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Hydrogen sulfide determines HNO-induced stimulation of trigeminal afferents

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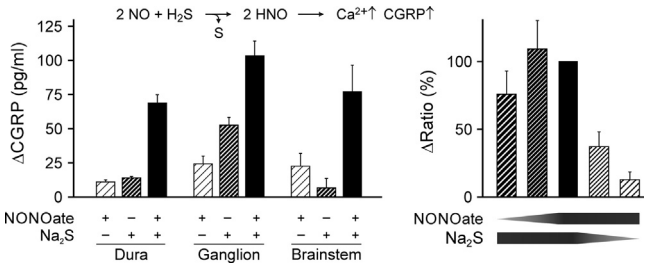
HIGHLIGHTS

- NO donors and sulfide combined act synergistic compared to either substance.
- This combined stimulation activates all parts of the primary trigeminal afferents.
- Sulfide rather than NO is the rate-limiting factor for activation.

GRAPHICAL ABSTRACT



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ABSTRACT

Endogenous NO and hydrogen sulfide form HNO, which causes CGRP release via TRPA1 channel activation in sensory nerves. In the present study, stimulation of intact trigeminal afferent neuron preparations with NO donors, Na_2S or both was analyzed by measuring CGRP release as an index of mass activation. Combined stimulation was able to activate all parts of the trigeminal system and acted synergistic compared to stimulation with both substances alone. To investigate the contribution of both substances, we varied their ratio and tracked intracellular calcium in isolated neurons. Our results demonstrate that hydrogen sulfide is the rate-limiting factor for HNO formation. CGRP has a key role in migraine pathophysiology and HNO formation at all sites of the trigeminal system should be considered for this novel means of activation.

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1. Introduction

1.1. HNO-TRPA1-CGRP

Nitroxyl (HNO) is a redox sibling of nitric oxide (NO) with cardioprotective effects by inducing CGRP release [1]. We have recently unraveled the underlying signaling pathway, HNO activates TRPA1 in sensory neurons and in turn releases CGRP [2]. The activation of expressed human TRPA1 was demonstrated by Angeli's salt which decomposes to nitrite and HNO, specificity was validated by the TRPA1 receptor antagonist HC030031. The activation by HNO can be clearly distinguished from NO given that the NO donor DEA-NONOate had no effect. In the trigeminal system, Angeli's salt increased CGRP release from rat and mouse dura mater but not in $TRPA1^{-/-}$ mice. Taken together, this confirms that TRPA1 activation is responsible for HNO-induced CGRP release.

The combined application of NO and H_2S caused calcium increase in DRG neurons, while both substances were inefficient when applied separately. Co-localization of neuronal NO syn-

Abbreviations: CGRP, calcitonin gene-related peptide; HNO, nitroxyl; NO, nitric oxide; NOS, nitric oxide synthase; SIF, synthetic interstitial fluid; STN, spinal trigeminal nucleus; TRPA1, transient receptor potential cation channel, subfamily A, member 1.

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thase (NOS) and cystathionine-β-synthase with TRPA1 supports the hypothesis [2,3].

Summarizing, data suggest an in vivo formation of HNO out of endogenous NO and H_2S, which activates neuronal TRPA1 channels causing CGRP release from sensory nerve fibers and endings.

1.2. Importance of CGRP

Calcitonin gene related peptide (CGRP) has a principle role in migraine pathophysiology. CGRP can induce delayed migraine attacks [4,5] and CGRP-receptor antagonists demonstrated efficacy in the acute treatment of migraine [6,7]. CGRP is abundant in trigeminal ganglion neurons [8], as well as in their peripheral [9] and central projections [10]. The neuropeptide is released upon activation of trigeminal afferents, and its effects depend on the localization of the corresponding CGRP receptors. Peripherally, CGRP receptors are located at vascular smooth muscles and mast cells in the meninges [11,12], where activation causes arterial vasodilatation and mild mast cell degranulation [13–15] but not excitation or sensitization of trigeminal afferents [16]. In the trigeminal ganglion, CGRP acts on neurons and satellite glial cells [17], which was shown to be followed by increased CGRP and NO release [18,19]. Centrally, CGRP receptors are located at primary afferent endings in the spinal trigeminal nucleus (STN) [20]. Evidence is mounting for an action at this site where CGRP seems to amplify the glutamatergic transmission [20–22] and experiments with the CGRP receptor antagonist olcegepant indicated that the site of action is located centrally of the trigeminal ganglion [19,23,24].

Due to the relevance of CGRP in migraine pathophysiology, we set out to probe the HNO-TRPA1-CGRP pathway throughout the trigeminal system. As previously demonstrated by our group, NO and Na_2S react to generate HNO [2]. We demonstrate that the latter is a potent agonist and causes more activation than the sum of Na_2S or an NO donor when applied separately. As index for activation we quantified the release of CGRP [25–27], which demonstrated activation at the dura mater, the trigeminal ganglion and the spinal trigeminal nucleus. Combined stimulation acted synergistic compared to stimulation with both substances alone, substantiating the essential generation of HNO for activation. Our previous experiments supplied both substances at a 1:1 molar ratio [2]. The current study varied this relation and demonstrates that hydrogen sulfide is the limiting factor.

2. Material and methods

2.1. Animals

Experiments were performed according to regulations and approved by the Animal Care Authority of the local district government (Ansbach, Germany).

2.2. CGRP release

As previously described, for brainstem slices Wistar rats of both sexes with a weight of 90–155 g were decapitated under sevofluorane inhalation anesthesia [27]. After dissection of the medullary brainstem, eight serial transverse slices (600 μm) were cut on a vibrating blade microtome in ice-cold synthetic interstitial fluid (SIF) [28]. Slices covering the range from cervical C2 segments to the rostral medullary brainstem were transferred into flat-bottom 6.5 mm diameter wells and placed in a 37 °C bath for 30 min. Trigeminal ganglia and hemisected sculls of rats were prepared as described earlier [25,26]. Brainstem slices and trigeminal ganglia were covered with 125 μl, hemisected sculls with 500 μl of carbogen-saturated SIF. Every 5 min 100 μl were removed to determine CGRP release, using a human CGRP enzyme immune assay (SPIbio, France) [29]. Both Na_2S and DEA-NONOate are not detected by the assay but affect CGRP determination in a concentration-dependent fashion, which was quantified by addition to SIF spiked with 100 pg/ml CGRP. A correction factor of 1.37 for Na_2S 300 μM and 1.51 for Dea-NONOate 200 μM was applied. *N* indicates the number of brainstem slices, all groups contain at least three independent animals and days.

2.3. Calcium imaging of trigeminal neurons

Trigeminal ganglia of C57Bl/6 were excised, exposed to collagenase and protease before dissociation, plated on poly-D-lysine-coated coverslips and cultured in serum-free neuronal medium with 100 ng/ml mouse NGF. After one day incubation at 37 °C and 5% CO_2, cells were stained by 3 μM Fura-2-AM and 0.02% pluronic. Coverslips were mounted on an inverse microscope and constantly superfused with extracellular fluid (in mM: NaCl 145, KCl 5, $CaCl_2$ 1.25, $MgCl_2$ 1, Glucose 10, Hepes 10) by a gravity-driven common-outlet superfusion [30]. Na_2S and DEA-NONOate (2 times the final concentration) were applied from different lines running at the same flow rate; this ensured mixing within the common outlet about one second before the solution reaches the cells. Fluorescence evoked by illumination at 340 nm and 380 nm was acquired by a CCD camera. After background subtraction the 340/380 nm ratio time series was calculated for individual cells, the mean ± SEM was visualized. Experimental groups were tested on at least two independent days.

2.4. Materials

Na_2S was dissolved as a 100 mM stock solution using argon-bubbled pure water and stored in glass vials at 4 °C until use for a maximum of one week. DEA-NONOate was dissolved at 10 mM in 15 mM potassium hydroxide. Both substances were diluted in SIF before the experiment. For 1:1 molar ratio between hydrogen sulfide and NO, it has to be considered that 2 molecules of DEA-NONOate release 3 molecules of NO. L-NMMA, oxamic acid and carvacrol were obtained from Sigma–Aldrich.

2.5. Statistical analysis

Directly adjacent brainstem slices were allocated into different experimental groups. Subsequent measurements and adjacent slices were compared with the Wilcoxon matched pairs test or with Student's *t*-test for $n \geq 10$. Analysis of variance and the HSD post-hoc test was used to evaluate multiple groups. Data are presented as mean ± SEM; $p < 0.05$ was considered significant.

3. Results

In hemisected scull preparations, DEA-NONOate 200 μM increased CGRP release by 11 ± 2 pg/ml compared to the control before (+72%, $p = 0.018$, $n = 8$, Fig. 1A), Na_2S 300 μM for 5 min increased CGRP release by 14 ± 1 pg/ml (+65%, $p = 0.028$, $n = 6$). The combined application of Na_2S and DEA-NONOate increased CGRP release by 69 ± 6 pg/ml, which is more than the sum of the effects of both substances alone (+190%, $p < 0.001$). Two subsequent 5 min stimulations with either of the substances alone showed substantial tachyphylaxis ($p \leq 0.027$, $n = 8$ each).

In trigeminal ganglia, DEA-NONOate 200 μM increased CGRP release by 24 ± 6 pg/ml (+103%, $p = 0.018$, $n = 8$, Fig. 1B), Na_2S 300 μM for 5 min increased CGRP release by 53 ± 6 pg/ml (+260%, $p = 0.018$, $n = 8$). The combined application of Na_2S and DEA-NONOate increased CGRP release by 103 ± 11 pg/ml, which is more

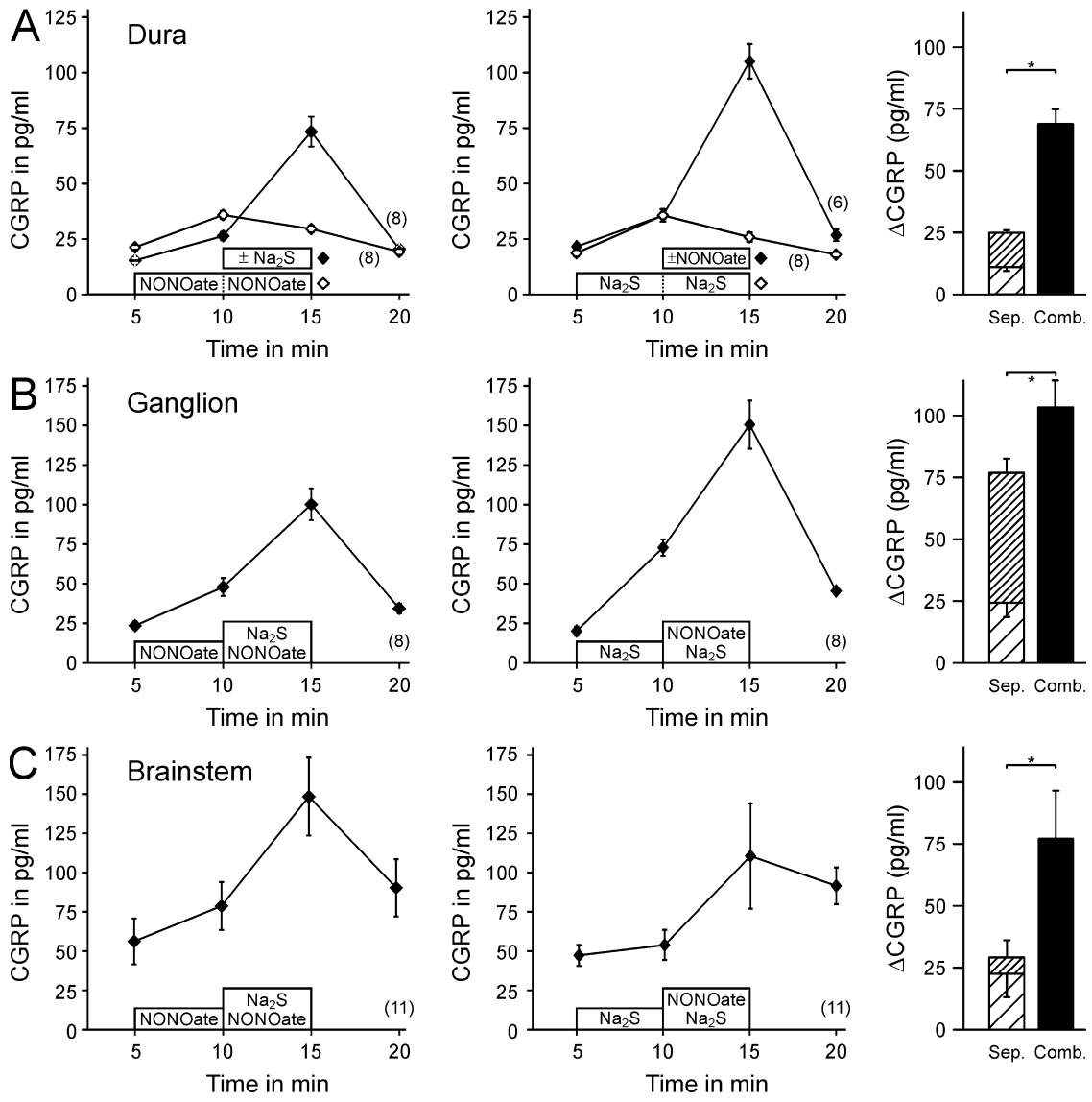

Fig. 1. Stimulated calcitonin gene-related peptide (CGRP) release in the trigeminal system. An identical protocol was applied for the dura mater (A), the trigeminal ganglion (B) and brainstem slices (C) of Wistar rats. After 5 min of baseline either DEA-NONOate (200 μM, NONOate, left panels) or sodium sulfide (Na_2S 300 μM, center panels) were applied before stimulation with the combination of both substances. This combined stimulation (Comb.) increased CGRP release more than the separate application (Sep., DEA-NONOate sparsely hatched and Na_2S densely hatched, right panels). Application of both substances at the dura for 10 min resulted in a substantial tachyphylaxis (open symbols, $n = 8$ each). Note that two molecules of DEA-NONOate release 3 molecules of NO. The number of preparations is given in brackets, data are mean ± SEM, $^*p < 0.05$.

than the sum of the effects of both substances alone (+462%, $p = 0.032$).

In rat brainstem slices, CGRP release in the presence of DEA-NONOate 200 μM was 79 ± 24 pg/ml compared to 56 ± 17 in the control before (+40%, $p = 0.28$, $n = 11$, Fig. 1C). In the presence of Na_2S 300 μM, the release was 54 ± 16 compared to 47 ± 14 in the control before (+14%, $p = 0.58$, $n = 12$). The combined application of Na_2S and DEA-NONOate increased CGRP release by 77 ± 20 pg/ml, which is more than the non-significant differences of both substances alone (+150%, $p = 0.003$ and 0.021).

Trigeminal neurons loaded with Fura-2 to track intracellular calcium were exposed to increasing concentrations of DEA-NONOate, Na_2S or both substances. In carvacrol-responsive cells, both substances alone did not elicit any effect, the combination increased intracellular calcium (Na_2S 1 μM + DEA-NONOate 0.67 μM: $p = 0.007$, Na_2S 10 μM + DEA-NONOate 6.7 μM: $p < 0.001$, Fig. 2A). A subsequent 10-fold higher application resulted in substantial or complete desensitization. Cells responding to the combined application of Na_2S 10 μM and DEA-NONOate 6.6 μM had a diameter of 18.7 ± 0.6 μM and were a subset (37%) of the carvacrol-responsive neurons (19.5 ± 0.3 μM). Both these populations were smaller than the average diameter of trigeminal neurons (23.8 ± 0.6 μM, $p < 0.001$ each).

In order to investigate the individual contribution of Na_2S and DEA-NONOate, both substances were applied continuously at 40 μM and 26.7 μM, the other substance was added at 10%, 31% and 100% of this concentration (considering 3 NO from 2 DEA-NONOate, Fig. 2B). The calcium response depends more on Na_2S compared to DEA-NONOate (10%: $p < 0.001$, 31%: $p = 0.001$). A similar experiment in the presence of oxamic acid 100 μM (pretreatment for 90 min) and L-NMMA 50 μM showed an identical response, which makes a contribution of endogenous NO and H_2S production unlikely (data not shown). Finally, due to the rapid breakdown of DEA-NONOate in aqueous solution, a sequential application by directly pipetting from the alkaline stock solution into the dish was performed. Note that with this interval of 20 s between application of DEA-NONOate 0.67 μM and Na2S 1 μM a substantially larger response was observed (Fig. 2C).

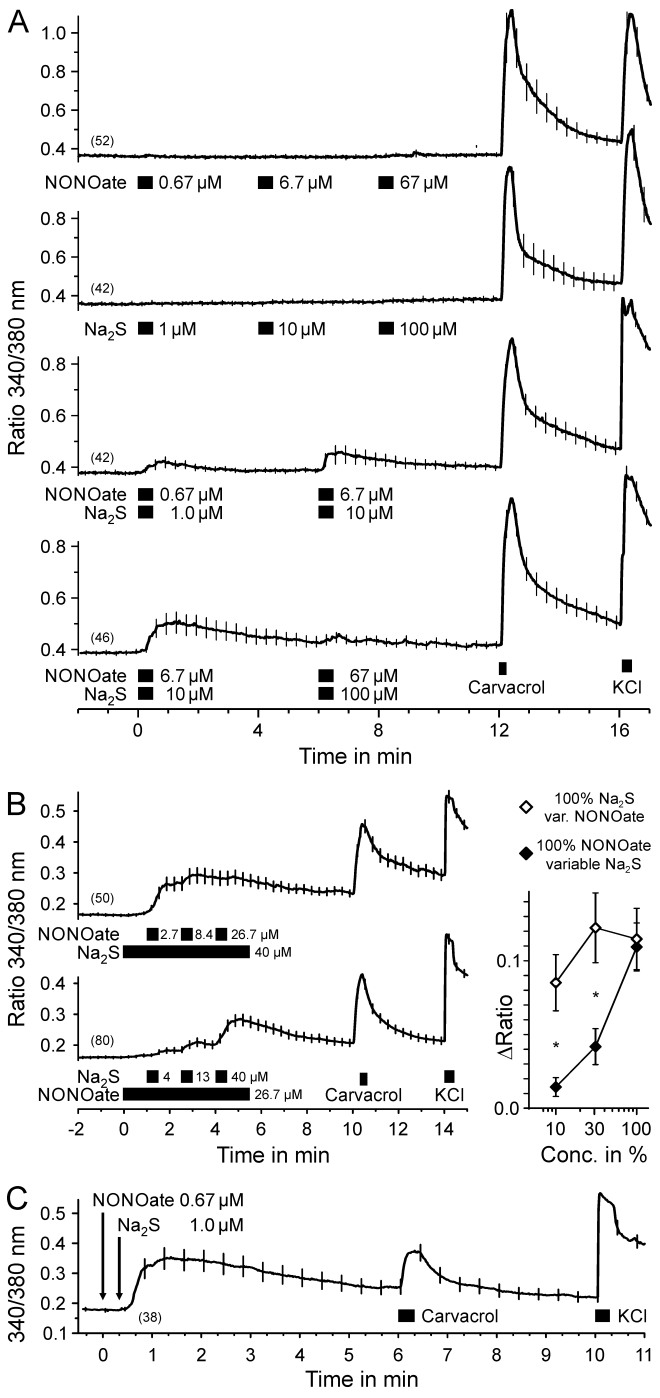

Fig. 2. Activation of mouse trigeminal ganglion neurons by sodium sulfide and an NO donor. (A) When applied alone, neither Na_2S nor DEA-NONOate (NONOate) in a concentration of up to 100 µM caused an increase in calcium in cells which respond to TRPA1 agonist carvacrol. When applied from separate tubes, the combination of both substances activates these neurons. The response shows substantial desensitization, also when comparing 10 µM each as second to 10 µM each as first application. Potassium chloride 60 mM (KCl) serves as reference. (B) Under continuous application of either 40 µM Na_2S or 26.7 µM DEA-NONOate, the coapplication of the other substance caused a calcium increase. The concentration-dependence, normalized to the continuous application in the right panel shows that Na_2S is rate-limiting for the calcium response. (C) Due to the rapid NO release and consumption, DEA-NONOate and 20 s later Na_2S from a 1000-fold stock solution were pipetted into the dish and carefully mixed. Note the amplitude of the response compared to panel A. The number of neurons is given in brackets, data are mean ± SEM. $^*p < 0.05$.

4. Discussion

We demonstrate that the stimulation with NO and sulfide causes neuropeptide release in freshly isolated preparations at all three trigeminal levels, the cranial dura mater, the trigeminal ganglion, and the medullary brainstem. The combined stimulation, which we previously demonstrated to form HNO [2], acted super-additive compared to both substances alone. This could be confirmed in a cellular model, which indicated that the sulfide is the rate-limiting factor in this combined activation of neurons sensitive to the TRPA1 agonist carvacrol.

Our recent findings indicate an HNO-TRPA1-CGRP signaling pathway in sensory nerves [2]. We demonstrated that HNO is endogenously formed from NO and sulfide, HNO specifically activates TRPA1 and, via calcium, releases CGRP. Based on the short half-life of NO, H_2S and HNO, circulating substances generating these molecules would need to penetrate the blood–brain barrier to affect central trigeminal sites. Both NO and H_2S can be produced endogenously from neurons and other cells within the blood–brain barrier: In the central nervous system, H_2S is mainly generated by cystathionine beta synthase [31], which is expressed in small pial arterioles, the trigeminal ganglion [32,33] and throughout the brain in neurons and astrocytes, including the STN [2,34,35]. NO can be produced by the endothelial, the neuronal and the inducible isoform of the NO synthase, respectively, eNOS, nNOS and iNOS [36]. The endothelial and inducible isoforms have been found in the dura mater, while their expression in the trigeminal ganglion and the STN is only marginal [3,37,38]. However, iNOS could have contributed to the endogenous NO generation in our cellular model, since the synthase can be induced by growth factors like NGF, which was added to the cell culture medium [39,40]. Furthermore, there is evidence about up-regulation of iNOS in culture conditions [41]. In contrast to the other isoforms, the neuronal NOS is present in all parts of the trigeminal system and might therefore be the major source of endogenous NO in the trigeminal nerve [3,38,42–44]. Taken together, both gasotransmitters can be produced endogenously in the trigeminal system and co-localization of their generating enzymes cystathionine beta synthase and NOS would allow a regulated formation of HNO in neurons [2].

In the dura mater and the trigeminal ganglion, both Na_2S and the NO donor DEA-NONOate increased CGRP release, the combination, however, caused a super-additive effect. In brainstem slices, only the combined stimulation was sufficient to increase CGRP release significantly. The increase in CGRP release caused by Na_2S in the trigeminal ganglion was the largest, possibly indicating a higher endogenous production of NO compared to the other preparations. The larger cytoplasm volume of the trigeminal cell bodies compared to the peripheral fibers might explain this. In addition, also satellite glial cells may produce NO [45], which might contribute to higher endogenous amounts of NO in the trigeminal ganglion. The results support no such differential production of sulfide.

Furthermore, these results were validated in a cellular model: In cultured trigeminal neurons, both Na_2S and NO had little effect on their own but were effective when combined in a 1:1 stoichiometry. To investigate the respective contribution, we varied the proportion of DEA-NONOate and Na_2S in combined stimulation. Compared to an equimolar ratio, a reduction of the Na_2S concentration significantly reduced the response, demonstrating that the sulfide formation is the rate-limiting factor for generation of HNO. In contrast, a similar reduction of the NO donor had less effect on the calcium influx.

When we minimized the time between diluting DEA-NONOate from the protective alkaline stock solution and the subsequent contact with hydrogen sulfide, a substantially larger response was observed compared to the majority of experiments, where solutions were prepared immediately before the experiment.

This indicates that a considerable fraction of the total HNO was degraded in most experiments before reaching the cells. An accurate concentration-response is difficult to perform for HNO, but the application from the NO-donor stock solution demonstrates the previously underestimated potency of the acutely formed chemicals for neuronal activation.

In the current study a synergistic action of NO and H_2S, which exceeds the individual effects, could be demonstrated at all levels of the trigeminal system. An involvement of NO in migraine pathophysiology is established, since the NO donor nitroglycerin can induce headaches and inhibition of NO generation was effective in a double-blind study [46]. However, the role of H_2S was not discussed in this context before and might contribute to the reported effects of NO in migraine. Indeed, a potentiation of nitroglycerin-induced headache by *N*-acetyl cysteine, which might be converted into H_2S via the cystathionine beta synthase, was described [47]. Efforts to detect the reaction products of HNO in migraine patients would be of interest. In summary, the presented results might pave the way to new therapeutic options in the trigeminal system, inhibition of the production of either substance as well as the inhibition of the targets of HNO.

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