Selective detection of endogenous H$_2$S in living cells and the mouse hippocampus using a ratiometric fluorescent probe

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As one of three gasotransmitters, the fundamental signalling roles of hydrogen sulphide are receiving increasing attention. New tools for the accurate detection of hydrogen sulphide in cells and tissues are in demand to probe its biological functions. We report the p-nitrobenzyl-based ratiometric fluorescent probe RHP-2, which features a low detection limit, high selectivity and good photostability. The emission intensity ratios had a good linear relationship with the sulphide concentrations in PBS buffer and bovine serum. Our probe was applied to the ratiometric determination and imaging of endogenous H$_2$S in living cells. Furthermore, RHP-2 was used as an effective tool to measure endogenous H$_2$S in the mouse hippocampus. We observed a significant reduction in sulphide concentrations and downregulated expression of cystathionine β-synthetase (CBS) mRNA and CBS protein in the mouse hippocampus in a chronic unpredictable mild stress (CUMS)-induced depression model. These data suggested that decreased concentrations of endogenous H$_2$S may be involved in the pathogenesis of chronic stress depression.

Hydrogen sulphide (H$_2$S) has been identified as an endogenous gaseous signalling molecule as well as a cytoprotectant$^{1,2}$. Endogenous H$_2$S is primarily produced in the mitochondria or cytosol from a cysteine substrate, or its derivatives, with catalysis by enzymes such as cystathionine β-synthetase (CBS), cystathionine γ-lyase (CSE) and cysteine aminotransferase (CAT)/3-mercaptopyruvate sulphurtransferase (3-MST)$^3$. Physiological concentrations of H$_2$S are associated with the regulation of diverse biological functions, including vasodilation, apoptosis, neurotransmission, ischemia/reperfusion-induced injury, insulin secretion and inflammation$^4$–$^9$. In addition, H$_2$S protects the cardiovascular and central nervous systems from oxidative stress$^{10}$. The misregulation of endogenous H$_2$S is present in many diseases$^{11}$–$^{14}$, such as Down syndrome, Alzheimer’s disease (AD), Parkinson’s disease (PD) and febrile seizures. However, the detailed molecular pathways of H$_2$S are not fully understood, partly due to the lack of non-invasive and real-time detection techniques for H$_2$S.

Current approaches to H$_2$S detection include the methylene blue method, the monobromobimane method (MBB), gas chromatography (GC) and the sulphide ion selective electrodes (ISE) method$^{15}$–$^{20}$. However, these methods often require the extraction of sulphide from tissues or cells. For the dynamic monitoring of H$_2$S in biological specimens, small-molecule fluorescent probes have recently emerged as an effective tool for the detection and imaging of H$_2$S. Detection mechanisms may involve trapping H$_2$S via nucleophilic addition, copper sulphide precipitation, H$_2$S-mediated reduction, or the thiolysis of dinitrophenyl ether by H$_2$S$^{21}$–$^{31}$. Compared with “turn-on” fluorescent probes, ratiometric fluorescent probes are more accurate for detecting H$_2$S, independently of variables in quantitative analysis such as excitation intensity variations, environmental factors, light scattering and probe concentrations$^{32}$.

As a result of the reduction of nitro groups to amino groups under hypoxic conditions, several research groups have reported nitro-based fluorescent probes for imaging hypoxic status via the detection of nitroreductase$^{33}$–$^{37}$. We were especially interested in the p-nitrobenzyl-based hypoxia probe (referred to as RHP) reported by Qian et al. that is synthetically accessible and has robust efficacy in vitro$^{38}$. Additionally, there are several reports on the development of H$_2$S-specific probes based on the nitro-reduction mechanism by H$_2$S$^{39}$–$^{42}$. Accordingly, we hypothesised that the p-nitrobenzyl moiety is applicable to H$_2$S identification under normoxic conditions. Herein, we describe the ratiometric fluorescent probe RHP-2 for the selective detection of H$_2$S, featuring the
same scaffold as the RHP probe by Qian (Fig. 1). Using the RHP-2 probe, we detected and imaged the endogenous H₂S in MCF-7 cells and measured endogenous H₂S in the mouse hippocampus.

Results

Synthesis and sensing mechanism. We employed 1,8-naphthalimide as an intramolecular charge transfer (ICT) fluorophore owing to its desirable spectroscopic properties and feasibility in structural modification. The nitro group served as the H₂S reaction site. Probe RHP-2 was constructed by connecting a p-nitrobenzyl group to 1,8-naphthalimide via a carbamate-linkage. The electron-withdrawing carbamate group weakened the ICT effect, resulting in blue shifts in emission. The ratiometric detection of sulphide, as anticipated, involved the group weakened the ICT effect, resulting in blue shifts in emission. The ICT mechanism of RHP-2 was further (Fig. 2A insert), indicating that RHP-2 is a ratiometric fluorescent probe for sulphide. The ICT mechanism of RHP-2 was further (Fig. 2A insert), indicating that RHP-2 is a ratiometric fluorescent probe for sulphide. The ICT mechanism of RHP-2 was further (Fig. 2A insert), indicating that RHP-2 is a ratiometric fluorescent probe for sulphide. The ICT mechanism of RHP-2 was further (Fig. 2A insert), indicating that RHP-2 is a ratiometric fluorescent probe for sulphide. The ICT mechanism of RHP-2 was further characterized by NMR spectroscopy and mass spectrometry (refer to the supplementary information). To assess the reaction mechanism of RHP-2 with sulphide, RHP-2 was incubated with Na₂S (a common hydrogen sulphide donor), resulting in a green fluorescent product that was identified as NAP-NH₂ based on fluorescence emission and H and 13C NMR spectra (refer to the supplementary information). The reaction between RHP-2 and sulphide proceeded as depicted in Figure 1.

Fluorescent properties of RHP-2. The sensitivity of RHP-2 (5 μM) to sulphide was determined at 37°C in 20 mM PBS buffer (pH 7.4). In the absence of Na₂S, RHP-2 displayed a fluorescence emission maximum at 467 nm (Φ = 0.12). Upon treatment of RHP-2 with a cascade of Na₂S (0–100 μM), the fluorescence intensity gradually decreased at 467 nm with the concomitant generation of a new emission peak at 532 nm (Φ = 0.13) (Fig. 2A). The fluorescence emission colour of the solution changed from blue to green (Fig. 2A insert), indicating that RHP-2 is a ratiometric fluorescent probe for sulphide. The ICT mechanism of RHP-2 was further demonstrated by the density functional theory (DFT) (refer to the supplementary information).

Figure 2B depicts elevated emission intensity ratios with increasing concentrations of Na₂S until a plateau is reached at 300 μM Na₂S, suggesting that complete reaction between RHP-2 and Na₂S occurs at the concentration ratio of 1:60. Enhancements of 27-fold and 26-fold in emission intensity ratios were observed, from 0.34 and 0.31 for PBS buffer and serum, respectively, in the absence of Na₂S to 9.0 and 8.2 for PBS buffer and serum, respectively, in the presence of 10 equiv. Na₂S (Fig. 2B). Furthermore, the emission intensity ratios showed an excellent linear relationship with Na₂S concentrations from 0–100 μM. The detection limit for sulphide was 270 nM and 280 nM in PBS buffer and foetal bovine serum, respectively (Supplementary Fig. S2). These results indicate that RHP-2 is sensitive to sulphide and is suitable for the quantitative analysis of endogenous H₂S in complex biological systems.

The reaction of RHP-2 with sulphide was completed in approximately 40 min (Supplementary Fig. S3). Under pseudo-first-order conditions, the rate constant for sulphide was 1.0 × 10⁻³ s⁻¹ (Supplementary Fig. S4). Furthermore, the plot of kobs vs [Na₂S] formed a straight line passing through the origin, suggesting that the reaction was overall second order with k₂ = 5.0 M⁻¹s⁻¹ (Supplementary Fig. S4). As shown in Figure S5, the maximum peaks of emission intensity ratios were between pH 6.2 and 9.0, and the minimum emission intensity ratios were between pH 4.2 and 5.0. These results could be due to the inhibition of carbamate cleavage from RHP-2 under acidic conditions. The ratios of the free probe exhibited almost no changes between pH 6.2 and 9.0. Thus, RHP-2 can serve as a fluorescent ratiometric probe for sulphide between pH 6.2 and 9.0.

Subsequently, the determination of the photostability of RHP-2 was conducted, in which an RHP-2 solution was exposed to visible light and UV light for 60 min, and no changes in the emission intensities of RHP-2 were observed (Supplementary Fig. S6).

Selectivity of RHP-2. The selectivity of RHP-2 for sulphide was examined. As shown in Figure 3A, RHP-2 was selective for sulphide in the absence of interference with biothiols, such as glutathione (GSH), cysteine (Cys) and homocysteine (Hcy). The remaining non-thiol amino acids (Ala, Glu, Trp, Met, Tyr, Leu, Val, Ser, Pro, Arg, Gly, Phe, His, Gln, Asn, Ile and Thr), inorganic salts (KCl, CaCl₂, NaCl, MgCl₂, FeCl₂, ZnSO₄ and NaH₂PO₄), reactive oxygen species (H₂O₂, *OCl*, *O₂* ·, OH and 'BuOOH), reactive nitrogen species (NO₂⁻ and NO), reducing agents (NADH and glucose), sulphur-containing inorganic ions (S₂O₃²⁻, S₂O₅²⁻, SO₄²⁻, S₂O₃²⁻, SO₃²⁻ and SCN⁻) and S-nitroso glutathione (SNG) showed negligible responses (Fig. 3B, 3C, 3D and S8). Additionally, competitive experiments revealed minimal interference with sulphide detection in the coexistence of various species and Na₂S. The emission intensity ratios decreased only in the presence of H₂O₂ and ZnSO₄ (Fig. 3C), which may be attributed to the oxidation of H₂S by H₂O₂ and sulphide precipitation of H₂S by ZnSO₄. Accordingly, RHP-2 is applicable to the selective determination of sulphide with minimal interference with these biological species.

Detection of H₂S in living cells. We tested the potential utility of RHP-2 for ratiometric fluorescence imaging of H₂S in living MCF-7 cells. Prior to cell imaging, MTT assays were conducted to evaluate the cytotoxicity of RHP-2 and NAP-NH₂. RHP-2 and NAP-NH₂ showed IC₅₀ values of 101.2 ± 1.3 and 82.6 ± 1.1 μM, respectively (Supplementary Figs. S9 and S10), indicating the low toxicity of RHP-2 and NAP-NH₂ in cultured MCF-7 cells. The cell viability of RHP-2 and NAP-NH₂ at 0, 6, 12, 18 and 24 hours further
that the fluorescence change in the cells arises from H2S, cells were
incubated for 40 min under a 365-nm UV lamp. (B) Emission intensity ratios of RHP-2 (5 μM) after 40 min of incubation with different concentrations of Na2S (0 to 400 μM) in PBS buffer (pH 7.4, 5% CH3CN) and foetal bovine serum (5% CH3CN). Insert: The linear relationship between the emission intensity ratios (F532/F467) and the concentrations of Na2S (0 to 100 μM) in PBS buffer and foetal bovine serum. Data are presented as the mean ± SD (n = 3).

We then explored the kinetics of the reaction between RHP-2 and sulphide in living cells. Fig. 4 (panel 4D) depicts a sulphide-induced increase in emission intensity ratios in regions a and f in Fig. 4 (panel 3C), with a plateau appearing at approximately 40 min. To verify that fluorescence change in the cells arises from H2S, the cells were pretreated with ZnCl2, which eliminates H2S (Supplementary Fig. S12, panel 6B) and showed that enzyme inactivation can suppress the production of endogenous H2S. These results established that RHP-2 is biocompatible and capable of ratiometrically imaging endogenous H2S in living cells.

**Figure 2 | Ratiometric fluorescence response of RHP-2 to sulphide.** (A) Fluorescence spectra of RHP-2 (5 μM) with Na2S (0, 2, 4, 8, 10, 15, 20, 30, 40, 60 and 100 μM) in PBS buffer (20 mM, pH 7.4, 5% CH3CN) at 37 °C for 40 min. Insert: Photograph showing the visual fluorescence of RHP-2 without (a) or with (b) Na2S under a 365-nm UV lamp. (B) Emission intensity ratios of RHP-2 (5 μM) after 40 min of incubation with different concentrations of Na2S (0 to 400 μM) in PBS buffer (20 mM, pH 7.4, 5% CH3CN) and foetal bovine serum (5% CH3CN). Insert: The linear relationship between the emission intensity ratios (F532/F467) and the concentrations of Na2S (0 to 100 μM) in PBS buffer and foetal bovine serum. Data are presented as the mean ± SD (n = 3).

**Figure 3 | Determination of endogenous sulphide in the mouse hippocampus.** Hydrogen sulphide plays important roles in regulating CNS function and is known to regulate LTP by activating NMDA receptors in neurons, eliciting Ca2+ waves, increasing Ca2+ levels and regulating intracellular pH in neurons and glial cells. Moreover, H2S has been reported to exert neuroprotective effects via various mechanisms, including antioxidative, anti-inflammatory and anti-apoptotic effects. In addition to its physiological roles as a neuromodulator and as a neuroprotector, H2S is also involved in the pathophysiology of the CNS. Currently, controversy remains as to the actual concentration of H2S in brain tissues, ranging from undetectable to over 100 μM, thus indicating that new methods for accurate determination of H2S are in high demand.
Prompted by the promising results of selectivity, sensitivity and linearity measurements, we utilised RHP-2 to determine sulphide concentrations in the mouse hippocampus. Accordingly, the measurement of sulphide concentrations in the mouse hippocampus was conducted based on our previous method. In brief, fresh hippocampus homogenate was centrifuged, and the supernatant was obtained. PBS buffer, RHP-2 and Na₂S (as the internal criterion) were incubated with the supernatant for 40 min at 37°C. As shown in Table 1 (Supplementary Fig. S15), the median sulphide concentration in the mouse hippocampus was 1.67 ± 0.08 μmol g⁻¹ protein. The validity of our present method was verified using our previous fluorescence probe SFP-2. The values obtained by RHP-2 were similar to those obtained by SFP-2, suggesting that RHP-2 is suitable for the determination of endogenous sulphide in biological tissues.

Determination of endogenous sulphide in the hippocampus in mouse models of CUMS-induced depression. Depression, a widespread incapacitating psychiatric condition, imposes a substantial health threat to society. Unlike studies addressing AD and PD, little is known about the role of endogenous H₂S in the pathogenesis of depression. H₂S has been reported to exert specific antidepressant-like and anxiolytic-like effects in behavioural models of depression and anxiety in mice. Thus, it would be interesting to investigate the pathological correlations between endogenous H₂S concentrations and depression. We established a chronic unpredictable mild stress (CUMS)-induced depression-like model in mice. Adult male Kunming mice weighing 20–25 g were randomised into three groups: control group (without treatment), model group (5-week CUMS induction) and NaHS group (5-week CUMS induction and intraperitoneal injection of NaHS at the dose of 5 mg/kg). At week 5, mice underwent the sucrose preference, forced swimming and tail suspension tests to evaluate depressive behaviour, followed by the determination of sulphide concentrations and CBS mRNA levels and CBS protein expression in the mouse hippocampus.

There was no significant difference in sucrose consumption among the three groups prior to CUMS induction (Supplementary Fig. 3). The selectivity of RHP-2 for sulphide. (A) Fluorescence responses of RHP-2 (5 μM) towards Na₂S (100 μM) and various thiols after 40 min of incubation. 1. Na₂S (0 μM); 2. Na₂S (10 μM); 3. Na₂S (100 μM); 4. Cys (1 μM); 5. Cys (1 mM); 6. Hcy (100 μM); 7. Hcy (1 mM); 8. GSH (1 mM); 9. GSH (10 mM); 10. Na₂S (10 μM) + Cys (100 μM); 11. Na₂S (100 μM) + Cys (100 μM); 12. Na₂S (10 μM) + Cys (1 mM); 13. Na₂S (100 μM) + Cys (1 mM); 14. Na₂S (10 μM) + Hcy (100 μM); 15. Na₂S (100 μM) + Hcy (100 μM); 16. Na₂S (10 μM) + Hcy (1 mM); 17. Na₂S (100 μM) + Hcy (1 mM); 18. Na₂S (10 μM) + GSH (1 mM); 19. Na₂S (100 μM) + GSH (1 mM); 20. Na₂S (10 μM) + GSH (10 mM); and 21. Na₂S (100 μM) + GSH (10 mM). (B) Fluorescence spectra of RHP-2 (5 μM) with Na₂S (100 μM) and various amino acids (1 mM) after 40 min of incubation. (C, D) Fluorescence responses of RHP-2 (5 μM) towards Na₂S (100 μM), ROS (1 mM), RNS (1 mM), sulphur-containing inorganic ions (1 mM), reducing agents (1 mM), inorganic salts (1 mM) and S-nitroso-glutathione (SNG, 1 mM) after 40 min of incubation. Data are presented as the mean ± SD (n = 3).
Fig. S17, p < 0.05). At the end of the 5-week stress, sucrose consumption in the CUMS mice was remarkably lower than that in the control group (Supplementary Fig. S17, p < 0.001). NaHS treatment significantly increased sucrose consumption compared to the CUMS group (Supplementary Fig. S17, p < 0.001). CUMS-induced depressive mice showed a significant increase in immobility duration in the tail suspension test (Supplementary Fig. S18, p < 0.001) and the forced swimming test compared to the control group (Supplementary Fig. S19, p < 0.001), demonstrating that CUMS successfully induced a depression-like state in mice. As shown in Figure 6 (Supplementary Table S1), sulphide concentrations (p < 0.001), expression of CBS protein (p < 0.001) and CBS mRNA (p < 0.001) in the hippocampus of the model group were significantly lower than those in the control group. However, there was significant
reduction in immobility duration in the NaHS group compared to the model group (Supplementary Fig. S18, \(p\), 0.001; Fig. S19, \(p\), 0.001). Moreover, NaHS administration attenuated CUMS-induced decreases of sulphide concentrations (Fig. 6A, \(p\), 0.001, Table S1), CBS protein expression (Fig. 6B, \(p\) < 0.001) and CBS mRNA levels (Fig. 6C, \(p\) < 0.001) compared to the model group. Consequently, (1) RHP-2 is an effective tool for the determination of different concentrations of endogenous sulphide in the mouse hippocampus; (2) the application of exogenous sulphide has an antidepressant-like effect in mice with CUMS-induced depression; and (3) a significant decrease in sulphide concentrations and the downregulated expression of CBS mRNA and CBS protein were observed in the hippocampus in a mouse model of chronic stress depression, preliminarily suggesting that the reduced production of endogenous H\(_2\)S may contribute to the pathogenesis of depression.

**Discussion**

Tissue sulphide concentrations depend on the homeostasis between enzymatically producing and consuming reactions\(^5\). However, determination conditions and sample pretreatment may have profound effects on sulphide production and consumption in tissues. Traditional detection methods are the methylene blue and ISE methods. These two methods measured biomolecule-bound sulphide rather than free sulphide due to harsh chemical treatment (strong acid or base, respectively) prior to analysis. Sample pretreatment in
mRNA levels in the mouse hippocampus. Data are presented as the mean ± SEM (n = 8). *p < 0.001 vs. control, **p < 0.001 vs. model. (B) CBS protein expression in the mouse hippocampus. Data are presented as the mean ± SEM (n = 3). *p < 0.001 vs. control, **p < 0.001 vs. model. (C) CBS mRNA levels in the mouse hippocampus. Data are presented as the mean ± SEM (n = 3). *p < 0.001 vs. control, **p < 0.001 vs. model. Full-length blots are presented in Supplementary Figures S20 and S21.

the fluorescent probe-based method does not involve sophisticated sample processing and the addition of chemicals\(^\text{46}\). Specifically, to minimise deviation from the actual value, the following precautions were taken: (1) sample pre-processing was performed in an ice bath to minimise the anabolism and catabolism of sulphide, and the homogenate supernatants were immediately used for the determination; (2) hippocampus tissues were isolated and immediately homogenised within 60 s in PBS buffer (pH 7.4) to trap free hydrogen sulphide as HS\(^-\); (3) each experiment was conducted at least in triplicate; and (4) we repeated the measurements with our previous probe(SFP-2) to compare with the data using RHP-2.

CUMS is a validated depression model. Throughout the CUMS induction, the animals were subjected to chronic and continuous low stress, and consequently exhibited the apparent behavioural deficits that are signs of human depressive states, such as despair, anhedonia and lack of activation\(^\text{52,53}\). CUMS induction successfully simulated a depressive-like status in mice by the reduction of sucrose intake and the increase of immobility duration in the forced swimming and tail suspension tests.

CBS, the major H\(_2\)S-producing enzyme in the brain, is highly expressed in the hippocampus. Interestingly, we noted that the endogenous H\(_2\)S concentrations were markedly reduced in the hippocampus in depressive-like mice after exposure to CUMS. Additionally, CBS mRNA and CBS protein expression were decreased. To the best of our knowledge, we are the first group to obtain preliminary data indicating that decreased levels of endogenous H\(_2\)S might be involved in the pathogenesis of depression. Moreover, treatment with NaHS significantly attenuated TS-induced downregulation of CSE and CBS expression, suggesting that the enhancement expression of CBS contributes to the elevation of H\(_2\)S concentrations in the mouse hippocampus. Surprisingly, the injection of NaHS resulted in a positive feedback for the enhanced expression of CBS. These results are consistent with several recent publications on the upregulation of CBS/CSE expression by exogenous sulphide. In the studies of Han et al, tobacco smoke exposure (TS) reduced the protein expression of CSE and CBS as well as the capacity for H\(_2\)S synthesis in mouse lungs, and treatment with NaHS attenuated TS-induced downregulation of CSE and CBS expression\(^\text{15}\). Park observed that unilateral ureteral obstruction (UO) attenuated TS-induced downregulation of CSE and CBS expression\(^\text{54}\). However, our studies demonstrated the antidepressant effects of H\(_2\)S. The present findings imply that H\(_2\)S might be a potential therapeutic target for chronic stress depression, and the application of exogenous H\(_2\)S and H\(_2\)S donors may be of therapeutic value in the treatment of depression.

To conclude, we synthesised and applied the ratiometric fluorescent probe RHP-2 for H\(_2\)S detection. Probe RHP-2 underwent a blue-to-green fluorescent emission colour change in response to sulphide. Advantages of this H\(_2\)S-specific probe include a low detection limit, high sensitivity and selectivity, good photostability and low cytotoxicity. The emission intensity ratios had a good linear relationship with sulphide concentrations in PBS buffer and bovine serum. This probe enabled the ratiometric fluorescence imaging of endogenous H\(_2\)S in living cells and the determination of sulphide in the mouse hippocampus. The results using RHP-2 suggest that decreased concentrations of endogenous H\(_2\)S may be involved in the pathogenesis of CUMS-induced depression.

**Methods**

**Synthesis of RHP-2.** A solution of NAP-NH\(_2\) (27 mg, 0.1 mmol) and 4-dimethylaminopyridine (DMAP) (37 mg, 0.3 mmol) in toluene (8 mL) was cooled to 0°C, which was followed by the dropwise addition of a solution of triphosgene (45 mg, 0.15 mmol) in toluene. The reaction mixture was heated under reflux for 6 h. When cooled to room temperature, the mixture was diluted with dehydrated CH\(_2\)Cl\(_2\) (8 mL), followed by the addition of 4-nitrobenzyl alcohol (20 mg, 0.13 mmol). The resulting solution was stirred overnight at room temperature. The reaction was quenched with water (5 mL) and extracted with EtOAc (3 × 10 mL). The combined organic layers were rinsed with water and saturated brine and dehydrated with Na\(_2\)SO\(_4\). The solvent was evaporated, and the crude product was purified by column chromatography on SiO\(_2\) to give the purified product, a white powder. Yield: 16 mg, 35.8%. TIC (silica, hexane:EtOAc:9:1:9): \(R_f = 0.4\); H NMR (400 MHz, DMSO-d\(_6\)): \( \delta \) 10.48 (s, 1 H, ArH), 8.70 (d, \( J = 8.8\) Hz, 1 H, ArH), 8.49 (d, \( J = 7.6\) Hz, 1 H, ArH), 8.46 (d, \( J = 8.4\) Hz, 1 H, ArH), 4.28 (s, \( J = 8.4\) Hz, 2 H, ArH), 4.18 (d, \( J = 8.0\) Hz, 1 H, ArH), 7.83 (t, \( J = 8.0\) Hz, 1 H, ArH), 7.76 (d, \( J = 8.8\) Hz, 2 H, ArH), 5.42 (s, 2 H, Ar\(_2\)N), 4.01 (t, \( J = 7.6\) and 7.2 Hz, 2 H, NCH\(_2\)), 1.56–1.63 (m, 2 H, NCH\(_2\)), 1.29–1.38 (m, 2 H, CH\(_2\)), 0.91 (t, \( J = 7.2\) Hz, 3 H, -CH\(_3\)\(_2\)). \(^{13}\)C NMR (100 MHz, DMSO-d\(_6\)): \( \delta \) 163.9, 163.3, 154.2, 147.6, 144.6, 140.9, 132.1, 131.4, 129.7, 129.0, 128.7, 124.3, 124.1, 122.7, 118.8, 117.7, 65.7, 39.3, 30.1, 20.2, 14.2; HRMS (m/z): [M-H]\(^-\) calcd for C\(_9\)H\(_{16}\)N\(_2\)O\(_4\): 446.1352, observed, 446.1359.

**Fluorometric analyses.** All fluorescence measurements were conducted at room temperature on a Hitachi F4600 Fluorescence Spectrophotometer. The RHP-2 probe (CH\(_3\)CN) was added to a quartz cuvette. With the probe diluted to 5 μM with 20 mM PBS buffer, Na\(_2\)S was added (Na\(_2\)S·9H\(_2\)O serving as the H\(_2\)S source in all experiments). The resulting solution was incubated for 40 min. The fluorescence
intensity was measured at $\lambda_{ex} = 415$ nm with the slit width of excitation and emission set at 5 nm. The emission spectra ranged from 430 to 550 nm at a velocity of 240 nm/min. The photomultiplier voltage was set at 550 V. Data are presented as the mean ± SD ($n = 3$).

Cell cultures and fluorescence confocal imaging. The MCF-7 cells were cultured in DMEM media with 10% (v/v) FBS (foetal bovine serum) and penicillin/streptomycin (100 U/mL) at 37°C in a 5% CO$_2$ incubator. Cells were permitted to grow to 80% confluence before harvesting and transferring to a confocal (Lab-Tek® II Chambered Coverglass, NaléNuc, Naperville, USA). The RHP-2 solution (1.0 mM stock solution in CH$_3$CN, final concentration 5 µM) was added to the cell media and incubated for 30 min with or without PGE (100 µg/mL; stock solution in 10 mM stock solution of DI H$_2$O, final concentration 1 µM) pretreatment. Afterwards, the cells were thrice rinsed with PBS solution (pH 7.4) to remove excess RHP-2, which was followed by the addition of Na$_2$S (20 mM stock solution in DI H$_2$O, final concentration 300 µM) or SNP (sodium nitroprusside, 20 mM stock solution in DI H$_2$O, final concentration 200 or 400 µM) in PBS (pH 7.4) to incubate at room temperature for 10 min. The samples were rinsed twice with PBS buffer prior to imaging. Confocal fluorescence imaging was performed on an Olympus FV1000 confocal laser scanning microscope with ×10 dry and ×60 oil objectives. The excitation wavelength was 425 nm. The blue fluorescent images were obtained from 445 to 495 nm, and the green fluorescent images were obtained from 520 to 580 nm. The images were obtained at 1024 × 1024 pixels. Images and data were analysed with Olympus software (FV10-ASW). All data are expressed as the mean ± SD ($n = 3$).

Determination of sulphide in the mouse hippocampus. For the measurement of sulphide, the mice were sacrificed, and the hippocampus was immediately isolated and homogenized without (w/o) HzO$_2$ (w/v) of ice-cold 100 mM PBS buffer (pH 7.4); the homogenate was centrifuged at 10,000 × g for 10 min at 4°C. All of the procedures were performed in an ice bath, and the homogenate supernatants were immediately transferred for sulphide determination. All fluorescence measurements were recorded on a Hitachi F4600 Fluorescence Spectrophotometer. Protein concentrations of the mouse hippocampus were determined with a Pierce BCA Protein Assay Kit. The determination of sulphide concentration in hippocampal homogenates spiked with Na$_2$S were used as an internal standard (X, X Protein Assay Kit. The determination of sulphide concentration in hippocampal homogenates spiked with Na$_2$S were used as an internal standard (X, X Proctol Redox. Signal. 17, 119–140 (2012).

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Author contributions

J.Z. and Y.L. conceived the idea and directed the work. J.Z. and L.Z. designed the experiments. I.Z., W.M. and L.L. performed the synthesis. I.Z. and L.Z. performed the in vitro fluorescence tests and quantitative tests in the mouse hippocampus. W.M. and C.L. performed the animal studies. Y.X. provided the molecular calculation data. All authors contributed to the data analysis and to writing the manuscript.

Additional information

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