

# H<sub>2</sub>S promotes developmental brain angiogenesis via the NOS/NO pathway in zebrafish

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## ABSTRACT

**Background** Hydrogen sulphide (H<sub>2</sub>S) is considered as the third member of the gasotransmitter family, along with nitric oxide (NO) and carbon monoxide. H<sub>2</sub>S has been reported to induce angiogenesis by promoting the growth, migration and tube-like structure formation of endothelial cells. Those studies were conducted in conditions of cell culture, mouse Matrigel plug assay model, rat wound healing model or rat hindlimb ischaemia model. Recent in vivo studies showed the physiological importance of H<sub>2</sub>S in muscle angiogenesis. However, the importance of endogenous H<sub>2</sub>S for brain angiogenesis during development remains unknown. We therefore aimed at determining the role of H<sub>2</sub>S in brain vascular development.

**Methods and results** Both knockdown and knockout of H<sub>2</sub>S-producing enzymes, cystathionine β-synthase (*cbs*) and cystathionine γ-lyase (*cth*), using morpholino oligonucleotides and clustered regularly interspaced short palindromic repeats/Cas9-mediated mutation, impaired brain vascular development of larval zebrafish. Incubation with the slow-releasing H<sub>2</sub>S donor GYY4137 alleviated the defects of brain vascular development in *cbs* and *cth* morphants. Quantitative analysis of the midbrain vascular network showed that H<sub>2</sub>S enhances angiogenesis without affecting the topological structure of the brain vasculature. Mechanically, nitric oxide synthase 2a (*nos2a*) expression and NO production were decreased in both *cbs* and *cth* morphants. Overexpression of *nos2a* by coinjection of *cbs* or *cth* MO with full-length zebrafish *nos2a* mRNA alleviated the brain vascular developmental defects in *cbs* and *cth* morphants.

**Conclusion** We conclude that H<sub>2</sub>S promotes brain developmental angiogenesis via the NOS/NO pathway in zebrafish.

## INTRODUCTION

Since found in the mammalian brain in 1989,<sup>1</sup> hydrogen sulphide (H<sub>2</sub>S) has been attracting continuous attention on its physiological and pathophysiological roles in the central nervous system (CNS). Along with nitric oxide (NO) and carbon monoxide, H<sub>2</sub>S has been considered to be the third member of the gasotransmitter family.<sup>2</sup> Cystathionine β-synthase (CBS) and cystathionine γ-lyase (CTH) are two main H<sub>2</sub>S-producing enzymes from cysteine and homocysteine in mammalian tissues.<sup>3</sup> CBS is abundantly expressed in

the brain,<sup>4</sup> especially in the hippocampus and the cerebellum.<sup>5</sup> In developing mouse brains, H<sub>2</sub>S is preferentially produced in radial glia/astrocyte lineages.<sup>6,7</sup> The expression of CTH is rather widely distributed among peripheral tissues including the liver, pancreas, uterus and intestine.<sup>8</sup> Western blotting experiments detected CTH rather than CBS in cerebral microvessels of newborn pigs<sup>9</sup> and CTH is the main H<sub>2</sub>S-producing enzyme in blood vessels.<sup>10</sup>

In the CNS, the well-studied functions of H<sub>2</sub>S include the modulation of neurotransmission<sup>4</sup> and neuroprotection.<sup>11</sup> In the peripheral vascular system, H<sub>2</sub>S is reported to induce vasorelaxation<sup>12</sup> and angiogenesis.<sup>13–15</sup> In vitro studies have demonstrated that exogenous H<sub>2</sub>S at physiologically relevant doses induced angiogenesis by promoting the growth, migration and tube-like structure formation of vascular endothelial cells (ECs),<sup>13,14</sup> while in situ studies showed that neovascularisation is promoted by H<sub>2</sub>S in the mouse Matrigel plug assay model,<sup>13</sup> rat wound healing model<sup>14</sup> and rat hindlimb ischaemia model.<sup>15</sup> Recently, two in vivo studies have shown the importance of H<sub>2</sub>S in muscle angiogenesis.<sup>16,17</sup> However, the importance of endogenous H<sub>2</sub>S for brain angiogenesis during development remains unknown.

The zebrafish is a well-established vertebrate model for in vivo study of vascular development.<sup>18</sup> Its accessible, small and transparent embryo facilitates high resolution in vivo imaging of the brain vasculature.<sup>19</sup> In the present study, we used larval zebrafish as a model and found that endogenous H<sub>2</sub>S plays an important role in the brain vascular development by promoting angiogenesis, and this effect is mainly mediated through the nitric oxide synthase (NOS)/NO pathway.

## METHODS

Detailed methods are available in the online supplemental material.

### In vivo confocal imaging

In vivo long-term serial confocal imaging of the midbrain vasculature was conducted in the same larva during 3–5 days post fertilisation (dpf). Larvae were embedded in 1.2% low-melting agarose (Invitrogen, 16520050) without anaesthetic at room temperature. After being imaged, the 3 or 4-dpf larvae were dug out for husbandry till subsequent imaging of the same larva was performed. Imaging was carried out with an Olympus Fluoview 1000 confocal microscope (Olympus, Japan). XLumplfl 20× (W/IR; NA, 0.95) objective lenses were used. The Z-step of imaging was 3 µm. Raw images were processed with ImageJ. Quantitative analysis of the midbrain vasculature morphology was conducted following the processes previously described.<sup>19</sup>

### Whole-mount in situ hybridization

Zebrafish whole-mount in situ hybridization was performed as previously described.<sup>20–21</sup> Probes for the *cbs* and *cth* mRNAs were synthesised and labelled with digoxigenin (Roche, Mannheim, Germany). Embryos were incubated with corresponding probes (1 ng/µL) at 68°C overnight. Antidigoxigenin AP-conjugated antibody (1:5000, Roche 11093274910) was used to detect digoxigenin and was further stained with nitro-blue-tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) solution (Roche, 11681451001).

### Microinjection

Zebrafish embryos were microinjected with 8 ng *cbs* morpholino oligonucleotide (MO), 0.5 ng *cth* MO, or equivalent Ctrl MO at one-cell stage. The MOs were purchased from Gene Tools (Philomath, Oregon, USA), and the sequences are as follows.

*cbs* MO: 5'-CTGGCATGGTTTACCCTGACTATCA-3'  
*cth* MO: 5'-GGCTGAGCTGTCGTTCTGCATCTCT-3'  
 Ctrl MO: 5'-CCTCTTACCTCAGTTACAATTATA-3'

### CRISPR/Cas9-mediated mutation of zebrafish *cbs* or *cth*

Mutations of zebrafish *cbs* or *cth* genes were induced by using the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system as previously reported.<sup>22–24</sup> Single guide RNA (sgRNA) sequences (*cbs*: 5'-GATGCTGACGATGTCTTCAA-3', *cth*: 5'-CGTTG-GTTCAGAGCCCCGAGC-3') were designed to target the sequences of mature *cbs* and *cth*. The paired oligonucleotides which contained the *cbs* or *cth* sgRNA sequence were annealed and cloned into a pT7-gRNA plasmid. Then, *cbs* sgRNA and *cth* sgRNA were synthesised with the MAXIscript T7 kit (Ambion, AM1312M). The synthesised sgRNA was purified with the mirVana miRNA isolation kit (Ambion, AM1560).<sup>25</sup> Coinjection of 600 pg Cas9 nuclease (NEB, M0386M) and 80 pg *cbs* or *cth* gRNA into zebrafish embryos were conducted at one-cell stage. The *cbs* or *cth* gene mutations in F0 embryos were examined by sequencing analysis after in vivo confocal imaging of the brain vasculature. The imaging data of F0 embryos carrying *cbs* or *cth* mutations were analysed.

The *cbs* or *cth* heterozygous mutant lines were generated by Nanjing XinJia Medical Technology Co. The two lines were separately outcrossed with the Tg(Flk1:eGFP) line. The crossed lines were further incrossed to observe the vascular development in homozygous mutant larvae at 3 and 5 dpf larvae. Genotyping was conducted after in vivo confocal imaging of the brain vasculature. The imaging data of embryos carrying *cbs* or *cth* homozygous mutations were analysed.

### Drug treatment

GGY4137 (SIGMA, SML0100) was dissolved in zebrafish culture medium at 1 dpf (2000 µM for *cbs* MO group and 1000 µM for *cth* MO group). The medium was refreshed once a day and phenotypes were characterised at 3–5 dpf.

### Measurement of NO production

Total NO production in the zebrafish larvae was examined with the total NO assay kit (Beyotime Institute of Biotechnology, S0023). It measured the concentration of nitrate and nitrite which are stable metabolites of NO.

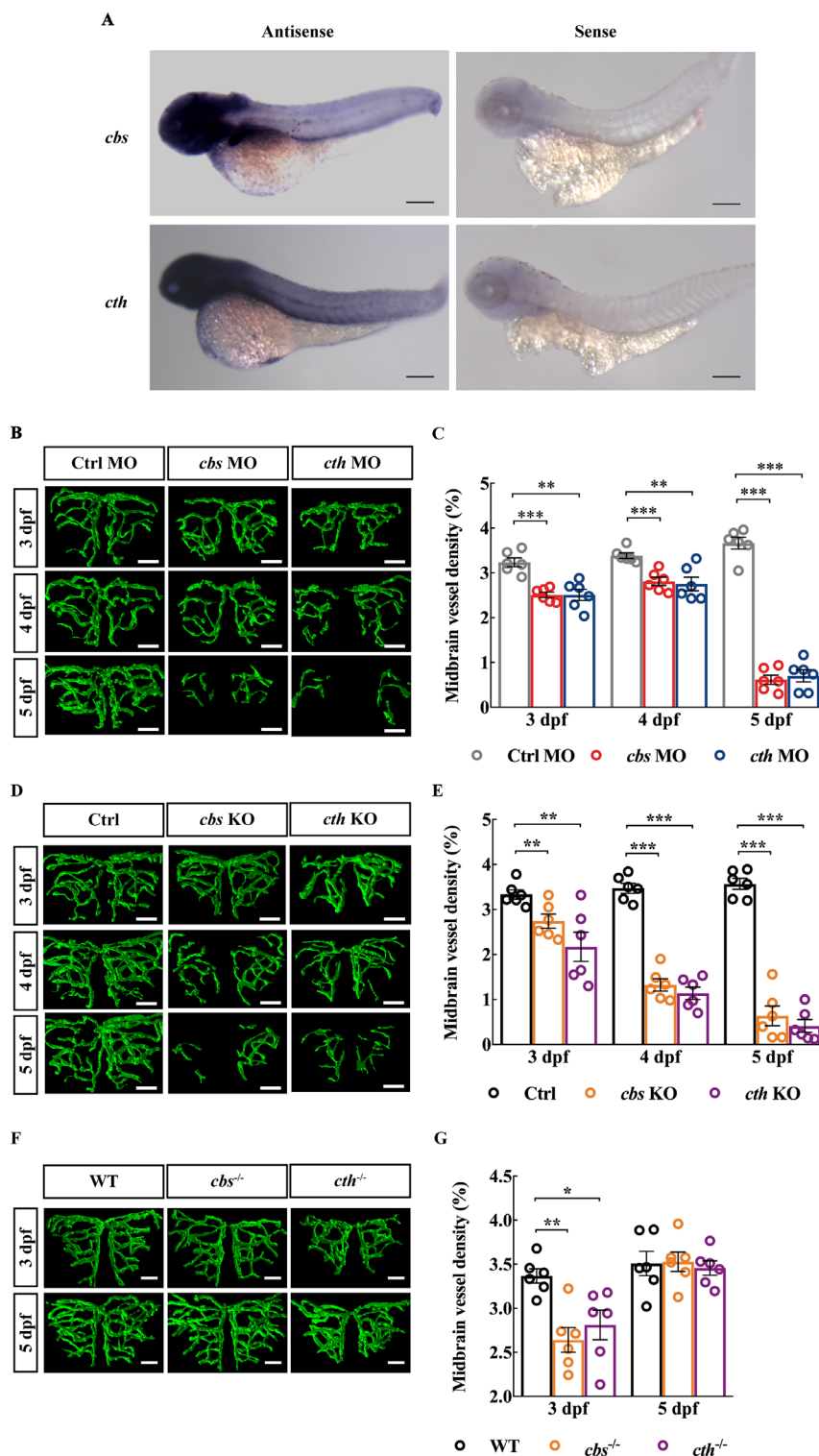
### Data analysis

All data were represented as mean±SEM. Statistical analysis was performed by using unpaired two-tailed Student's t-test or One-way analysis of variance.

## RESULTS

### Endogenous H<sub>2</sub>S is necessary for the brain vascular development in zebrafish

To examine the expression pattern of *cbs* and *cth* in zebrafish, we conducted whole-mount in situ hybridisation in larval zebrafish at 3 dpf and found that both *cbs* and *cth* were ubiquitously expressed in the zebrafish brain (figure 1A). To examine whether H<sub>2</sub>S is involved in brain vascular development, we first downregulated the expression of *cbs* and *cth* using MO-based knock-down. The expression of *cbs* and *cth* in zebrafish larvae were efficiently downregulated by the MOs (online supplemental figure S1) and H<sub>2</sub>S production were also significantly reduced in *cbs* and *cth* morphants (online supplemental figure S2). We performed in vivo long-term serial confocal imaging of the midbrain vasculature during 3–5 dpf in the same transgenic zebrafish Tg(Flk1:eGFP)<sup>26</sup> larva, in which vascular ECs express enhanced green fluorescent protein (eGFP). Compared with embryos injected with a control MO ('Ctrl MO'), both the *cbs* and *cth* morphants displayed impaired brain vascular development (figure 1B, online supplemental figure S3A), as evidenced by reduced midbrain vessel density during 3–5 dpf (figure 1C; p<0.01). Meanwhile, the brain size (indicated by the width of the optic tectum<sup>27</sup>) and cell apoptosis in *cbs* and *cth* morphants showed no significant difference compared with the Ctrl group at 3 dpf (online supplemental figure S4A,B,D and F), indicating that the impaired brain vascular development in *cbs* and *cth* morphants were not caused by the delayed brain development or



**Figure 1** Knockdown and knockout of *cbs* or *cth* impair brain vascular development of larval zebrafish. (A) In situ hybridisation of whole zebrafish larvae at 3 dpf showing the ubiquitous expression of *cbs* and *cth* in the brain (lateral view). (B and C) Effects of morpholino oligonucleotide-mediated *cbs* or *cth* knockdown on brain vascular development. (B) Representative midbrain vessel structures reconstructed from confocal images of *cbs* or *cth* morphants at 3–5 dpf of the same larva. (C) Summary of data. The experiments were repeated three times, and six embryos were examined for each group at each time. (D and E) Effects of *cbs* or *cth* knockout (F0) on brain vascular development. (D) Representative midbrain vessel structures reconstructed from confocal images of the same F0 mutant larva at 3–5 dpf. (E) Summary of data. Six embryos were examined for each group. (F and G) Effects of *cbs* or *cth* homomutants on brain vascular development. (F) Representative midbrain vessel structures reconstructed from confocal images of the same *cbs* or *cth* homomutant larva at 3 and 5 dpf. (G) Summary of data. Six embryos were examined for each group. Scale bar, 300  $\mu$ m (A), 25  $\mu$ m (B, D, F). Error bars, SEM. \* $P < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  (unpaired two-tailed Student's *t*-test).

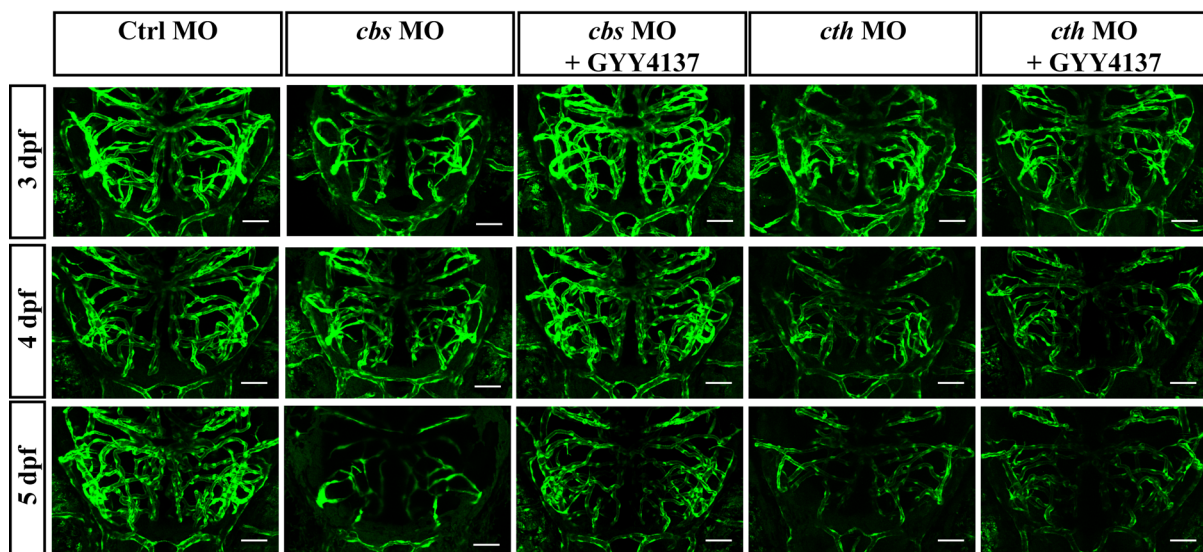


neuron death. Moreover, *cbs* and *cth* mutations (online supplemental figure S5) created by coinjecting *cbs* or *cth* sgRNA and Cas9 nuclease also caused a significant decrease in the midbrain vessel density during 3–5 dpf in F0 embryos (figure 1D,E, online supplemental figure S3B;  $p < 0.01$ ). Furthermore, *cbs* and *cth* homozygous mutants (online supplemental figure S6), obtained by incrossing the stable heterozygous mutant lines with the Tg(Flk1:eGFP) background, also displayed impaired brain vascular development at 3 dpf, with the relative normal vascular development at 5 dpf possibly due to the compensatory effect of the stable lines (figure 1F,G, online supplemental figure S3C;  $p < 0.05$ ). Although brains of *cbs* and *cth* homomutants were slightly smaller

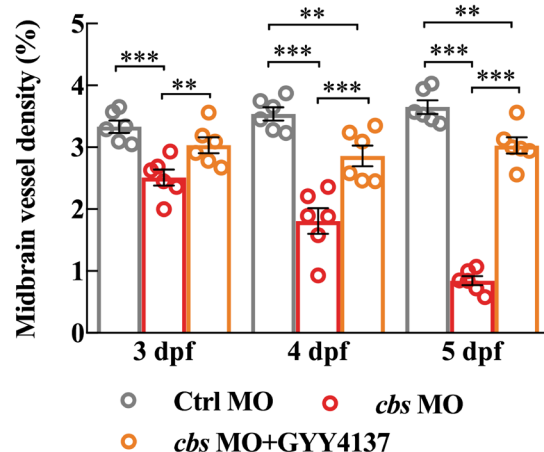
at 3 dpf online supplemental figure S4C;  $p < 0.05$ ), they did not show increased cell apoptosis (online supplemental figure S4E,G).

Next, we conducted rescue experiments on *cbs* and *cth* morphants by bath application of GYY4137 (200  $\mu$ M), a slow-releasing  $H_2S$  donor which is able to yield stable levels of  $H_2S$  at about 5–10  $\mu$ M at least for 7 days.<sup>28</sup> Embryos injected with MOs were exposed to Hanks' solution diluted with GYY4137 since 24 hours post fertilisation. GYY4137 treatment alleviated the brain vascular developmental defects in both *cbs* and *cth* morphants (figure 2). It is worth mentioning that administration of GYY4137 did not increase the midbrain vessel density in control fish (online supplemental figure S7). Taken

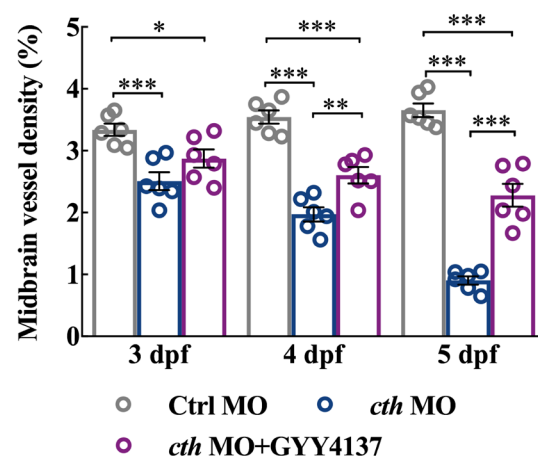
A



B



C



**Figure 2** Rescue effect of GYY4137 on the defects of brain vascular development in *cbs* and *cth* morphants. (A) Representative projected confocal images showing that GYY4137 treatment ameliorated the impaired brain vascular development in *cbs* and *cth* morphants. Confocal images were taken at 3–5 dpf of the same larva. (B) Summary of the rescue effect of GYY4137. Six embryos were examined for each group. Scale bar, 50  $\mu$ m (A). Error bars, SEM. \* $P < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  (one-way analysis of variance). MO, morpholino oligonucleotide.

together, these results indicate that endogenous  $H_2S$  is important for the brain vascular development.

### $H_2S$ promotes brain angiogenesis rather than topological structure formation

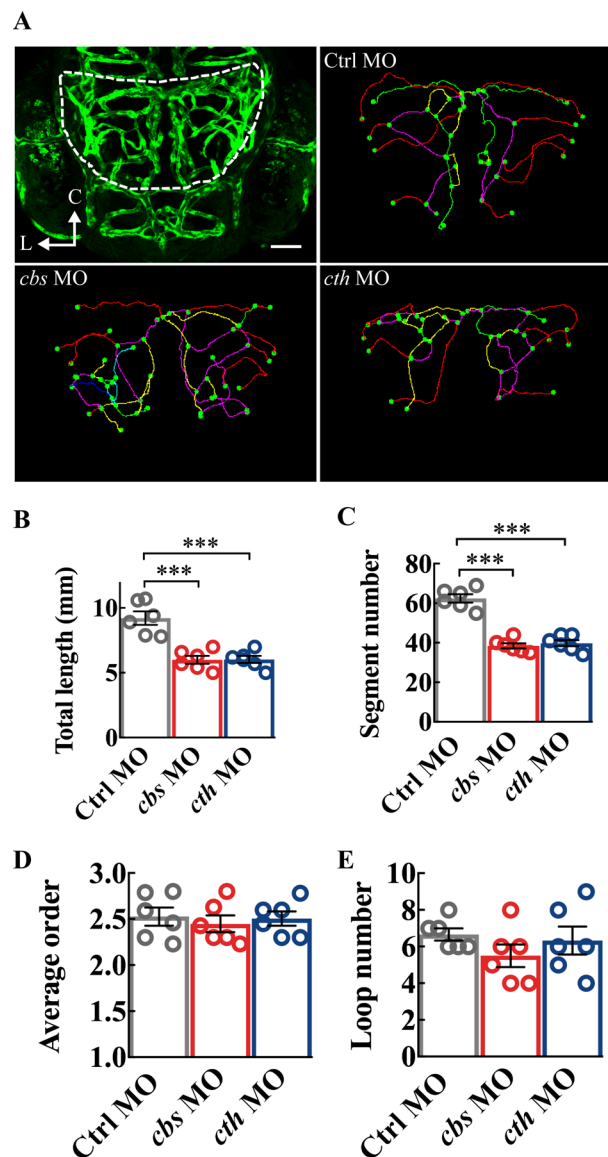
Maturation of vascular networks involves complicated processes including vasculogenesis, angiogenesis and followed with vessel formation, stabilisation, branching, pruning and specialisation.<sup>29</sup> It can be generalised into two major aspects: vessel growth (via angiogenesis) and topological structure formation (ie, branching and pruning). To figure out the detailed roles of  $H_2S$ , we thus analysed quantitatively the characteristics of the midbrain vasculature. We used the total length and vessel segment number to, respectively, quantify vessel elongation and new vessel addition through angiogenesis, and the average order of vessel segments and vessel loop number to quantify the complexity of the vascular network.<sup>19</sup> Both the total length and vessel segment number were markedly decreased in 3-dpf *cbs* and *cth* morphants (figure 3A–C;  $p < 0.001$ ), while the average order and loop number had no significant change (figure 3A,D and E). These results indicate that  $H_2S$  enhances angiogenesis without affecting the topological structure during brain vascular development in zebrafish.

### NOS/NO pathway is involved in the proangiogenic effect of $H_2S$

NO is formed from the reaction of L-arginine with  $O_2$ , a reaction catalysed by NOS. Three NOS isoforms have been identified in mammals<sup>30</sup>: neuronal NOS (NOS1), inducible NOS (NOS2) and endothelial NOS (NOS3). While in the zebrafish genome, there are one *nos1* homolog gene and two *nos2* homolog genes (*nos2a* and *nos2b*).<sup>31</sup> We found that there was a decrease in *nos2a* expression in both *cbs* and *cth* morphants, but an increase in *nos2b* in *cth* morphants which might be due to a compensatory effect (figure 4A;  $p < 0.05$ ). Meanwhile, the expression of *nos1* was not affected (figure 4A). Consistently, NO production was also decreased in both *cbs* and *cth* morphants (figure 4B;  $p < 0.01$ ). We then performed rescue experiments by coinjection of *cbs* or *cth* MO with full-length zebrafish *nos2a* mRNA, and found that the decreased NO production and brain vascular defects caused by *cbs* or *cth* knockdown were significantly alleviated (figure 4C,D;  $p < 0.01$  for NO production,  $p < 0.05$  for vascular density). It is noteworthy that the expression of ERK1/2 (p44/42 mitogen-activated protein kinase), phosphorylated ERK1/2 and vascular endothelial growth factor (VEGF) was not significantly changed in both *cbs* and *cth* morphants (figure 4E,F). Taken together, these results suggest that the NOS/NO signalling pathway plays an important role in the proangiogenic effect of  $H_2S$  in brain vascular development.

### DISCUSSION

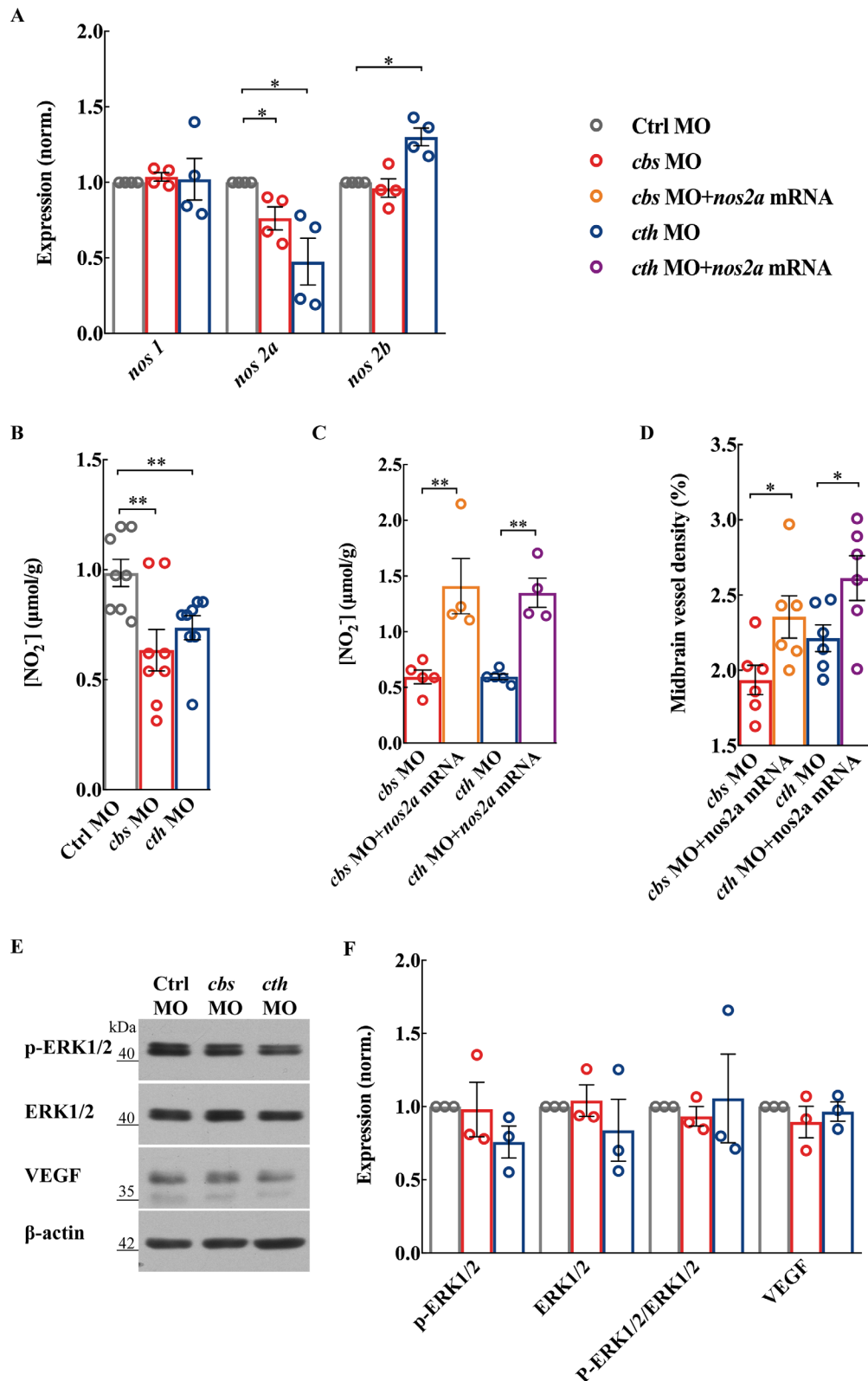
$H_2S$  has been shown to exert potent proangiogenic effects in vitro and in pathological models.<sup>14 15 32–35</sup> Recently, two in vivo studies showed the importance of  $H_2S$  in muscle



**Figure 3** Structure analysis of the midbrain vasculature in *cbs* and *cth* morphants. (A) Image of a 3-dpf larva showing the midbrain position delineated with dashed lines (upper left), and representative midbrain vasculature centerlines of 3-dpf larvae of Ctrl morpholino oligonucleotide (MO; upper right), *cbs* MO (lower left), and *cth* MO (lower right). C, caudal; L, lateral. (B–E) Summary of changes in the total vessel length (B), vessel segment number (C), weighted average segment Strahler order (D) and internal vessel loop number (E) of the midbrain vasculature in groups of Ctrl MO, *cbs* MO and *cth* MO. Six embryos were examined for each group. Error bars, SEM. \*\*\* $P < 0.001$  (unpaired two-tailed Student's t-test).

angiogenesis.<sup>16 17</sup> By in vivo confocal imaging of zebrafish larvae, our study reveals the role of endogenous  $H_2S$  in developmental angiogenesis of the brain.

The signalling pathways involved in the proangiogenic effect of exogenous  $H_2S$  on peripheral vessels has been well studied with in vitro preparations.<sup>14 15 32–34</sup> The proangiogenic effect of  $H_2S$  is mediated via: (1) EC-related angiogenic properties through a  $K_{ATP}$  channel/MAPK



**Figure 4** The nitric oxide synthase (NOS)/nitric oxide (NO) pathway is involved in *cbs* and *cth* knockdown-induced defects of brain vascular development. (A and B) Effects of *cbs* and *cth* knockdown on the NOS/NO signalling pathway. Summary of *nos1*, *nos2a* and *nos2b* RNA expression (A) and total NO production (B) in 3-days post fertilisation (dpf) embryos. (C and D) Rescue effect of *nos2a* mRNA on the brain vascular developmental defects in *cbs* and *cth* morphants. Summary of increased total NO production (C) and midbrain vessel density (D) in 3-dpf embryos coinjected with *cbs* or *cth* MO and *nos2a* mRNA in comparison with those injected with *cbs* or *cth* MO. Six embryos were examined for each group. (E and F) No effect of *cbs* and *cth* knockdown on the protein expression of p-ERK1/2, ERK1/2 and VEGF. Representative blots (E) and summary (F) of Western blotting data. Error bars, SEM. \* $P < 0.05$ , \*\* $p < 0.01$  (unpaired two-tailed Student's t-test).



pathway<sup>14</sup>; (2) upregulation of VEGF expression and release from cells<sup>36 37</sup>; (3) stimulation of the NOS/NO signalling pathway. In the present study, we examined ERK1/2 phosphorylation and VEGF expression and found no change of these signals in *cbs* and *cth* morphants. NO and H<sub>2</sub>S share many similar regulatory roles including promotion of angiogenesis, vasodilation, attenuation of apoptosis and antioxidant actions.<sup>32 38</sup> H<sub>2</sub>S is reported to exert proangiogenic functions via both NO-dependent and NO-independent mechanisms.<sup>32 34</sup> Exogenously administered H<sub>2</sub>S increases NOS expression<sup>33</sup> and phosphorylation<sup>34</sup> in cultured ECs and stimulates the production of NO. In zebrafish larvae, expression of *nos2a* was mainly detected in the head, eyes and gut regions,<sup>31</sup> consistent with the expression pattern of *cbs* and *cth* we observed. In the present study, depletion of H<sub>2</sub>S resulted in a reduction in *nos2a* expression and NO production. Injection of full-length zebrafish *nos2a* mRNA alleviated the brain vascular developmental defects in *cbs* and *cth* morphants. Thus, H<sub>2</sub>S promotes brain angiogenesis via the NOS/NO pathway in zebrafish.

Studies demonstrating the proangiogenic effect of endogenous H<sub>2</sub>S were mostly conducted through manipulating CTH,<sup>14 17 39</sup> and the cellular target of H<sub>2</sub>S is believed to be ECs as stated above. However, our study found that CBS-derived and CTH-derived H<sub>2</sub>S both played vital roles in brain developmental angiogenesis. Indeed, in the brain, H<sub>2</sub>S production in the astrocyte is almost 10 times as much as that in other brain cell types,<sup>6</sup> making the astrocyte an important target for H<sub>2</sub>S functioning and CBS a significant source of H<sub>2</sub>S in the brain. We speculate that H<sub>2</sub>S promotes brain angiogenesis by functioning on ECs through direct and indirect ways. The cellular mechanisms of H<sub>2</sub>S promoting brain angiogenesis require further study.

Currently, numerous animal and cellular studies have showed that H<sub>2</sub>S impacts ischaemic stroke outcomes due to its neuroprotective abilities.<sup>40</sup> Taking advantage of slow-releasing H<sub>2</sub>S donors, H<sub>2</sub>S is emerging as a potential therapy for cerebral ischaemia. Our study reveals the role of endogenous H<sub>2</sub>S in brain angiogenesis, making H<sub>2</sub>S-based therapies for cerebral ischaemia even more promising.

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