

RESEARCH PAPER

Meningeal blood flow is controlled by H₂S-NO crosstalk activating a HNO-TRPA1-CGRP signalling pathway

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Received

5 December 2014

Revised

17 March 2015

Accepted

10 April 2015

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BACKGROUND AND PURPOSE

Meningeal blood flow is controlled by CGRP released from trigeminal afferents and NO mainly produced in arterial endothelium. The vasodilator effect of NO may be due to the NO-derived compound, nitroxyl (HNO), generated through reaction with endogenous H₂S. We investigated the involvement of HNO in CGRP release and meningeal blood flow.

EXPERIMENTAL APPROACH

Blood flow in exposed dura mater of rats was recorded by laser Doppler flowmetry. CGRP release from the dura mater in the hemisected rat head was quantified using an ELISA. NO and H₂S were localized histochemically with specific sensors.

KEY RESULTS

Topical administration of the NO donor diethylamine-NONOate increased meningeal blood flow by 30%. Pretreatment with oxamic acid, an inhibitor of H₂S synthesis, reduced this effect. Administration of Na₂S increased blood flow by 20%, an effect abolished by the CGRP receptor antagonist CGRP₈₋₃₇ or the TRPA1 channel antagonist HC030031 and reduced when endogenous NO synthesis was blocked. Na₂S dose-dependently increased CGRP release two- to threefold. Co-administration of diethylamine-NONOate facilitated CGRP release, while inhibition of endogenous NO or H₂S synthesis lowered basal CGRP release. NO and H₂S were mainly localized in arterial vessels, HNO additionally in nerve fibre bundles. HNO staining was lost after treatment with L-NMMA and oxamic acid.

CONCLUSIONS AND IMPLICATIONS

NO and H₂S cooperatively increased meningeal blood flow by forming HNO, which activated TRPA1 cation channels in trigeminal fibres, inducing CGRP release. This HNO-TRPA1-CGRP signalling pathway may be relevant to the pathophysiology of headaches.

Abbreviations

CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; Cy3, cyanine dye 3; DAF, 4-amino-5-methylamino-2',7'-difluoresceine diacetate; HNO, nitroxyl; iNOS, inducible NOS; L-NMMA, L-NG-monomethylarginine acetate; MMA, middle meningeal artery; MST, mercaptopyruvate sulfurtransferase; nNOS, neuronal NOS; NONOate, diethylamine-NONOate, DEANONOate; ODQ, 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one; sGC, soluble GC; SIF, synthetic interstitial fluid; TRPA1, transient receptor potential ankyrin 1 channel

Tables of Links

TARGETS	
Ion channels^a	Enzymes^c
TRPA1 channel	CBS, cystathionine β -synthase
GPCRs^b	CSE, cystathionine γ -lyase
CGRP receptor	MST, mercaptopyruvate sulfur transferase
	nNOS
	eNOS

LIGANDS
CGRP
CGRP ₈₋₃₇
HC030031
NO
ODQ, 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (^{a,b,c}Alexander *et al.*, 2013a,b,c).

Introduction

Neurovascular regulation in the meninges involving CGRP and NO

Meningeal blood flow is regulated by neuronal (Owman, 1990) and epithelium-dependent metabolic mechanisms (Faraci and Heistad, 1991) acting primarily on arterial blood vessels. Meningeal arteries are surrounded by a dense network of autonomic and trigeminal sensory nerve fibres, a considerable proportion of which contains vasoactive peptides (Edvinsson and Uddman, 1981; Keller and Marfurt, 1991; Messlinger *et al.*, 1993). Upon their activation, trigeminal afferents secrete vasodilatory neuropeptides like CGRP, the most potent intracranial vasodilator (Edvinsson *et al.*, 1987). The vasodilatory function of CGRP release in the dura mater has been shown by many experiments *in vitro* (Gupta *et al.*, 2006) and *in vivo* employing video microscopy (Williamson *et al.*, 1997) or laser Doppler flowmetry (Kurosawa *et al.*, 1995). Arterial vasodilatation and blood flow increase caused by CGRP release have therefore widely been used to assess the activation of the trigeminal system in animal experiments (Williamson *et al.*, 1997; Dux *et al.*, 2003; Messlinger *et al.*, 2011). CGRP binds to GPCRs of arterial smooth muscle cells inducing muscular relaxation through intracellular cAMP and cGMP increase (Gray and Marshall, 1992; Brain and Grant, 2004).

The putatively most important metabolic factor mediating vasodilatation in the dura mater is NO, which in arterial smooth muscle cells binds to soluble GC (sGC) increasing intracellular cGMP levels (Brian *et al.*, 1996). Under normal conditions, the main source of NO is most likely the arterial endothelium, which can be demonstrated by NOS immunohistochemistry or NADPH-diaphorase activity (Hope *et al.*, 1991). The continuous production of endothelial NO seems to be important for the basal relaxation of arterial vessels maintaining the basal blood flow in the meninges (Messlinger *et al.*, 2000). The inducible NOS (iNOS) is expressed, for example, during inflammation by macrophages invading the dura mater (Reuter *et al.*, 2001). Furthermore, some nerve fibres accompanying intracranial arteries, both of trigeminal and parasympathetic origin, have been identified by immunohistochemistry as positive for the neuronal NOS (nNOS) (Nozaki *et al.*, 1993).

Interaction of CGRP and NO in meningeal blood flow

In earlier experiments, we and other groups recognized that the vasodilatory effects of NO clearly depended partly on the action of CGRP and *vice versa* (Messlinger *et al.*, 2000; Akerman *et al.*, 2002). Electrical or chemical stimulation of meningeal afferents increasing meningeal blood flow through CGRP release was less effective when the production of NO was inhibited, and the blood flow increasing effect of NO donors was decreased when CGRP receptors were inhibited (Strecker *et al.*, 2002a). This indicated a synergistic or permissive effect of NO, the mechanism of which was not clear. We concluded that CGRP receptor activation is necessary to fully enable the vasodilatory function of NO but we could not adequately explain the mechanisms behind this synergistic effect.

However, NO can also be converted to nitroxyl (HNO), a protonated, one electron-reduced derivative of NO (Fukuto *et al.*, 2005; Fukuto and Carrington, 2011), which is also a vasodilator (Ellis *et al.*, 2000). In addition to its vasodilator effects, HNO exerts positive inotropic and lusitropic effects on heart, initially assigned to CGRP release (Paolocci *et al.*, 2001; 2003). Recently, we have demonstrated that HNO activated the transient receptor potential ankyrin 1 (TRPA1) cation channel which, on opening, allows calcium influx and consequent exocytosis of CGRP from primary afferent terminals (Eberhardt *et al.*, 2014). Using Angeli's salt as an HNO donor, we observed Angeli's salt-induced CGRP release from the cranial dura mater and increased meningeal blood flow.

The pathway(s) for endogenous generation of HNO has long been a matter of debate (Fukuto *et al.*, 2005; Flores-Santana *et al.*, 2011; Fukuto and Carrington, 2011) but recent studies of the crosstalk between NO and H₂S suggested their potential role in endogenous HNO generation (Yong *et al.*, 2010; 2011; Filipovic *et al.*, 2012; 2013; Miljkovic *et al.*, 2013). H₂S has emerged as a new endogenous gasotransmitter that can regulate vascular tone (Yang *et al.*, 2008). H₂S is produced by the action of at least three enzymes: cystathionine β -synthase (CBS), cystathionine γ -lyase (CSE) and mercaptopyruvate sulfurtransferase (MST) (Kabil and Banerjee, 2014; Kabil *et al.*, 2014). Differently expressed in different tissues (and in different intracellular compartments), these enzymes control H₂S production with different efficiency.

Most recently, we unambiguously showed that NO and H₂S do react directly and that this reaction represents an endogenous source of HNO (Eberhardt *et al.*, 2014), which then activates a HNO-TRPA1-CGRP signalling cascade. This is in agreement with previous observations of H₂S-induced TRPA1-dependent effects on skin blood flow, mesenteric artery dilatation and CGRP release from the trachea (Pozsgai *et al.*, 2010; 2012).

The experiments described here were designed to explain our previous observations of NO-dependent CGRP release (Messlinger *et al.*, 2000; Akerman *et al.*, 2002; Strecker *et al.*, 2002a) to extend our recent findings about the HNO-TRPA1-CGRP signal cascade, activated by the reaction of NO with H₂S (Eberhardt *et al.*, 2014) and to dissect this cascade in the trigeminovascular system of the cranial meninges. To measure changes in meningeal blood flow, we used laser Doppler flowmetry to determine NO- and HNO-induced arterial dilatation. We employed a selective ELISA method to directly measure CGRP release from the rat dura mater *in vitro*. In addition, we utilized different histochemical methods using immunofluorescence and new fluorescence sensors to demonstrate the presence of NO, HNO and H₂S and sources of NO involved in the physiological HNO production in the dura mater. We describe, step-by-step, the whole cascade that could underlie the NO-induced activation of trigeminal afferents and the consequent increase in meningeal blood flow resulting from neurogenic vasodilatation of arterial blood vessels. Beyond these vasodilatory processes induced by this HNO-TRPA1-CGRP signalling pathway, we discuss its possible role in meningeal nociception and the generation of headaches.

Methods

Animals

All animal care and experimental procedures complied with the guidelines for the welfare of experimental animals of the Federal Republic of Germany and the European Commission (Directive 2010/63/EU), and those of the International Association for the Study of Pain. They were reviewed and approved by the animal protection authorities of the District Government of Mittelfranken (54-2532.1-21/12). Animal studies are described in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 96 animals were used in the experiments described here.

For *in vivo* and *in vitro* experiments, adult male Wistar rats weighing 300–350 g were used. The animals were bred and housed in the animal facility of the Institute of Physiology under a 12-12 h light-dark cycle with food and water *ad libitum*.

Blood flow recordings *in vivo*

Rats were anaesthetized initially by inhalation of 4% isoflurane (Forene; Abbott, Wiesbaden, Germany) in a closed box. Then they were tracheotomized and artificially ventilated throughout the experiment with 2% isoflurane in oxygen-enriched room air. End-expiratory CO₂ was monitored and held at 3–3.5%. Systemic blood pressure was recorded with a pressure transducer via a cannula inserted into the femoral

artery. A venous catheter introduced into the femoral vein served for the systemic administration of substances. The body temperature of the animals was recorded with a thermoprobe inserted into the rectum, and was kept at 37–37.5°C with a feedback-controlled heating pad. A cranial window for the measurement of dural blood flow was prepared according to previous studies (Kurosawa *et al.*, 1995; Dux *et al.*, 2002; see Figure 1B). The head of the animal was fixed in a stereotaxic frame, the scalp was removed, and the parietal bone was exposed. The skull was trepanized using a saline-cooled drill. The experiments were terminated using an overdose of *i.v.* thiopentone (Trapanal®; Altana, Konstanz, Germany).

The cranial recording window was filled with 50 µL of synthetic interstitial fluid (SIF) containing (in mM): 108 NaCl, 3.48 KCl, 3.5 MgSO₄, 26 NaHCO₃, 11.7 NaH₂PO₄, 1.5 CaCl₂, 9.6 Na⁺ gluconate, 5.55 glucose and 7.6 sucrose; pH 7.4. Meningeal blood flow was measured with needle-type probes of a laser Doppler flowmeter (Moore DRT4; Moore Instruments, Axminster, UK). Probes were positioned over branches of the middle meningeal artery (MMA) lying distant from visible cortical blood vessels (see Figures 1B and 2A right hand). Perfusion of the dura mater (measured in perfusion units) and systemic blood pressure of the animals were stored and processed with the DRT4 program. Substances were applied either topically with a micropipette onto the exposed dura mater or *i.v.* through the femoral catheter. Changes in meningeal blood flow after topical or *i.v.* application of agonists [Na₂S and diethylamine-NONOate (DEANONOate)] were determined by comparing the mean flow values within the 10 min application period with the baseline measured as the mean flow during the 5 min period prior to drug application and expressed as % change. The effects of antagonists (oxamic acid, HC030031, CGRP₈₋₃₇ and L-NMMA, see below) were determined by comparing the blood flow response to the agonists before and after the application of antagonist in each experiment. Effective doses and application periods of drugs were chosen according to previous or pilot experiments.

Measurement of CGRP release *in vitro*

Measurement of CGRP released from dural afferents was performed by the method described by Ebersberger *et al.* (1999). Rats were killed by CO₂ inhalation and decapitated. After removal of the skin and muscles, the skull was divided into halves along the midline and the cerebral hemispheres were removed (see Figure 3). Skull halves with intact dura mater were superfused at room temperature for 30 min with SIF. Skull halves were placed in a humid chamber to maintain their temperature at 37°C. The cranial fossae were filled with 500 µL SIF. Consecutive samples of the superfusate were collected at periods of 5 min by carefully removing the content of the skull halves with a micropipette. All samples were transferred to plastic cups (Eppendorf, Hamburg, Germany), 100 µL of each sample was taken, diluted with 20 µL EIA buffer (SPIbio, Paris, France), and immediately processed for analysis of the CGRP content using a specific enzyme-linked immunoassay (CGRP-EIA; SPIbio). The absorbance of the reaction product representing the CGRP concentration of the sample was determined photometrically using a microplate reader (Opsys MR; Dynex, Denkendorf, Germany). The minimum detection limit of the assay is specified as 5 pg·mL⁻¹

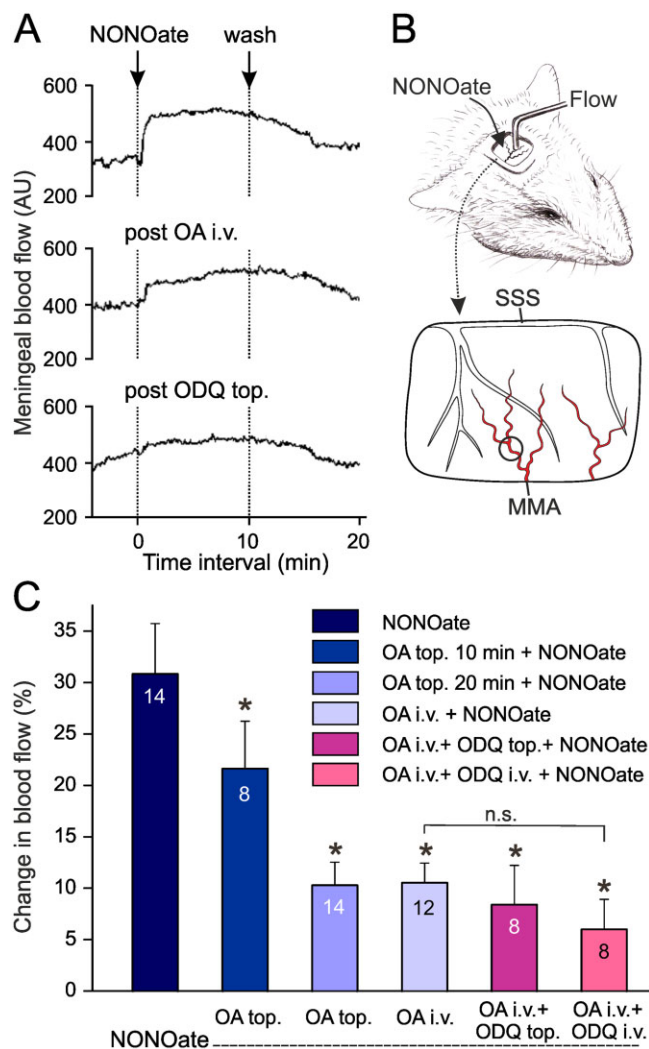


Figure 1

Increases in meningeal blood flow within 10 min periods following topical application of DEANOate (NONOate, 200 μ M) onto the dura mater. (A) Segments of an original recording showing increases in blood flow induced by NONOate before and after i.v. administration of oxamic acid (post OA i.v.) and, in addition, of ODQ topically applied onto the dura mater (post ODQ top.). (B) Experimental set-up showing the position of the recording probe on the MMA visible in the cranial window (same experiment as in A). SSS, superior sagittal sinus with cortical veins visible through the dura. (C) Increases in meningeal blood flow relative to the baseline prior to substance application; numbers of experiments are indicated in the bars. Effect of NONOate following topical (top., 2 mM) and systemic (i.v., 10 mg·kg⁻¹) pre-administration of oxamic acid (OA) and the sGC inhibitor ODQ (50 μ M or 1 mg·kg⁻¹ respectively). **P* < 0.05, significantly different from NONOate-evoked flow increase without pretreatment; n.s., not significant.

by the manufacturer. The CGRP concentration was calculated in pg·mL⁻¹, allowing for the added volume of EIA buffer. Raw data were used for statistical comparison.

Histochemistry

Rats deeply anaesthetized by breathing 5% isoflurane and i.p. injection of thiopentone (Trapanal®, 150–200 mg·kg⁻¹) were

thoracotomized and perfused transcardially with physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The skin and muscles of the skull were removed and the skull was divided into halves along the midline. After removal of the brain, samples of the dura mater were harvested and processed for microscopic examination.

For indirect immunofluorescence, whole mounts of the dura mater were rinsed in PBS (0.01 M, pH 7.4), pre-incubated for 2 h at room temperature with a solution of 5% goat serum (Dianova, Hamburg, Germany), 0.5% Triton X-100 and 1% BSA containing PBS, rinsed in PBS, and incubated overnight with rabbit polyclonal antisera raised against rat CGRP 1:1000 (Peninsula T 4032; Bachem, Weil/Rhein, Germany) or rat nNOS 1:1000 (Calbiochem ST 1995; Merck Millipore, Darmstadt, Germany). For TRPA1 immunostaining, unfixed whole mounts of rat dura mater were treated accordingly and incubated with rabbit polyclonal antiserum raised against rat TRPA1 1:500 (ab58844; Abcam, Cambridge, UK). Then whole mounts were washed with PBS, incubated with goat anti-rabbit IgG conjugated with indocarbocyanine 1:200 (Cy3; Dianova 111-165-144) for 2 h at room temperature, rinsed again and coverslipped in Fluoromount G (Science Services, München, Germany). Some samples were mounted with Roti®-Mount Fluor Care (Roth, Karlsruhe, Germany) containing DAPI for nuclear DNA staining.

For NADPH-diaphorase histochemistry, the dura mater was pre-incubated in 0.1 M PBS containing 0.3% Triton X-100 for 60 min and then incubated in reaction solutions for NADPH-diaphorase activity (1 mg·mL⁻¹ nitroblue tetrazolium, 0.4 mg·mL⁻¹ β -NADPH, 0.3% Triton X-100 in 0.1 M PBS) at 37°C for 15 min. Dura samples were mounted on glass slides, air dried and coverslipped.

For detection of NO, H₂S and HNO by fluorescence sensors, isolated (unfixed) dura mater was incubated for 15 min at 37°C with DAF (4-amino-5-methylamino-2',7'-difluoresceine diacetate) for NO, with WSP-1 (Cayman, distributed by Alexis, Grünberg, Germany) for H₂S, or with the HNO-selective fluorescent dye CuBOT1, synthesized as previously described by Rosenthal and Lippard (2010), then washed with SIF and further incubated in SIF for additional 30 min at 37°C. In other experiments, the isolated dura mater was pre-incubated with L-NMMA (2 mM) or with oxamic acid (2 mM) for 45 min before the above sensors were applied. The green fluorescence was visualized with the FITC filter setting using the LSM 780 confocal microscope.

In order to examine the production of NO and H₂S from the dura, isolated rat dura was incubated with the NO sensor DAF for 30 min following pretreatment with oxamic acid (2 mM) for 30 min at 37°C or, in the same way, with the H₂S sensor WSP-1 following L-NMMA (2 mM). The fluorescence of the supernatant was analysed with Jasco spectrofluorometer (Jasco Analytical Instruments, Gross-Umstadt, Germany) with λ_{ex} 485 and λ_{em} 538 nm for DAF-FM-DA and λ_{ex} 465 and λ_{em} 515 nm for WSP-1. The intensities (arbitrary units per wet weight of tissue) compared with the value of the contralateral dura half, incubated without pretreatment.

Microscopy

The stained dura mater was analysed using an LSM 780 light and confocal microscope (Carl Zeiss MicroImaging GmbH,

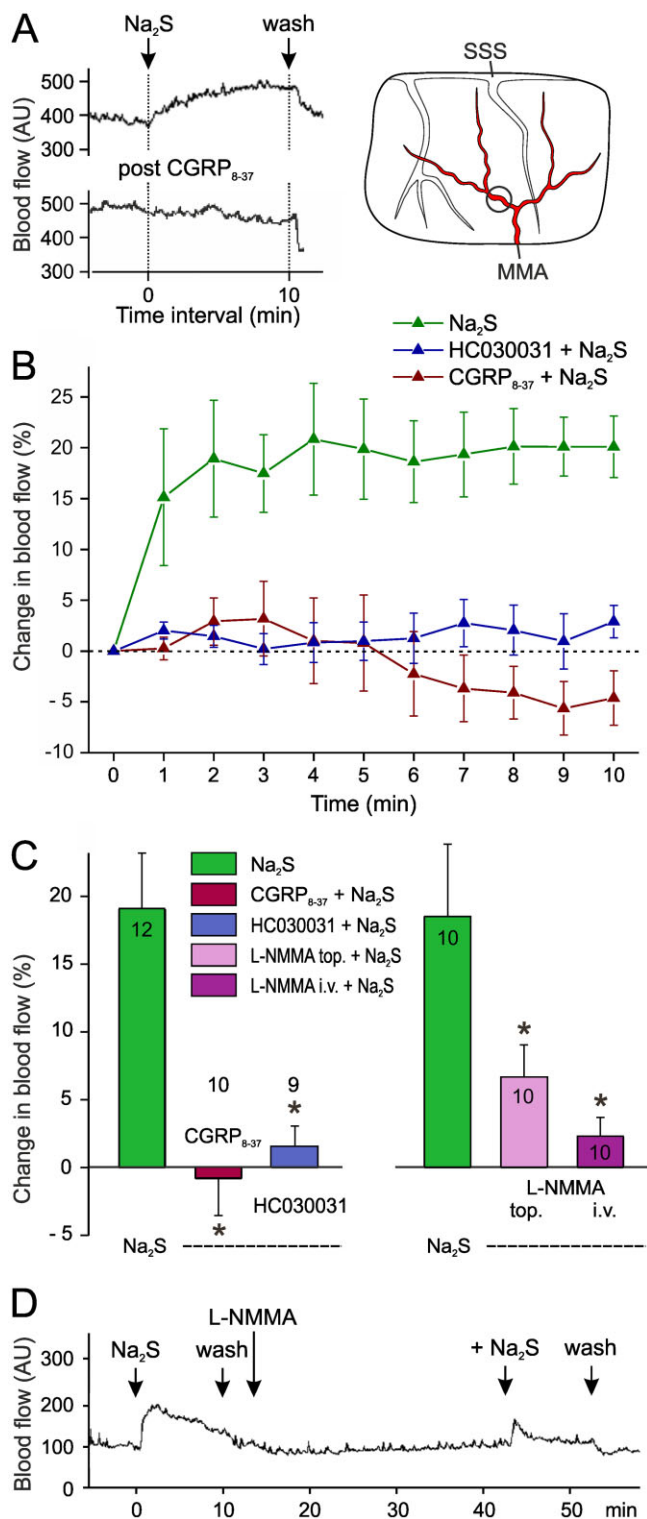


Figure 2

Changes in meningeal blood flow within 10 min periods following topical application of Na₂S (300 μM) onto the dura mater. (A) Segments of an original recording showing an increase in blood flow induced by Na₂S, which is abolished after topical administration of CGRP₈₋₃₇. The position of the flow probe is indicated in the sketch of the cranial window. (B) Time course of changes in blood flow following application of Na₂S with and without preapplication of CGRP₈₋₃₇ (100 μM) or the TRPA1 antagonist HC030031 (50 μM). The flow after Na₂S is significantly different from the baseline from the second minute (repeated measures ANOVA and LSD *post hoc* test, $P < 0.01$) and different from the Na₂S-evoked flow after pre-administration of HC030031 or CGRP₈₋₃₇ (two-way ANOVA, $P < 0.001$). (C) Changes in blood flow relative to the baseline induced by Na₂S prior to and after pre-administration of CGRP₈₋₃₇ or HC030031 (left panel, same measurements as in B) and after topical (1 mM) and i.v. (12 mg·kg⁻¹) pre-administration of L-NMMA (right panel). Mean values from the numbers of experiments indicated within the bars. * $P < 0.05$, significantly different from effects of Na₂S alone. (D) Original recording of an experiment showing the effect of Na₂S prior to and after pre-administration of L-NMMA.

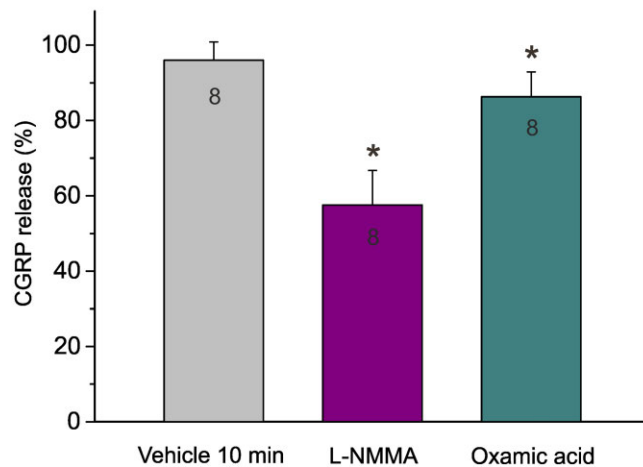
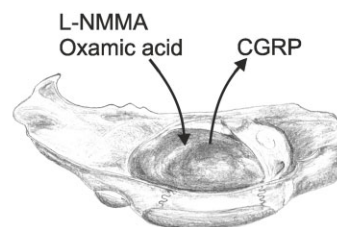


Figure 3

CGRP release from the dura mater in the hemisected rat head (inset) determined as the concentration of CGRP collected in the superfusate within 5 min after treatment with vehicle, L-NMMA (2 mM) or oxamic acid (2 mM) for 10 min, normalized to the first release value prior to treatment. * $P < 0.05$, significantly different from vehicle.

Jena, Germany) mounted on an inverted Axio Observer Z1. Two dry objective lenses (10× and 20× with numerical apertures of 0.3 and 0.8) were used. Fluorescent structures were observed in the light path mode using red and green filters. Confocal images were taken using filter settings for Cy3

(excitation 514 nm, emission 530–566 nm) and for FITC (excitation 488 nm, emission 505–520 nm). The number of image pixels was 1024 × 1024 or 512 × 512. Pictures were converted to a 12-bit RGB tiff file using confocal assistant software ZEN 2010. The final layout was made with Corel-Draw (Corel, Dublin, Ireland).

Data analysis

All values are expressed as means \pm SEM. Statistical analysis of the data was performed using Statistica 7 (StatSoft, Tulsa, OK, USA). The Student's *t*-test was used for group sizes of $n \geq 10$. The Wilcoxon matched pairs test was used for dependent measurements and the Mann–Whitney *U*-test for independent measurements of group sizes <10 . ANOVA was applied for analysing the time course of the flow increase after application of Na₂S. A probability level of $P < 0.05$ was regarded as statistically significant.

Materials

The NO donor DEANONOate purchased from Sigma (St. Louis, MO, USA) was prepared in 10 mM KOH. The H₂S source, Na₂S, was purchased from Sigma as the anhydrous form and stored in an atmosphere with <1 p.p.m. O₂ and H₂O. A stock solution of 100 mM was prepared using argon-bubbled nanopure water and stored in glass vials with PTFE septa at 4°C until use, for a maximum of 1 week. Adequate volumes taken from the vial with a small syringe were diluted in SIF immediately before use. The TRPA1 channel antagonist HC030031 (Tocris, Bristol, UK) was stored as a 10 mM stock solution in DMSO. The CGRP receptor antagonist CGRP₈₋₃₇ (Tocris), the NOS inhibitor L-N^G-monomethylarginine acetate (L-NMMA; Sigma) and oxamic acid (Sigma) were dissolved in saline, the sGC inhibitor 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ, Calbiochem, distributed by Merck, Schwalbach, Germany) as a 25 mM stock solution in ethanol. All stock solutions were diluted with SIF immediately before use, except for L-NMMA which was administered i.v., in saline.

Results

Role of NO in meningeal blood flow

Topical administration of the NO donor DEANONOate at a concentration of 200 μ M onto the exposed dura mater for 10 min caused an increase in meningeal blood flow in each experiment, on average by about 30% above baseline (Figure 1A and 1C), consistent with previous findings (Strecker *et al.*, 2002b). In addition, the increase in flow has already been shown to be reduced (albeit not totally abolished) by blockade of CGRP receptors (Strecker *et al.*, 2002a). This suggests that besides the classical NO-cGMP signalling pathway, a CGRP receptor-mediated arterial vasodilatation is involved in the blood flow elevating effect of DEANONOate. Because there is no mechanism known by which NO can directly activate CGRP receptors (Miranda *et al.*, 2003), we assumed that the NO derivative, HNO, may have been responsible for this effect (Eberhardt *et al.*, 2014). Knowing that HNO can be formed by the reaction of NO with H₂S, and the formation of H₂S is accelerated by endogenous CBS and CSE, we examined if inhibition of these enzymes by oxamic acid has an influence on the DEANONOate-evoked blood flow.

Pretreatment of the dura mater with oxamic acid (2 mM), a non-selective inhibitor of both CBS and CSE (Asimakopoulou *et al.*, 2013), reduced the blood flow increase evoked by DEANONOate (200 μ M) in a time-dependent

manner (Figure 1A and C). When oxamic acid (10 mg·kg⁻¹) was given i.v. a comparable inhibition of the increased blood flow was observed, though with a faster onset. The basal meningeal blood flow was not significantly changed by oxamic acid alone (5.2 \pm 2.0% increase after topical, $n = 14$; 4.5 \pm 2.9% increase after i.v. administration; $n = 12$).

The remaining blood flow increase induced by administration of DEANONOate was presumably due to the direct sGC activating effect of NO. However, in experiments with additional topical or systemic administration of the sGC inhibitor ODQ following i.v. oxamic acid treatment, the effect of DEANONOate was not significantly different compared with the DEANONOate effect after oxamic acid alone (Wilcoxon, $n = 8$, $P > 0.5$), although it tended to be further reduced (Figure 1A and C). The basal flow was not changed by adding ODQ after i.v. oxamic acid. Topical administration of oxamic acid or ODQ had no significant effect on the mean systemic blood pressure, which was 85.4 \pm 5.4 mmHg throughout the measurements.

Taken together, these results suggested that DEANONOate was fully effective in increasing meningeal blood flow only if there was endogenous production of H₂S, suggesting that the blood flow increase was due to the formation of HNO. In contrast, sGC activation linked to cGMP production seemed to play a minor role in the DEANONOate-induced effect in rat meningeal blood vessels. We therefore wanted to explore if supply of exogenous H₂S could further activate the synthesis of HNO from endogenously produced NO.

Role of H₂S in meningeal blood flow

Topical application of the H₂S source Na₂S (300 μ M) to the dura mater induced a rapid increase in meningeal blood flow, within a few minutes and outlasting the application period of 10 min (Figure 2A and B). The mean flow increase within the 10 min period (about 19%) is shown in Figure 2C. Repeated application of H₂S at intervals of 30 min after washout of the substance caused similar blood flow increases (data not shown).

Assuming that the effect of H₂S was mediated through the reduction of the endogenous NO to HNO, followed by HNO-induced activation of TRPA1 channels to release CGRP, which eventually caused the increased blood flow, as suggested by Eberhardt *et al.* (2014), we inhibited each step of this signal cascade. Pretreatment of the dura mater with the CGRP receptor antagonist CGRP₈₋₃₇ (100 μ M) or the TRPA1 channel antagonist HC030031 (50 μ M) abolished the blood flow increase following application of Na₂S (Figure 2A, B and C). CGRP₈₋₃₇ or HC030031 given alone had no significant effects on the basal meningeal blood flow.

The contribution of endogenous NO production in the Na₂S-induced blood flow was tested by topical and systemic administration of the non-specific NOS inhibitor L-NMMA (Figure 2C right side and 2D). Topical application of L-NMMA (1 mM) significantly decreased the basal meningeal flow by 21.2 \pm 4.4% (*t*-test, $n = 10$, $P < 0.01$) but had no effect on the arterial pressure. At the end of the 10 min application period of L-NMMA, the new baseline was stable, but the effect of topical Na₂S was markedly less (Figure 2C). Intravenous administration of L-NMMA (12 mg·kg⁻¹) increased the systemic blood pressure by 31.2 \pm 7.2% and the basal meningeal blood flow by 22.6 \pm 7.1% (*t*-test, $n = 10$, $P < 0.01$), which was

stable after some minutes. Under these conditions, the blood flow increase following topical Na_2S was abolished.

Role of NO and H_2S in CGRP release from the dura mater

In the hemisected rat head preparation with adhering dura mater *in vitro*, the basal release of CGRP in the incubation fluid (0.5 mL SIF), which was applied for 5 min at 37°C , was around $15 \text{ pg}\cdot\text{mL}^{-1}$ with small variations depending on the batch of the ELISA. After application of the non-specific NOS inhibitor L-NMMA (2 mM) twice for 5 min each, the CGRP release was about 50% of basal release, which is close to the detection level of the assay (Figure 3, see also Figure 4B). After application of the CBS/CSE inhibitor oxamic acid (2 mM), for two periods of 5 min each, the basal CGRP release was only slightly decreased, while vehicle applied in the same way had no significant effects (Figure 3).

Administration of Na_2S (0.1–1 mM) significantly increased CGRP release over mean baseline values of $15.0\text{--}17.8 \text{ pg}\cdot\text{mL}^{-1}$ (Figure 4A) but the Na_2S -induced increases in CGRP release were not significantly different between the concentrations (Mann–Whitney *U*-test, $P = 0.7$; Figure 4A). For the subsequent experiments, we used Na_2S at the intermediate concentration of $300 \mu\text{M}$.

To test if the release of CGRP by Na_2S depended on the presence of NO, we inhibited endogenous NO production by pre-treatment with the non-specific NOS inhibitor, L-NMMA (2 mM). After this pre-treatment, the CGRP release evoked by Na_2S ($300 \mu\text{M}$) was not different from baseline (Figure 4B). Conversely, to examine if the Na_2S -evoked CGRP release was influenced by a synergistic action of NO, Na_2S ($300 \mu\text{M}$) was co-administered with DEANOate ($200 \mu\text{M}$) and compared with the effect of Na_2S alone, applied to the contralateral skull half. Mean basal CGRP levels were $15\text{--}15.3 \text{ pg}\cdot\text{mL}^{-1}$ and Na_2S alone raised CGRP release to about double this level. Although DEANOate did not affect basal levels of CGRP release, it did potentiate the increase induced by Na_2S (Figure 4C).

Histochemical evidence for HNO-TRPA1-CGRP signalling in the dura mater

In the whole mount preparations of the dura mater, a dense network of CGRP-immunofluorescent nerve fibres was observed surrounding the MMA and its branches, indicating that CGRP can be released close to the arterial vessel wall (Figure 5A). We indirectly visualized different sources for NO; NADPH-diaphorase, a specific marker for NO-producing cells, was localized by its activity mostly in the wall of the MMA and arterioles up to the transition into capillaries and at a low intensity in some nerve fibre bundles accompanying the artery (Figure 5B). Some nerve fibres immunoreactive for the neuronal subtype of NOS were identified accompanying branches of the MMA (Figure 5C). TRPA1 channel immunoreactivity was detected in perivascular nerves of the dura mater staining several axons in a patchy manner (Figure 5D).

The sensors for NO and H_2S stained the endothelium of the MMA and its branches but not venous vessels or other structures of the dura mater (Figure 5E and F). This may be due to the low sensitivity of the used sensors coupled with

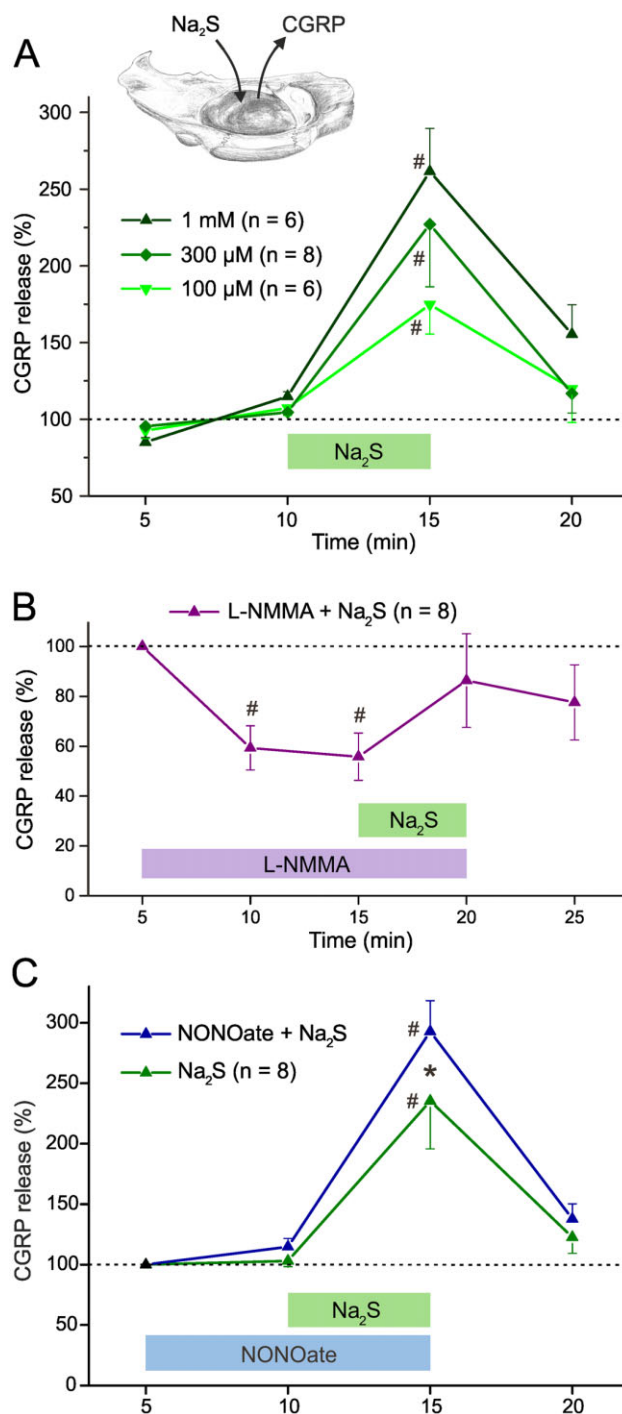


Figure 4

CGRP release from the dura mater, measured as the concentration of CGRP collected in the superfusate of the hemisected head (inset) at periods of 5 min. # $P < 0.05$, significantly different from baseline at 5–10 or 5 min. (A) Effect of different concentrations of Na_2S applied for 5 min. (B) CGRP release decreased during administration of L-NMMA (2 mM). Na_2S ($300 \mu\text{M}$) after L-NMMA administration did not increase CGRP release above baseline. (C) Application of NONOate ($200 \mu\text{M}$) tends to increase CGRP release (at 10 min) while co-administration of Na_2S ($300 \mu\text{M}$) causes a synergistic effect significantly different from Na_2S alone. * $P < 0.05$, significant effect of NONOate.

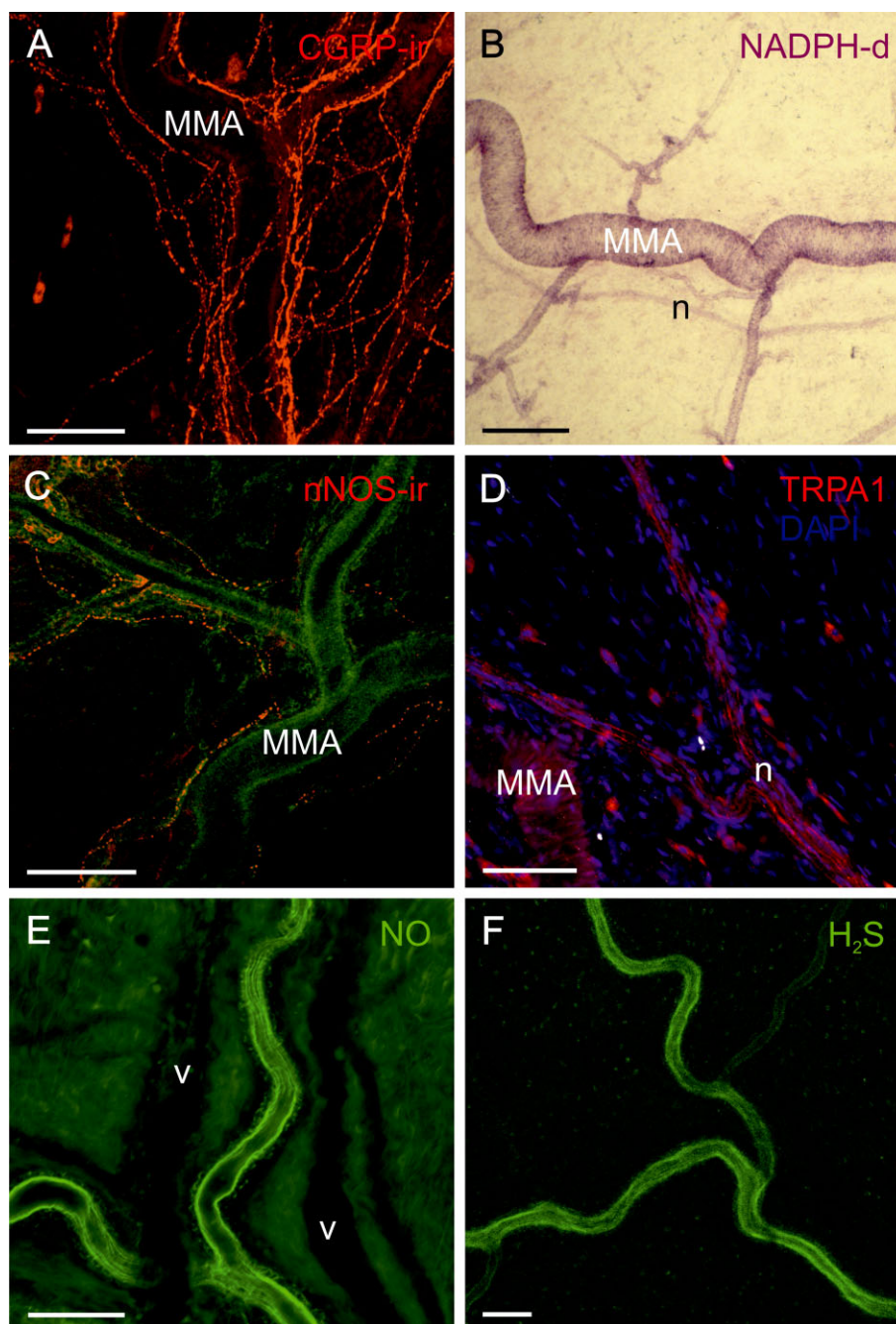


Figure 5

Histochemical demonstration of components of HNO-CGRP signalling in the dura mater; size bars 100 μm . (A) Confocal immunofluorescence image of CGRP-immunoreactive nerve fibres densely surrounding the MMA and its branches. (B) Transmission light microscopic image showing NADPH-diaphorase staining of the MMA and some arterioles up to the transition into capillaries, indicative for the production of NO. An adjacent nerve bundle (n) is weakly stained. (C) Confocal image of nerve fibres immunoreactive for nNOS surrounding the MMA, which is visible in the green background channel. (D) Confocal image showing TRPA1-immunoreactive nerve fibres in a nerve bundle (n) adjacent to the MMA. (E) Confocal image showing arterial endothelium of the MMA stained by DAF indicating NO. Two unstained accompanying veins are visible in the background as dark shadows. (F) Confocal image showing endothelial staining of the MMA by WSP1 demonstrating the presence of H_2S .

relatively low steady-state levels of both NO and H_2S in the neuronal cells. HNO staining was visible in the arterial vessel walls and particularly in nerve fibre bundles adjacent to the arterial vessels (Figure 6A). Pretreatment of the dura mater

with oxamic acid significantly reduced the fluorescence after incubation with the HNO sensor (Figure 6B). Pretreatment with L-NMMA or a combination of oxamic acid and L-NMMA abolished the staining (Figure 6C and D).

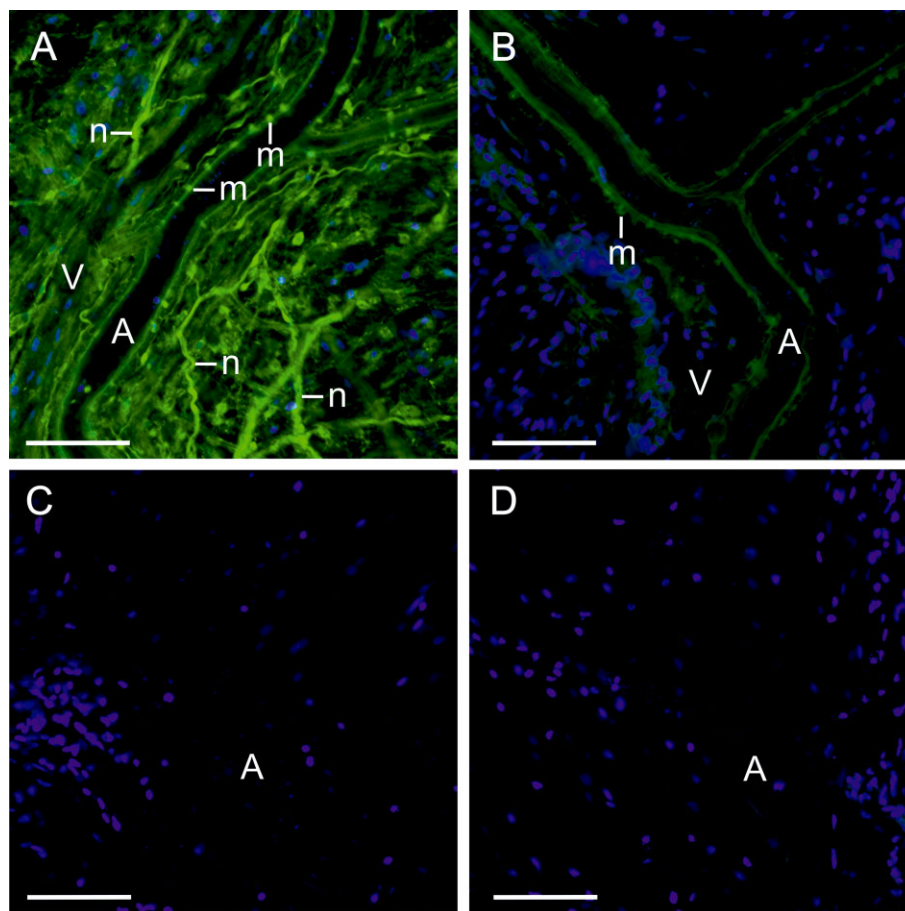


Figure 6

Confocal images of dura mater stained by CuBOT1 indicative of HNO (green colour). Branches of the MMA (A) are accompanied by a venous vessel (V) and bundles of nerve fibres (n). Nuclei are stained with DAPI (blue); size bars 100 μm . (A) Control dura. Nerve bundles and smooth muscle cells (m) of the artery as well as other perivascular cells appear HNO positive. (B) The HNO staining was significantly reduced after pretreatment with oxamic for 45 min. After pretreatment with L-NMMA (C) or the combination of oxamic acid and L-NMMA (D), the staining was lost indicative of blocked HNO production.

In addition, we assessed the intensity of fluorescence in the supernatant after incubating the dura with the NO sensor or H₂S sensor as a measure of NO and H₂S generation from this tissue. Following pretreatment with oxamic acid, the fluorescence of the supernatant created by the NO fluorescence sensor was increased by $51.4 \pm 9.2\%$ compared with the contralateral control dura, indicating that less NO was consumed in the tissue when the H₂S production was inhibited. Similarly, after pretreatment with L-NMMA, the fluorescence of the H₂S sensor in the supernatant was increased by $32.9 \pm 10.8\%$ compared with the control side indicative of less H₂S consumption in the dura upon inhibition of the NO synthesis.

Discussion and conclusions

CGRP release upon formation of HNO inducing meningeal blood flow

The present results reveal an important role for NO and H₂S crosstalk in controlling the release of CGRP from afferent

endings, which is involved in the regulation of meningeal blood flow. We have recently described the cellular mechanism underlying the interaction of these two gasotransmitters (Filipovic *et al.*, 2012; 2013; Miljkovic *et al.*, 2013; Eberhardt *et al.*, 2014). NO and H₂S generate HNO, which is an activator of TRPA1 cation channels by modifying cysteine residues of the receptor protein to form disulfide bonds, which opens the receptor channels and the consequent influx of Ca²⁺ activates the exocytosis of CGRP (Eberhardt *et al.*, 2014). In the histochemical preparations shown here (Figures 5 and 6), we localized endothelial and neuronal sources for NO in the dura mater, while H₂S production was predominantly visible in arterial endothelium. CSE, CBS and MST are the key enzymes of H₂S production. CSE has been found in endothelium and tunica media cells of extra- and intracranial arteries (Chertok and Kotsyuba, 2012) and CBS in the brain (Kimura, 2002). In addition, our previous results showed that CBS immunoreactivity in small trigeminal neurons partly co-localizes with TRPA1 channel immunoreactivity (Eberhardt *et al.*, 2014). The clearly demonstrated co-dependence of these two gasotransmitters is reflected in

the data presented here showing that higher amounts of NO leave the dura mater when H₂S production is inhibited and that higher concentrations of H₂S can be detected when NO production is inhibited. This confirms that these two gas-transmitters react to form HNO, which has been previously shown to increase intracellular Ca²⁺ levels by opening TRPA1 channels in primary afferent neurons and to thus activate CGRP exocytosis (Eberhardt *et al.*, 2014). Indeed, for the first time, the ongoing basal HNO production could also be visualized in a tissue using the HNO sensitive dye CuBOT1. The strong staining, which is due to the high sensitivity of the HNO sensor, is clearly reduced after treatment of the dura mater with L-NMMA or oxamic acid, which inhibit the production of NO or H₂S respectively (see Figure 6). In the dura mater, the close apposition of HNO sources and meningeal arteries surrounded by CGRP expressing trigeminal afferents provide the morphological substrate for this putative HNO-TRPA1-CGRP signalling path, which was substantiated by our observation that blockade of either TRPA1 channels or CGRP receptors abolished the blood flow increasing effect of Na₂S (Figure 2).

Our experimental results indicate that the modest basal release of CGRP in the dura mater measured in the hemi-sectioned skull preparation is partly the result of constitutive HNO production, as both inhibition of NO production by L-NMMA and inhibition of the H₂S synthesis by oxamic acid, reduced the (unstimulated) basal level of CGRP release (Figures 3 and 4B). Application of additional Na₂S, i.e., exogenous H₂S, induced CGRP release from the dura mater, which was strongly dependent on the NO/HNO production, as it was abolished by the NOS inhibitor L-NMMA. Conversely, providing additional NO through the NO donor DEANONOate further increased the Na₂S-induced CGRP release.

The amount of CGRP released under physiological conditions is probably below the level needed to maintain vasodilatation, because application of the CGRP receptor antagonist CGRP₈₋₃₇ did not change the basal meningeal blood flow. On the other hand, the flow increase induced by H₂S in the presence of endogenous NO was fully antagonized by CGRP₈₋₃₇, suggesting that HNO formed by H₂S and NO exerts its vasodilator effects largely through CGRP release. Furthermore, the CGRP release evoked by HNO depends on the activation of TRPA1 channels, because the H₂S-induced flow increase was abolished by inhibition of these channels with HC030031 (Figure 2B).

The proposed pathway of NO-TRPA1-CGRP signalling in the dura mater with the experimental interventions is summarized in Figure 7. We assume that under natural conditions, most of the NO is produced in the endothelial cells of meningeal arteries by endothelial NOS. NO diffuses into perivascular trigeminal nerve fibres and is converted to HNO in the presence of H₂S, which is formed by CBS, an enzyme that co-localizes with TRPA1 channels, HNO then activates TRPA1 channels inducing Ca²⁺ influx and consequent CGRP release. CGRP activates CGRP receptors of smooth arterial muscle cells causing vasorelaxation through up-regulation of cyclic monophosphates followed by increased perfusion of the meninges. This fits nicely with the data presented here showing that H₂S-induced increase of meningeal blood flow was inhibited by TRPA1 channel and CGRP receptor antagonists, as well as by the NOS inhibitors (Figures 1 and 2).

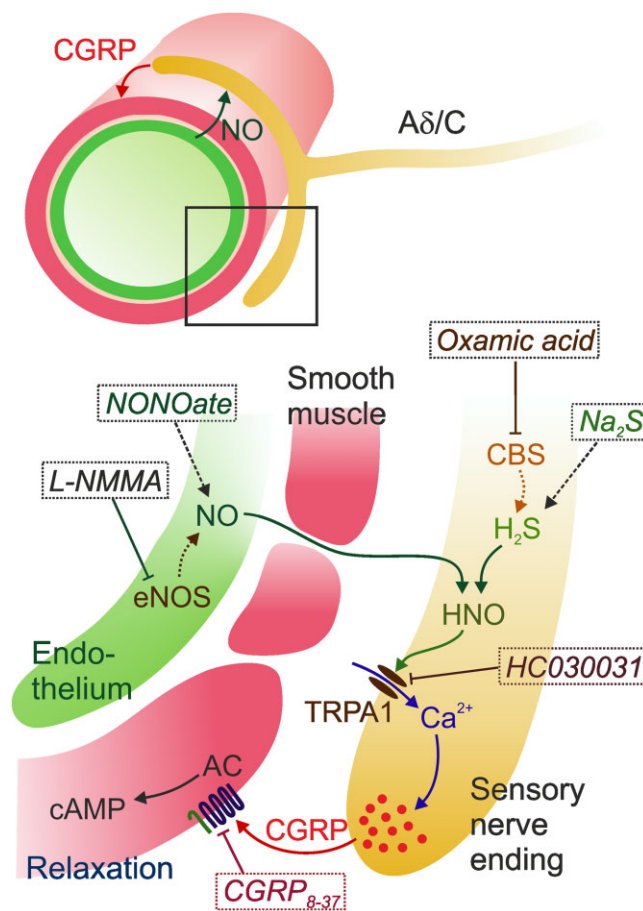


Figure 7

Scheme illustrating the proposed HNO-TRPA1-CGRP signalling pathway at meningeal arteries innervated by trigeminal afferent nerve fibres (Aδ/C). NO mainly produced by the endothelial NOS (eNOS) diffuses into perivascular nerve fibres, where HNO is formed together with H₂S produced in the presence of neuronal CBS. HNO activates TRPA1 channels leading to Ca²⁺-induced CGRP release. CGRP activates its receptors in arterial smooth muscle cells causing vasodilatation upon up-regulation of the AC-cAMP and other pathways. Experimentally used NO and H₂S donors (NONOate, Na₂S) and blockers of the NO and H₂S generation (L-NMMA, oxamic acid), as well as blockers of TRPA1 channels or CGRP receptors (HC030031 and CGRP₈₋₃₇) are indicated in italics.

Other factors mediating NO-induced blood flow increase

Following inhibition of the endogenous H₂S production by oxamic acid and thereby of HNO, DEANONOate still induced an increase in meningeal blood flow by about 10% above baseline (Figure 1A and C). This component was obviously not mediated by CGRP but may have resulted partly from the direct cGMP regulating effect of NO. Indeed, some further reduction of the blood flow increase was seen after blockade of cGMP production with ODQ. Nevertheless, a 6–8% increase in blood flow was maintained. The maintained increase in flow could have been due to a low, persistent production of H₂S. Activation of K_{ATP} channels by H₂S medi-

ating relaxation of smooth muscle cells has previously been shown (Mustafa *et al.*, 2011; Eberhardt *et al.*, 2014). Furthermore, Na₂S relaxed isolated aortic rings by maintaining a tonic inhibitory effect on the phosphodiesterase type 5 in endothelial cells, thereby inhibiting the degradation of cGMP and permitting physiological cGMP signalling (Coletta *et al.*, 2012). However, this mechanism of vasorelaxation seems to play a minor role in the meningeal vasodilatation, as blocking the TRPA1-CGRP pathway almost completely abolished the blood flow increase induced by Na₂S and inhibition of NO production significantly reduced the effect. This differs from recently examined functions of HNO in the heart, where Angeli's salt increased coronary blood flow and left ventricular inotropy (Chin *et al.*, 2014). Scavenging of HNO and inhibition of sGC but not of CGRP receptors reduced these effects.

Role of CGRP in meningeal nociception and headache

According to vascular and neuroinflammatory theories of migraine, the dilatation of meningeal arteries has been considered to account for the generation of headaches (Moskowitz *et al.*, 1989). Neurogenic vasodilatation in the dura mater induced by the release of potent vasodilators like CGRP is still considered as an important process in the pathophysiology of primary headaches such as migraine (Geppetti *et al.*, 2012). Although these hypotheses have widely been modified and amended by central migraine theories (May and Goadsby, 1999), it is evident that nociceptive events in the trigeminovascular system causing the sensation of headaches are linked to vascular processes (Olesen *et al.*, 2009). The concept of the neurovascular generation of headaches results from the observation that infusion of vasodilatory mediators can induce headaches which resemble spontaneously occurring primary headaches, such as migraine (Olesen, 2008). Most intriguingly, infusion of CGRP induced acute and delayed migraine-like headaches in migraineurs (Lassen *et al.*, 2002), an effect which was prevented by the CGRP receptor antagonist olcegepant, concomitant with an inhibition of the dilatation of extracranial arteries (Petersen *et al.*, 2005). Contrariwise, the infusion of agents such as vasoactive intestinal polypeptide (Rahmann *et al.*, 2008) and adrenomedullin (Petersen *et al.*, 2009), which dilate blood vessels but do not induce headaches, has been used to argue against vascular migraine theories (Panconesi *et al.*, 2009).

Finally, we have to state that currently no theory based on acute peripheral CGRP effects in migraine generation is satisfying for the following reasons. Nociceptive afferents in the meninges, the activation of which is regarded as the primary source of headache generation, are neither activated directly by CGRP nor by vasodilatation induced by CGRP (Levy *et al.*, 2005; Bhatt *et al.*, 2015). Injection of CGRP or the CGRP receptor antagonist olcegepant into the ganglion did not change the activity of spinal trigeminal neurons with meningeal afferent input (Covasala *et al.*, 2012). In this respect, acute CGRP release and vasodilatation during experimental activation of meningeal afferents (Zagami *et al.*, 1990) or during attacks of primary headaches (Edvinsson and Goadsby, 1995) may rather be consequences than causes of

meningeal afferent activation. On the other hand, the application of CGRP antagonists or antibodies seems to be very efficient in preventing migraines (Edvinsson, 2001; Elvidge, 2014; Bigal *et al.*, 2015). It appears possible that long-term effects of CGRP can trigger trigeminal sensitization and the generation of primary headaches. CGRP receptors have immunohistochemically been located to a major proportion of trigeminal ganglion neurons and satellite cells, noticeably not co-localized with CGRP-immunoreactive neurons (Lennerz *et al.*, 2005; Eftekhari *et al.*, 2010). In trigeminal ganglion cell cultures, CGRP has been shown to up-regulate the expression of iNOS and NO production in glial cells (Li *et al.*, 2008), involving MAPK pathways (Vause and Durham, 2009). NO donors in turn increased CGRP promoter activity and CGRP release, which surprisingly was not dependent on the cGMP signalling pathway (Bellamy *et al.*, 2006), possibly indicating a vicious cycle of CGRP and NO production in the trigeminal ganglion. The mechanism of CGRP release may be the same as in the dura mater, namely conversion of NO to HNO catalysed by CBS in the presence of H₂S followed by HNO-induced TRPA1 channel activation and Ca²⁺ influx. Apart from CGRP release, Ca²⁺-activated gene expression could thus increase neuronal sensitivity.

Possible role of HNO-TRPA1-CGRP signalling in meningeal nociception and headache

Activation of TRPA1 channels expressed in nociceptive afferents is likely not only to release CGRP but also to contribute to the activation of the nociceptive system, as shown by recent patch-clamp recordings of rat trigeminal afferent projection to the dura mater and by behavioural studies (Edelmayer *et al.*, 2012). Regarding meningeal afferents, we have currently no final results but there is evidence that TRPA1 channel agonists cause at least transient activation in rodents. Activation of TRPA1 channels of the trigeminal afferents innervating the nasal mucosa is considered as a pathophysiological mechanism involved in the generation of headache induced by inhaled irritants (Kunkler *et al.*, 2011). TRPA1 channels are present in 36% of trigeminal neurons and they are frequently co-localized with CGRP (Story *et al.*, 2003; Huang *et al.*, 2012). Thus, the described activation of TRPA1 channels by HNO as a result of the interaction of the two gasotransmitters NO and H₂S may be highly relevant as a headache-provoking mechanism. Indeed, increased plasma levels not only of CGRP but also of NO metabolites have been found during migraine attacks (Sarchielli *et al.*, 2000). Furthermore, some researchers have described higher plasma levels of homocysteine in patients suffering from migraine with aura, which could be indicative of an increased metabolism of H₂S for HNO formation (Moschiano *et al.*, 2008). It would be worthwhile to look further for metabolites of the NO-H₂S-HNO pathway in primary headache patients.

These putative processes are probably not restricted to the periphery but are present also in the spinal trigeminal nucleus, where CGRP release from the central terminals of sensory neurons seems to facilitate glutamatergic neurotransmission (Storer *et al.*, 2004) and the activation and/or sensitization of the nociceptive pathway, a matter which is currently under investigation in our laboratory. In the spinal system, the contribution to central sensitization of CGRP has long

been shown in a variety of animal experiments (Sun *et al.*, 2009). The release of CGRP and other neurotransmitters from presynaptic neuronal elements is likely to be controlled by HNO. Nitroglycerin is able to activate and sensitize spinal trigeminal neurons (Lambert *et al.*, 2000; Jones *et al.*, 2001), and this could be reversed and prevented by inhibition of CGRP receptors (Koulchitsky *et al.*, 2009; Feistel *et al.*, 2013), which is probably, at least in part, a central effect. We do not yet know to which extent the central CGRP release depends on the proposed HNO-TRPA1 signalling but all elements of this pathway, NOS, CBS, TRPA1 channels, CGRP and its receptor, are present in the spinal trigeminal nucleus (Lennerz *et al.*, 2005; Schlechtweg *et al.*, 2009; Eberhardt *et al.*, 2014). It is tempting to speculate that CBS-produced H₂S modifies nitroglycerin to form HNO and activates the TRPA1-CGRP process, thus stimulating a migraine attack.

Therapeutic considerations

Consistent with above-mentioned findings in animal experiments, drugs that block the release (triptans) or the receptor binding of CGRP (antagonists, antibodies) are all therapeutic in migraine and partly in other primary headaches (see Bigal *et al.*, 2013). However, there is a great deal of scientific discussion around the question, on which level of the trigeminal system these inhibitory effects take place. In particular, the newly approved CGRP antibodies and also triptans and CGRP receptor antagonists will not readily pass the blood brain barrier, so that a peripheral action (i.e. in the meninges or the trigeminal ganglion) appears likely. Because the HNO-TRPA1-CGRP signalling may take place on all levels of the trigeminal system, the trigeminal ganglion seems to be the most likely site of interaction. In line with the discussed signal cascade, inhibition of NO generation by L-NMMA has also proven to be therapeutic in migraine, while specific inhibitors of iNOS were ineffective (see Olesen, 2008; Hoffmann and Goadsby, 2012). The role of H₂S production has never been addressed before in the context of migraine, so this might be the missing link between NO, CGRP and migraine attacks. Regarding possible therapeutic options by targeting the generation of H₂S and HNO, further preclinical investigations are necessary to elucidate the role of the HNO-TRPA1-CGRP signalling cascade in meningeal nociception and headache generation.

Acknowledgements

We like to thank Jana Schramm, Annette Kuhn and Susanne Haux-Oertel for excellent technical assistance. The project was supported by the Friedrich-Alexander-University Erlangen-Nürnberg (Emerging Fields Project: Medicinal Redox Inorganic Chemistry), the Humboldt Foundation (travel grant to M. D.) and the FP7 project EUROHEADPAIN (Grant Agreement No. 602633). This paper originated from the 8th International Symposia on the CGRP Family; CGRP, Adrenomedullin, Amylin, Intermedin and Calcitonin.

Author contributions

M. D., C. W., B. V., M. R. F. and K. M. performed the research. M. R. F. and K. M. designed the research study. M. D., C. W. and B. V. analysed the data. M. D., M. R. F. and K. M. wrote the paper.

Conflict of interest

The authors declare no conflicts of interest.

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