif-1* is the sole *C. elegans* orthologue of the highly conserved bHLH transcription factor HIF (the hypoxia inducible factor), which mediates a broad transcriptional response to low oxygen (hypoxia) in metazoans (Semenza, 2000; Jiang, 2001). *hif-1* is required for all early transcriptional responses to H_2S in *C. elegans* (Miller, 2011). Although *hif-1* plays important roles in activating transcription in both hypoxia and H_2S, there is little overlap in the *hif-1*-dependent genes that are regulated in each condition (Miller, 2011).

To explore the role of *hif-1* in H_2S survival, we asked whether rescuing HIF-1 in specific tissues would permit a wild-type H_2S response. HIF-1 protein accumulates in most, if not all, cells when animals are exposed to hypoxia or H_2S (Budde, 2010; Shen, 2003). However, *hif-1* is only required in neurons to regulate hypoxia-induced developmental diapause (Miller, 2009). Thus, we considered the possibility that there would be tissue-specific requirements for *hif-1* in H_2S.

We exposed strains that expressed an activated HIF-1 only in specific tissues to H_2S to determine where HIF activity was required in H_2S. As expected, *hif-1(ia4)* mutant

animals all died when exposed to H_2S. Rescuing expression of HIF-1 in muscles did not improve survival in H_2S (Fig 1B). In contrast, animals expressing HIF-1 exclusively in neurons (*hif-N*) all survived H_2S (Fig 1A and B). This result indicates that HIF-1 stabilization in neurons is sufficient to activate the H_2S survival response.

Since *hif-1* is required for all early transcriptional responses to H_2S, even of non-neuronal targets (Budde, 2011), we next asked if *hif-1* target genes were upregulated in *hif-N* animals exposed to H_2S. We found that mRNA abundance of 6 known *hif-1* target genes was increased by exposure to H_2S in *hif-N* mutants in a manner indistinguishable from wild-type controls (Fig. 1C). Because neurons constitute a small percentage of the total mass of *C. elegans* and the mRNA signal increase in animals exposed to H_2S is robust, it is unlikely that the increase in mRNA abundance seen in *hif-N* animals represents genes turned on exclusively in neurons. We examined expression of one known *hif-1* target, *sqrd-1* (Budde, 2011), and found that *hif-N* animals show expression of *sqrd-1::gfp* throughout the hypodermis and body wall muscle (Fig. 1D). This further supports our hypothesis that *hif-1* expression just in neurons is sufficient to activate an organism-wide H_2S response program.

Cholinergic signaling is required for survival in H_2S.

Based on the finding that neurons play a role in H_2S responding, we reasoned that neuronal signaling might be involved in the response. To identify neuronal factors involved in mediating the response to H_2S, we performed a candidate screen of mutants with a variety of neuronal defects. We reasoned that if any mutant animals died when exposed to H_2S it would suggest a potential role for the corresponding neuronal factor in controlling the response to H_2S.

We screened 31 mutant strains with defects in neurotransmission, neurotransmitter synthesis, dense core vesicle trafficking, and other neuronal functions. Of these candidates, defects in cholinergic signaling (*cha-1(p1152), unc-17(e245)*) had the greatest effect on survival in H_2S (Fig 2A), though we also noted a sensitivity of mutant animals with defects in GABA synthesis and trafficking. *cha-1* is the worm homologue to choline acetyltransferase, which metabolizes choline into acetylcholine, and *unc-17* encodes the vesicular acetylcholine transporter, responsible for packaging acetylcholine into the vesicle (Rand, 1984).

Based on our candidate screen, we hypothesized that other mutations that disrupted cholinergic signaling would also cause H_2S lethality. We first measured survival in H_2S of several other alleles of *cha-1*. As expected, animals with the *p1152* or *y226* alleles died when exposed to H_2S (Fig. 2C). However, *cha-1(p503)* mutant animals survived exposure to H_2S. The *p503* allele is hypomorphic, and retains 10% of normal CHA-1 activity (Rand, 1984). In contrast, both *p1152* and *y226* alleles decrease CHA-1 activity by at least 99%. This result suggests that the residual CHA-1 activity in animals with the *p503* allele is sufficient for the H_2S response. The rate-limiting step in cholinergic neurotransmission is the choline transporter

(Bazalakova, 2006) encoded by worm *cho-1* (Okuda, 2000). We found that *cho-1(ok1069)* mutant animals died when exposed to H_2S (Fig 2B). These findings support our conclusion that cholinergic signaling is an essential aspect of the response to H_2S.

We reasoned that cholinergic signaling in the H_2S response would involve one or more acetylcholine receptors (AChRs). Like mammals, *C. elegans* have two main classes of AChRs: muscarinic and nicotinic AChRs. Muscarinic AChRs (mAChRs) are G protein-coupled receptors (Eglen 2006). *gar-1, gar-2,* and *gar-3* are homologous with vertebrate mAChRs (Lee, 2000&1999), though *gar-3* shares the most structural and pharmacological similarities with the mammalian mAChRs (Park 2003). We found that animals with mutations in any of these three genes survived exposure to H_2S (Fig. 3B, see also supplemental table 1). We conclude that mAChRs are not involved in the H_2S response.

In contrast to mAChRs, nicotinic AChRs (nAChRs) are ionotropic receptors. When two ACh ligands bind a nAChR, the channel changes conformation and allows ions to flow through nonselectively. This typically results in Na^+ entry, which causes cell depolarization and facilitates firing (Albuquerque, 2009). *C. elegans* has at least 27 nAChR subunits (Jones, 2004) compared to sixteen known mammalian nAChR subunits (Albuquerque 2009). In the worm, nAChR subunits are divided into five classes based on sequence similarity. We screened animals with mutations in each of the five classes to determine which were important for survival in H_2S. We found that both *unc-38* and *unc-29* were necessary to survive exposure to H_2S (Fig 3A). Mutants with defects in *acr-8(ok1240)*, *acr-16(ok789)*, or *deg-3(u662)* survived H_2S like wild-type controls (Fig 3A). These results suggest that two classes of AChR receptors are involved in the response to H_2S.

nAChR receptors are composed of five subunits (Hernando, 2012). UNC-38, an α-subunit, and UNC-29, a non-α subunit, are components of the levamisole-sensitive nAChR (L-AChR). Functional L-AChRs require at least one α and one non-α subunit, and may include any or all of: LEV-1 (non-α), LEV-8 (α), ACR-8 (α), and UNC-63 (α) in addition to UNC-38 and UNC-29 (Hernando, 2012; Culetto, 2004; Towers, 2005). We did not observe a requirement for *lev-1*, *lev-8*, *acr-8*, or *unc-63* in H_2S (Fig. 3B). This result does not preclude a central role for L-AChRs in H_2S, however, because functional channels can form in Xenopus oocytes expressing only *unc-38* and *unc-29* (Fleming, 1997). Consistent with a role for L-AChRs in the H_2S response, animals with mutations that disrupt trafficking (*unc-50(ok1847);* (Eimer, 2007)) or maturation (*ric-3(hm9);* (Halevi, 2002)) of nAChRs also died when exposed to H_2S (Fig 3C). We conclude that cholinergic signaling through L-AChRs is an essential mediator of the response to H_2S.

Balanced cholinergic signaling at the NMJ is required for the H_2S response

Cholinergic signaling at the NMJ plays an important role in locomotion (Sarter, 1997). As movement is compromised in H_2S (Budde, 2011), we considered the possibility that cholinergic signaling is required at the NMJ for the response to H_2S. To test this hypothesis, we exposed *unc-3(e151)* mutant animals, which lack expression of all cholinergic genes in motor neurons (Kratsios, 2011), to H_2S. We observed high lethality of *unc-3(e151)* mutant animals exposed to H_2S, consistent with the suggestion that cholinergic signaling at the NMJ is required for H_2S survival (Fig 4A).

When *C. elegans* are exposed to H_2S, they initially display increased movement (Budde, 2011), which is consistent with the idea that cholinergic activity at the NMJ increases in H_2S. Moreover, when wild-type animals are exposed to high H_2S (> 150 ppm H_2S), they become paralyzed long before they die (Budde 2011). We noted that animals paralyzed in H_2S resemble animals paralyzed with levamisole, a potent L-AChR agonist (Lewis, 1980; Petzold, 2011). Thus, excessive cholinergic signaling may contribute to H_2S toxicity at high concentrations. We found that *ace-1; ace-2* double mutant animals, which disrupt the acetylcholinesterases that break down ACh (Combes, 2000), died when exposed to H_2S (Fig. 4B), which supports this notion. Together, these experiments suggest that H_2S exposure activates L-AChRs at the NMJ.

Cholinergic signaling and *hif-1* act in genetically separate H_2S-response pathways.

When *hif-1(ia4)* mutants are exposed to H_2S they become paralyzed long before they die, similar to wild-type animals exposed to high H_2S (Budde, 2011). This could indicate an excess of ACh in the NMJ. However, we found that *unc-29(ok2450)* mutant animals, which do not have a functional L-AChR (Culetto, 2004), also became paralyzed when exposed to H_2S. This result suggests the possibility that H_2S could induce paralysis through multiple mechanisms.

If *unc-29(ok2450)* and *hif-1(ia4)* mutant animals become paralyzed through the same mechanism in H_2S, we would expect both mutant animals to become paralyzed at similar rates. In contrast, we found that adult *unc-29(ok2450)* mutant animals became paralyzed much faster in H_2S than *hif-1(ia4)* mutant animals (Fig. 5A). This is consistent with our suggestion that there are at least two mechanisms by which the response to H_2S can lead to paralysis, one of which requires signaling through L-AChR and another that occurs in the absence of L-AChR activation.

We reasoned that if delayed paralysis in the *hif-1* mutant animal required activation of L-AChRs, then the *hif-1(ia4);unc-29(ok2450)* double mutant worms would become paralyzed rapidly, just like the *unc-29(ok2450)* mutant alone. To test this, we constructed the *hif-1(ia4);unc-29(ok2450)* double mutant and measured the rate of paralysis in H_2S. We found that the *hif-1(ia4);unc-29(ok2450)* double mutant animals became paralyzed at the same rate as *unc-29(ok2450)* single mutants (Fig.

5A). This result supports the hypothesis that in H_2S, *hif-1* mutant animals become paralyzed as a result of L-AChR signaling.

The fact that *unc-29* is epistatic to *hif-1* when measuring paralysis in H_2S suggests the possibility that L-AChR signaling may contribute to activation of HIF-1 in H_2S. Moreover, the L-AChR receptor subunit *unc-29(ok2450)* was not identified as a transcriptional target of HIF-1 in microarray studies (Shen, 2005), suggesting that it is not a downstream target of HIF-1. We reasoned that if L-AChR activation is required to activate HIF-1 in H_2S, then disrupting *unc-29* would abrogate *hif-1*-dependent upregulation of target genes in H_2S. However, we found the transcriptional response to H_2S in *unc-29(ok2450)* animals was indistinguishable from wild-type controls (fig 5B). This result suggests that cholinergic signaling is not upstream of *hif-1* signaling in the H_2S response. We conclude that *hif-1* acts independently of cholinergic signaling in the response to H_2S.

Nutrient restriction abrogates the requirement for cholinergic signaling in H_2S.

Endogenous H_2S is produced as a result of amino acid metabolism, and H_2S plays a role mediating physiological effects of dietary restriction (Hine, 2015). This led us to consider the possibility that the response to H_2S could vary depending on nutrient status.

When *C. elegans* are in nutrient-poor conditions they can enter into an alternative third larval stage, dauer, where no food is required and animals are extremely stress-resistant (Hu, 2007). Cholinergic signaling plays a role in regulating dauer, as ACh synthesis (*cha-1(p1152)*) and degradation (*ace-1;ace-2(p1000;g72)*) mutant animals are dauer constitutive and dauer defective, respectively (Lee, 2014). Dauer larvae contain up to five times as much L-AChR protein as non-dauer animals (Lewis, 1987), and acetylcholine production is greatly decreased (Lee, 2014). This would reduce the fraction of L-AChRs bound to ACh. Consistent with this view, dauer larvae are relatively immobile (Hu, 2007).

We hypothesized that reducing cholinergic signaling in dauer larvae would reduce viability in H_2S, similar to our observations with fourth-stage (L4) cholinergic mutant larvae. However, we found that *unc-29(ok2450)* dauer larvae maintained high viability in H_2S (Fig. 6A). This result suggests that the dauer stage is protective against H_2S exposure. Consistent with this conclusion, we found that *hif-1(ia4)* dauer larvae also had increased survival in H_2S.

When animals exit dauer, they continue on to L4 (Hu. 2007). However, postdauer animals up- or down-regulate over 2000 genes and show extended lifespans and increased brood-sizes compared with control animals which never pass through the dauer stage (Hall, 2010). We wondered if passing through dauer has long-lasting protective effects against H_2S toxicity. We found that *unc-29(ok2450)* mutant animals that had been through dauer survived exposure to H_2S, whereas controls that developed directly to L4 had low viability (Compare figs 3A and 6B). In contrast,

postdauer *hif-1(ia4)* mutant animals died when exposed to H_2S, though the lethality was slightly less severe than in animals that develop directly to L4 (fig 6B). This result indicates that activation of the dauer pathway eliminates the requirement for cholinergic signaling in H_2S.

Two main genetic pathways control entry into dauer: insulin/IGF-like signaling (IIS) and TGFβ. IIS is not required for increased lifespan or thermotolerance in H_2S (Miller, 2007). Similarly, we found that *daf-16(m26)* mutant animals, which cannot form dauer larvae, did not die when exposed to H_2S as L4 (see supplemental table 1). We conclude that the protective effect of dauer is not a result of IIS. In contrast, we found that animals with mutations in the TGFβ pathway *dbl-1(wk70)*, *sma-3(e491)*, *sma-4(e729)*, and *sma-9(tm572)* died when exposed to H_2S (Fig. 6C). This result suggests that activation of TGFβ signaling in dauer larvae reduces the necessity of cholinergic signaling in H_2S, even once animals have recovered to the L4 stage.

Discussion

Our results indicate that neurotransmission is an essential element of the H_2S response. Previous studies have shown that *C. elegans* undergo transcriptional changes in response to H_2S exposure, and that *hif-1* is of principle importance for coordinating this response (Miller, 2011). Here, we extend what is known about the organismal response to H_2S by identifying a variety of genes that are required for optimal H_2S responses. By using a candidate-gene approach we were able to find phenotypes (<100% death) that unbiased screening approaches would not have been sensitive enough to pick up (Budde, 2011). Our study increases the number of factors known to be required for survival in H_2S from 6 to 44.

A recent report showed that endogenously produced H_2S modulates cholinergic transmission at the mouse NMJ. The authors found that H_2S acts exclusively at the presynapse, increasing the frequency of miniature endplate potentials without altering their amplitude (Gerasimova, 2015). A similar model of H_2S modulation of cholinergic activity in our study is appealing. Because no cholinergic genes were found in a microarray assessment of transcriptional changes following H_2S exposure (Miller, 2011), we favor the hypothesis that H_2S increases cholinergic quantal release rather than abundance of any cholinergic genes. Supporting this possibility is the finding that *C. elegans* exposed to H_2S show an almost immediate increase in movement (Budde, 2011), which would be too fast for a transcriptional response.

Our finding that engaging the dauer pathway confers lasting resistance to H_2S toxicity reveals a curious interplay between nutrient-responsive signaling and H_2S-response circuitry. Cholinergic signaling is naturally decreased in dauer, which could explain why a different response pathway is used during this developmental stage (Lee 2014). One simple possibility is that the known stress-resistance of dauers extends to reduce toxic effects of H_2S. Since both *unc-29(ok2450)* and *hif-1(ia4)* mutant dauers have increased survival in H_2S, this suggests that the dauer

protection mechanism is epistatic to both of these pathways. Our genetic data suggest that the TGFβ signaling pathway may mediate the protective effects of dauer in H_2S. Nakano et al (2015) recently found that fasting blocks ischemia/reperfusion injury in mouse cardiac tissue, and that this relies on H_2S production (Nakano, 2015). Another study showed similar effects, where blocking endogenous H_2S production eliminates the ischemia/reperfusion effects of dietary restriction (Hine 2015). With our studies, these data suggest a complex interplay between nutrient signaling and H_2S signaling that could have significant therapeutic ramifications.

H_2S is gaining attention as a potent neuromodulator which may influence a variety of neurodegenerative diseases (Kimura, 2002; Hu, 2010). In nematodes, H_2S protects against the aggregation of proteins associated with neurodegeneration (Fawcett, 2015). Our studies here provide new evidence of the nature in which H_2S influences neuronal functions. The gene products we have identified are highly conserved in mammals. Thus, our results provide a new foundation to explore the potential of modulating H_2S signaling therapeutically.

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In figures: Use Hr, not Hrs (abbreviations are never plural in scientific writing)

Also, the A, B, C used to designate different panels in the figures should be in upper right corner not lower right corner.

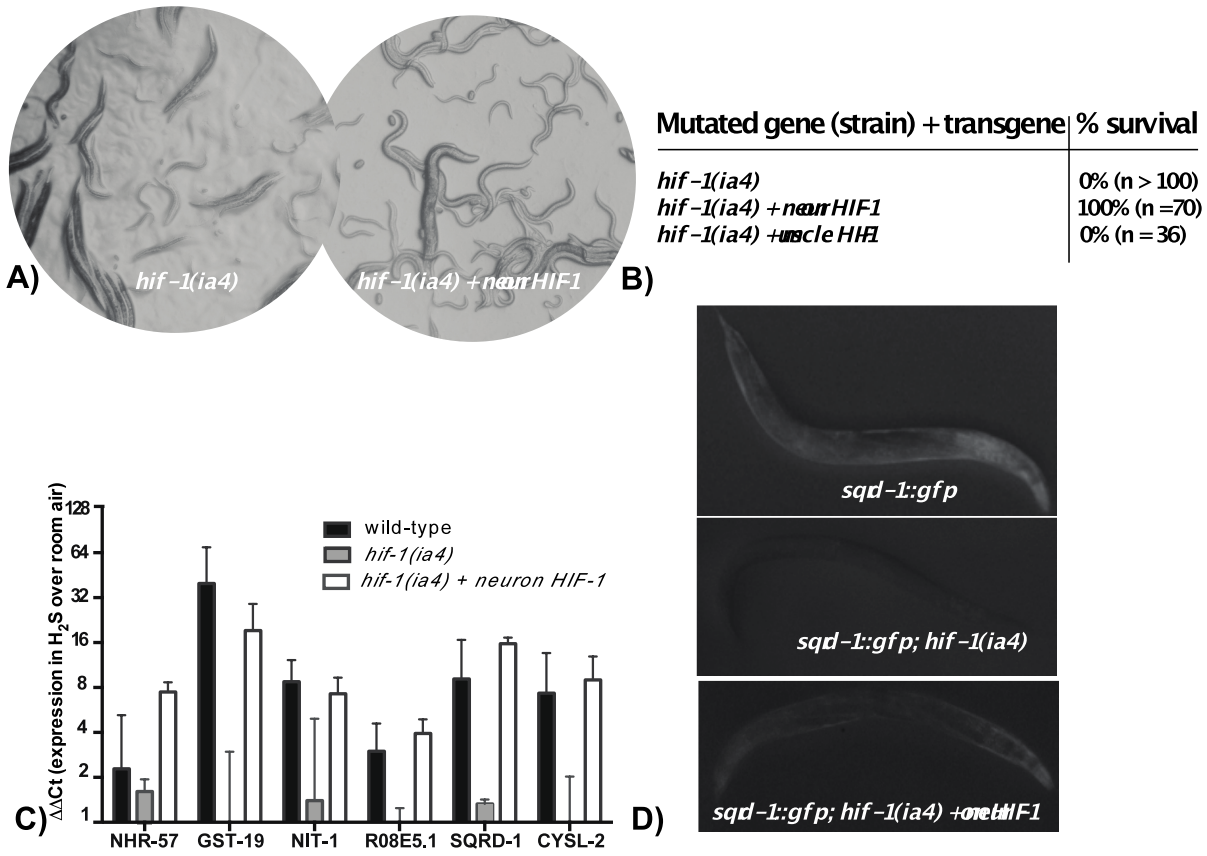

Figure 1. *hif-1* is only required in neurons to coordinate the whole organism H_2S response.
(A and B) Rescuing *hif-1* in neurons (pictured in a, quantified in b) allowed survival in H_2S ($p<0.0001$ comparing *hif-1* and *hif-1 + neuron HIF1*) Rescuing in muscle (not pictured) had no effect. (C) qPCR results show that *hif-1* target genes are turned on in H_2S in wild-type and neuronal *hif-1* transgenic animals in a *hif-1* mutant background, but not *hif-1* mutants. (D) *sqrd-1* is activated by *hif-1* in H_2S. *sqrd-1* is fused with a GFP reporter, and is activated in *hif-1* mutant animals expressing neural HIF-1, but not in *hif-1* mutant animals.

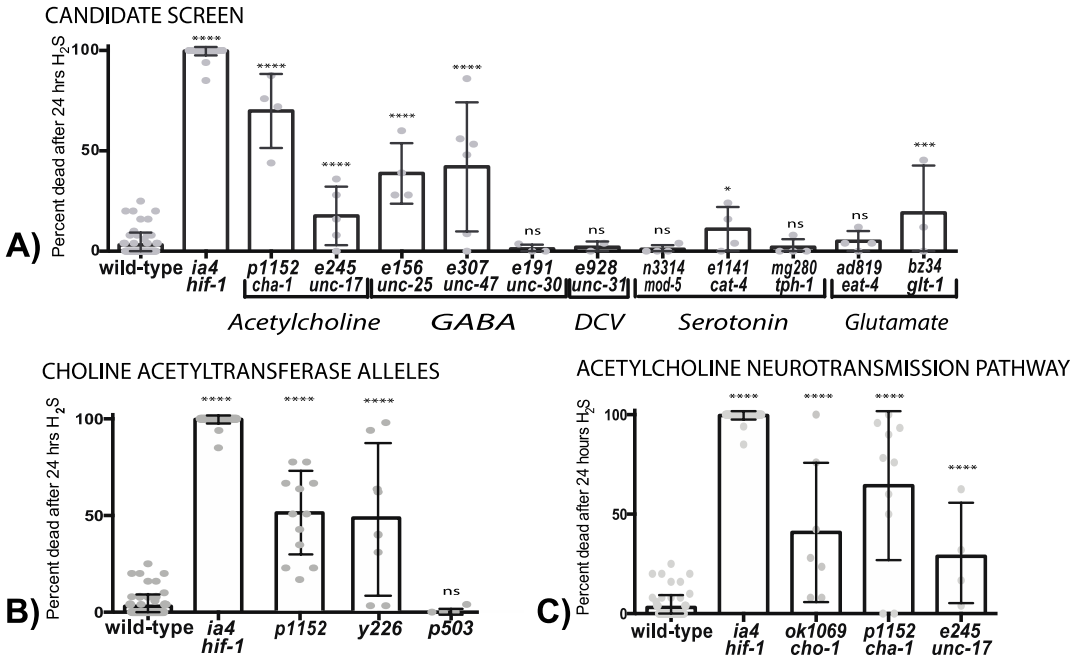

Figure 2. Involvement of neurotransmitters in H_2S signaling.
Mutants deficient in cholinergic signaling die in H_2S. (A-C) Percent dead out of an average of 25 animals per trial. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Wild-type and ia4 positive and negative control data (respectively) is identical in all graphs.
Mean +/- SD (error bars). Points represent individual trials; minimum of 3 trials per mutant.
(A) In an initial mutant screen, *C. elegans* homozygous for mutations in acetylcholine synthesis and trafficking machinery die in H_2S. n =/> 3 trials per mutant. (B) Different cha-1 mutant alleles have death in H_2S phenotypes. p503 does not show H_2S toxicity. n > 3 trials. p1152 shown here is a distinct strain (MT3516) from that shown in B (PR1152).

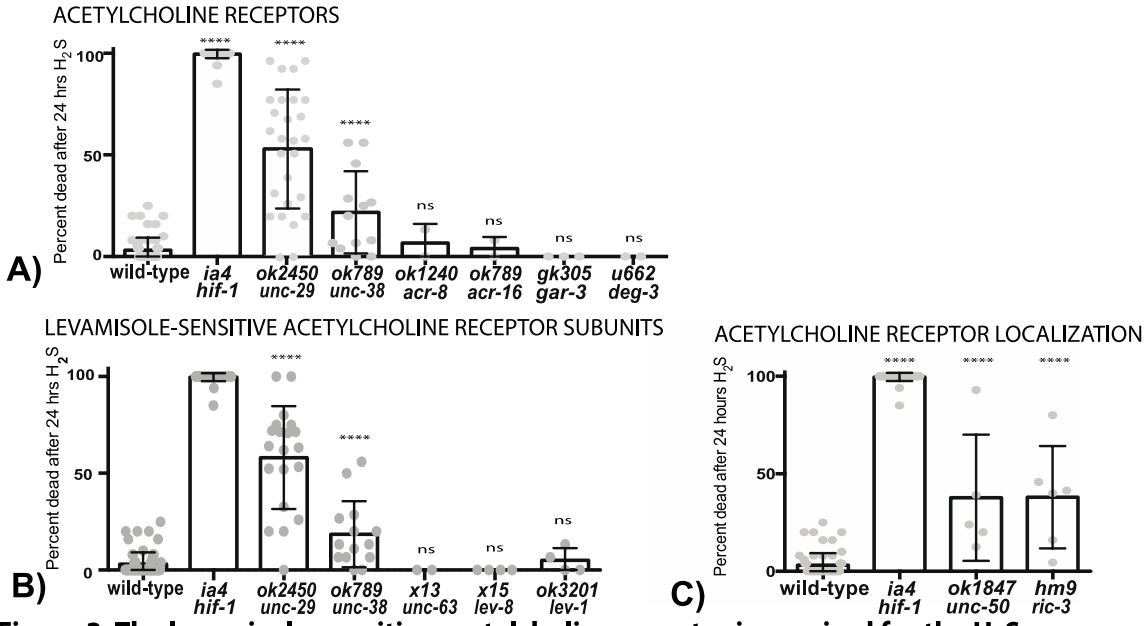

Figure 3. The levamisole-sensitive acetylcholine receptor is required for the H_2S response

(A-C) Percent dead out of an average of 25 animals per trial. ****p<0.0001. Wild-type and ia4 positive and negative control data (respectively) is identical in all graphs. (A) *unc-29*- and *unc-38*-like acetylcholine receptors are required for H_2S survival. These genes encode beta and alpha (respectively) subunits of the levamisole-sensitive acetylcholine receptor. Mutations in other acetylcholine receptor subtypes do not cause death in H_2S. n=/>2 trials per mutant. (B) *unc-29* and *unc-38* are two of five subunits that comprise the levamisole-sensitive nicotinic acetylcholine receptor. Mutating other subunits of this receptor has no effect on H_2S survival. (C) *unc-50* and *ric-3* are responsible for chaperoning acetylcholine receptors to the cell surface. Mutating these genes causes death in H_2S. n > 5 trials per mutant.

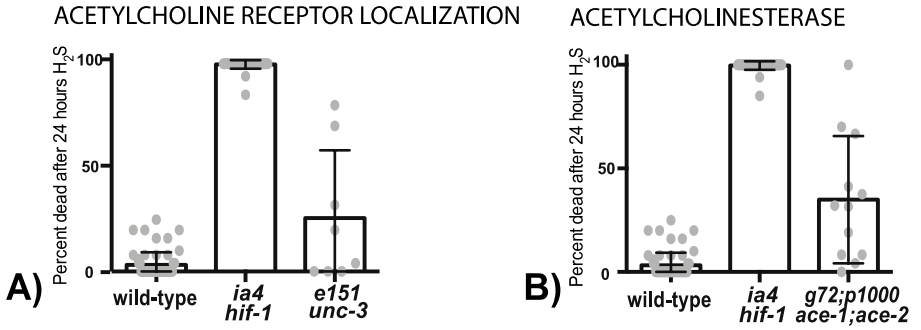

Figure 4. Balanced cholinergic signaling at the NMJ is required in H_2S

(A&B) Percent dead out of an average of 25 animals per trial. ****p<0.0001. Wild-type and ia4 positive and negative control data (respectively) is identical in all graphs. (A) *unc-3,* which is required for expression of cholinergic genes in motor neurons, is required for H_2S survival. (p<0.0001 compared to wild-type, n = 8) (B) *ace-1;ace-2* mutant animals are susceptible to H_2S toxicity, indicating that too much cholinergic signaling is also detrimental (n=12).

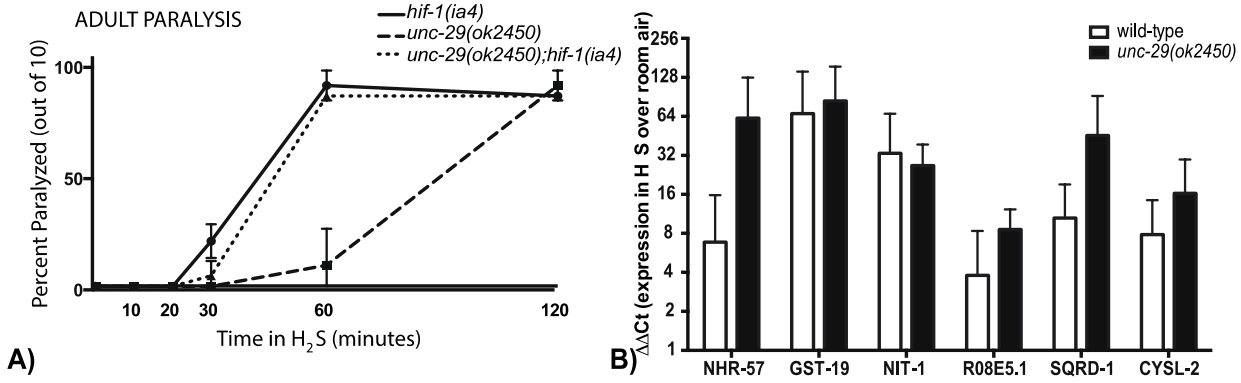

Figure 5. Characterizing the cholinergic mutant H_2S response.
(A) Adult *C. elegans* with mutations in *unc-29* get paralyzed more slowly in H_2S than *hif-1* mutants. We performed a 2 (genotype: *hif-1* vs *unc-29*) by 7 (time: 10, 20, 30, 60, 120, 180, 240 minutes) ANOVA and found a significant main effect of genotype, time, and an interaction effect ($p<0.0001$). 17.70% total variation is due to interaction effects. *unc-29;hif-1* double mutants get paralyzed at the same rate as *unc-29* mutants (B) qPCR results show that *hif-1* target genes are turned on in H_2S in *unc-29* mutants.

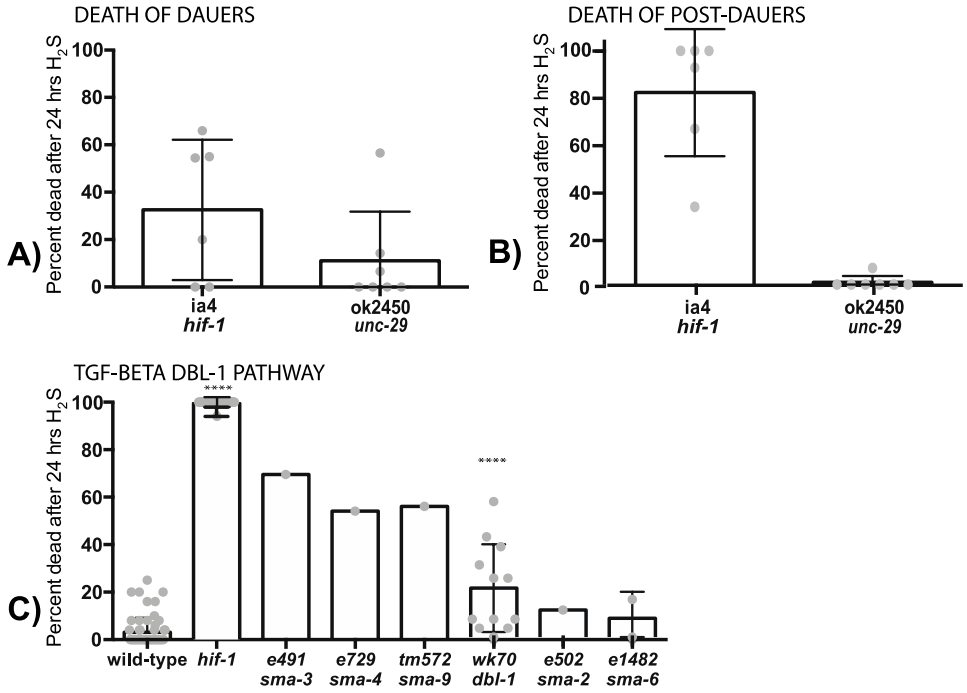

Figure 6. Nutrient restriction alters the H_2S signaling pathway.
(A) Putting mutant animals into the alternative stress-resistant developmental stage dauer reduces H_2S toxicity (*hif-1* dauer are different than non-dauer *hif-1*, $p<0.0001$; *unc-29* dauer are different than non-dauer *unc-29* , $p = 0.0015$, using data from figure 3A). (B) Post-dauer *unc-29* animals are not susceptible to H_2S toxicity, while *hif-1* animals are somewhat desensitized (*hif-1* post-dauer are different than non-dauer *hif-1*, $p<0.0001$; *unc-29* post-dauer are different than non-dauer *unc-29* , $p = 0.0005$, using data from figure 3A). (C) Percent dead out of an average of 25 animals per trial. ****$p<0.0001$. Wild-type and ia4 positive and negative control data (respectively) is identical in all graphs. Components of the TGF-beta dauer signaling pathway are required for H_2S survival.

Table S1.

allele	gene	% death in H_2S +/- SD
ad1110	*eat-18*	41.66 +/- 40.70
ad1116	*eat-2*	17.65 +/- 24.95
ad446	*bas-1*	4.783 +/- 4.114
ad465	*eat-2*	13.06 +/- 14.82
ad819	*eat-4*	5.0 +/- 2.52
ad820	*eat-18*	21.57 +/- 26.04
b404	*unc-63*	0 +/- 0
bz34;ok206	*glt-1;glt-3*	85 +/- 7.071
ch1	*ceh-14*	24.40 +/- 30.28
ch3	*ceh-14*	4.767 +/- 8.084
ctls40	*dbl-1*	38.05 +/- 37.05
e1112	*cat-2*	0 +/- 0
e113	*unc-17*	2.5 +/- 5.59
e1141	*cat-4*	11.0 +/- 5.51
e1141;e1490	*cat-4;him-5*	11 +/- 12
e120	*unc-4*	0.89 +/- 1.79
e1265	*unc-104*	4.0 +/- 2.828
e1265;jsls1	*unc-104*	2 +/- 2.31
e1295	*unc-104*	5.39 +/- 6.09
e1372	*daf-7*	0 +/- 0
e1386	*daf-5*	0 +/- 0
e1393	*daf-8*	10 +/- 14.14
e1402	*unc-15*	67.94 +/- 32.45
e1416	*unc-86*	27.20 +/- 32.95
e1482	*sma-6*	8.335 +/- 11.79
e151	*unc-3*	25.75 +/- 32.59
e156	*unc-25*	38.75 +/- 7.54
e1562	*vab-7*	10.17 +/- 3.49
e169	*unc-31*	0 +/- 2
e191	*unc-30*	1.2 +/- 1.2
e193	*unc-29*	16.28 +/- 13.69
e211	*lev-1*	3.335 +/- 4.716
e245	*unc-17*	22.30 +/-22.29
e264	*unc-38*	21.8 +/- 20.24
e307	*unc-47*	50.19 +/- 31.87
e408	*unc-43*	55.17 +/- 4.476

e491	*sma-3*	69.57 +/-
e502	*sma-2*	12 +/-
e51	*unc-13*	14.0 +/- 2.0
e729	*sma-4*	54.17 +/-
e883	*unc-74*	6.223 +/- 6.01
e928	*unc-31*	2.0 +/- 2.0
eg28	*snf-6*	12.0 +/- 2.83
g72;p1000	*ace-1;ace-2*	34.90 +/- 30.71
gb503	*kal-1*	35.64 +/- 33.66
gk395	*exc-9*	3.34 +/- 4.72
gk668	*unc-4*	14.33 +/- 14.59
gk794	*aptf-1*	20.01 +/- 19.96
gk8	*kpc-1*	42.82 +/- 28.69
hd31	*lad-2*	0 +/- 0
hm9	*ric-3*	37.95 +/- 26.26
ia4	*hif-1*	99.65 +/- 2.07
ia4 + neural HIF-1	*hif-1 + neural HIF-1*	0.2703 +/- 1.644
jh113	*cng-3*	33.18 +/- 44.58
ks86	*ceh-36*	33.7 +/- 36.41
ky5	*eat-4*	0 +/- 0
ky20	*adp-1*	5.33 +/- 10.05
ky651	*cfi-1*	47.23 +/- 35.14
m185	*dyf-3*	0 +/- 0
m26	*daf-16*	0 +/- 0
m40	*daf-1*	0 +/- 0
mg280;gr1321	*tph-1;cam-1*	2.0 +/- 2.0
mgDf90	*daf-3*	9.528 +/- 11.4
ms23	*ceh-23*	12.67 +/- 25.18
my8	*klp-6*	0 +/- 0
n1075	*egl-46*	0 +/- 0
n1107	*egl-49*	17.91 +/- 17.74
n1226	*egl-46*	3.34 +/- 4.72
n1438	*ham-1*	9.55 +/- 4.14
n2656	*egl-2*	88.79 +/-13.55
n2669	*exc-9*	3.333 +/- 6.665
n3314	*mod-5*	1.0 +/- 1.0
n4438	*mir-273*	21.56 +/- 31.49
n498	*unc-43*	100 +/- 0

n582	*egl-19*	10.64 +/- 0.84
n686	*egl-30*	55.14 +/- 25.35
n693	*egl-2*	0 +/- 0
n848	*unc-86*	24.17 +/- 29.06
n995	*egl-42*	0 +/- 0
ok1056	*kal-1*	4.44 +/- 7.67
ok1069	*cho-1*	46.97 +/- 28.37
ok1240	*acr-8*	6.665 +/- 9.426
ok1285	*acr-18*	0 +/- 0
ok1314	*acr-21*	0 +/- 0
ok1519	*lev-8*	0 +/- 0
ok1847	*unc-50*	37.69 +/- 32.33
ok2049	*acr-3*	0 +/- 0
ok2450	*unc-29*	58.81 +/- 31.18
ok2450;ia4	*unc-29;hif-1*	100 +/- 0
ok2804	*acr-23*	0 +/- 0
ok2896	*unc-38*	13.33 +/- 6.665
ok3117	*acr-6*	5.557 +/- 7.794
ok3201	*lev-1*	6.665 +/- 9.426
ok3204	*lad-2*	0 +/- 0
ok367	*acr-12*	0 +/- 0
ok3749	*dbl-1*	78.74 +/- 14.34
ok409	*ida-1*	0 +/- 0
ok769	*gcy-35*	5.557 +/- 5.092
ok769	*gcy-35*	12 +/- 11.31
ok769	*gcy-35*	3.34 +/- 4.72
ok789	*acr-16*	0 +/- 0
ot22	*ttx-3*	18.56 +/- 1.40
oy21	*cmk-1*	12.22 +/- 24.38
p1152	*cha-1*	66.49 +/- 36.2
p1152;e1275	*cha-1;lin-1*	50.75 +/- 21.67
p503	*cha-1*	1 +/- 2
p672	*che-1*	7.55 +/- 7.007
p696	*che-1*	53.46 +/- 42.87
pk362	*gpa-10*	30.83 +/- 42.46
s69	*unc-13*	14 +/- 2.83
sa307	*egl-9*	0 +/- 0
sa734	*goa-1*	30.28 +/- 33.45

tm351	*bas-1*	12.20 +/- 27.25
tm572	*sma-9*	54.17
tm863	*acr-7*	0 +/- 0
u282	*lin-32*	3.785 +/- 4.39
u38	*deg-1*	0 +/- 0
u506	*deg-1*	3.33 +/- 4.71
u662	*deg-3*	0 +/- 0
wk70	*dbl-1*	21.72 +/- 19.36
x13	*unc-63*	0 +/- 0
x15	*lev-8*	0 +/- 0
x20	*unc-38*	45.05 +/- 24.11
x30	*unc-29*	38.50 +/- 33.57
y226	*cha-1*	48.69 +/- 39.63