Research article

Anxiolytic-like effect of hydrogen sulfide (H$_2$S) in rats exposed and re-exposed to the elevated plus-maze and open field tests

Alberto Ferreira Donatti$^{a,*}$, Renato Nery Soriano$^b$, Christie Ramos Andrade Leite-Panissi$^c$, Luiz G.S. Branco$^c$, Albert Schiaveto de Souza$^d$

$^a$ Department of Psychology, Federal University of Mato Grosso do Sul, MS, Brazil
$^b$ Division of Physiology and Biophysics, Department of Basic Life Sciences, Federal University of Juiz de Fora, Governador Valadares, MG, Brazil
$^c$ Department of Morphology, Physiology and Basic Pathology, Dental School of Ribeirão Preto, University of São Paulo, SP, Brazil
$^d$ Department of Morphophysiology, Federal University of Mato Grosso do Sul, MS, Brazil

HIGHLIGHTS
- H$_2$S exerts anxiolytic-like effects in the open field (OF) test.
- H$_2$S does not cause alteration of locomotor activity in the OF test.
- H$_2$S modulates aversive learning in the elevated plus-maze (EPM) test.
- H$_2$S causes a decrease in risk assessment behavior in the EPM retest.

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ABSTRACT

Hydrogen sulfide (H$_2$S), an endogenous gaseous mediator, modulates many physiological functions in mammals but evidence of its involvement in emotional and behavioral aspects is currently scarce. We hypothesized that this gas plays a modulatory role in behavioral parameters in rats submitted to tests (for 5 min) in the open field (OF) and elevated plus-maze (EPM – test and retest). Male Wistar rats (200–250 g) were intraperitoneally injected with saline or Na$_2$S (a H$_2$S donor; 4, 8 and 12 mg/kg) either once or for 8 days, and submitted to the OF test or to the EPM test and retest. A third group (naive) was not injected but exposed to the same experimental protocols. In the OF test, Na$_2$S injected for 8 days caused a decrease in self-cleaning (4, 8 and 12 mg/kg) and freezing behaviors (8 and 12 mg/kg), and a rise in the rate of line crossings in the central part of the arena (12 mg/kg). In the EPM test and retest, Na$_2$S at 12 mg/kg for 8 days caused an increase in the number of open arm entries and in the percentage of time spent on open arms. Our data are consistent with the notion that H$_2$S exerts anxiolytic-like effects in rats submitted to the EPM and OF tests. Moreover, this gaseous modulator reduces aversive learning in the EPM retest.

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1. Introduction

Hydrogen sulfide (H$_2$S) has been referred to as the third gaseous mediator, alongside nitric oxide (NO) and carbon monoxide (CO), and plays a modulatory role in mammalian physiological systems/functions [1]. Regarding nociception, pulmonary ventilation, and thermoregulation, Donatti and colleagues have documented that endogenous H$_2$S plays a pronociceptive role [2], downregulates ventilatory responses to hypoxia [3], and is permissive for hypoxia-induced hypothermia [4].

It is well known that the hypothalamic-pituitary-adrenal (HPA) axis is activated in response to psychological stress, such as that one induced by exposure to tests in the open field (OF) or elevated plus-maze (EPM) [5,6]. The EPM retest (24 h after test) has been shown to blunt the anxiolytic effect of anxiolytic drugs (benzodiazepines), and this phenomenon has been considered to be dependent on learning from the first trial (known as aversive learning) [5]. Importantly, H$_2$S has been reported to modulate the HPA axis, affecting the hypothalamic release of CRF (corticotropin-releasing factor) [7,8]. The effects of H$_2$S (inhibitory vs. stimulatory) have been shown to be dependent on the type of stress.
Fig. 1. Experimental Protocols. Protocol 1—some rats were injected once with saline (vehicle) or a H2S donor (Na2S; 4, 8, 12 mg/kg, i.p.); one hour later, they were submitted to the open field or to the EPM test (evaluated for 5 min), and 24 h later re-submitted to the EPM (retest; re-evaluated for 5 min). Protocol 2—other rats were injected for eight consecutive days with saline (vehicle) or a H2S donor (Na2S; at the same doses as the protocol 1); one hour later, they were submitted to the open field test or to the EPM test (evaluated for 5 min), and 24 h later re-submitted to the EPM (retest; re-evaluated for 5 min). The naïve group received no treatment (but it was submitted to the same tests, and evaluated for 5 min as well).

2. Materials and methods

2.1. Animals

Adult male Wistar rats (220–280 g) were group-housed (four to five animals per cage, dimensions: 1394 cm²) and acclimated (25 °C; 12:12-h light-dark cycle) for 1 week before the experiments. They had free access to water and food. All procedures were approved by the Animal Care and Use Committee of the Federal University of Mato Grosso do Sul, Brazil (Protocol number: 618/2014), and were carried out in compliance with the recommendations by the SBCAL (Sociedade Brasileira de Ciências em Animais de Laboratório) and by the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Experiments were performed on fully conscious, freely moving animals. Efforts were made to minimize animal suffering, and the experiments were performed between 11:00 AM and 05:00 PM.

2.2. Drugs

Sodium sulfide (Na2S, a H2S donor) was purchased from Sigma-Aldrich (St. Louis, MO), and freshly dissolved in sterile saline (vehicle; 0.9%).

2.3. The elevated plus-maze (EPM) test

The EPM is an animal model of anxiety. It consists of two open arms (50 × 10 cm) and two enclosed arms (50 × 10 cm), 50-cm-high walls. The same type arms were opposite each other, and the maze was raised 50 cm from the floor. Tests were performed during the light phase of the light-dark cycle. The rats were individually placed on the center of the EPM facing an enclosed arm, and were allowed free exploration for 5 min. Behavioral aspects were recorded by a video camera positioned above the EPM, thus allowing discrimination of all behaviors. Images were relayed to a monitor in another room via a closed circuit of TV camera. The EPM was thoroughly cleaned after each test session.

The performance of each animal in the EPM was analyzed taking into consideration behavioral parameters reported elsewhere [11,13]. Standard measurements recorded in each section (enclosed and open arms): frequency of open and enclosed arms entries (an arm entry or exit being defined as all four paws into or out an arm, respectively), total arm entries and the amount of time spent in each section of the maze. In addition, the frequencies of the following “novel ethological categories” were measured: (1) head dipping:
dipping of the head below the level of the maze floor; (II) stretched-attend postures: when the animal stretches to its full length for the forepaws (keeping the hind paws in the same place) and turn back to the anterior position; (III) end-arm exploration: the number of times the rat reached the end of an open arm [14,15].

2.4. The open field (OF) test

The OF test is commonly used for evaluating locomotor activity and some behavioral parameters. It consists of an opaque, white polyethylene cylindrical arena. The cylinder (50-cm radius) has its floor divided into 19 roughly equal regions demarcated by three concentric circles of different radii (4, 12 and 20 cm). The animal was placed on the center of the arena, and its behavior was observed for 5 min.

The OF test was performed during the light phase of the light-dark cycle, and the behavior was recorded by a video camera positioned above the arena. The arena was thoroughly cleaned after each test session.

The following behavioral parameters were recorded: (I) Motor Activity: locomotion frequency (number of quarters in which the animal crosses with four legs), rearing (relying only on its hind legs, with the trunk perpendicular to the floor of the arena); (II) Behavioral Activity: grooming (protracted washing of the coat), number of times on the central part of the arena, freezing (the number of times the animal remains static only with the movement of breathing and vibrissae for more than 6 s) [15].

Fig. 2. Effect of the H2S donor (Na2S; 4, 8, 12 mg/kg, i.p.) on locomotor activity of rats submitted to the open field test. Panels A and C: number of line crossings. Panels B and D: number of rearing behavior. No statistical difference was found. Values are means ± SE. Number of animals in each group is shown in parenthesis.

2.5. Experimental procedures

2.5.1. Protocol 1

We assessed the putative effects of H2S on behavior of rats submitted to test and retest in the EPM (Fig. 1, upper panel). The animals' behavior was evaluated for 5 min (test or retest). The rats were randomly divided into five groups: (i) Naive: animals not treated were submitted to test, and 24 h later to retest in the EPM (n = 7); (ii) Vehicle: animals were injected with saline, 1 h later were submitted to test, and 24 h later to retest in the EPM (n = 8); Groups (iii), (iv), and (v): animals were injected with Na2S (4, 8 or 12 mg/kg, respectively), 1 h later submitted to test, and 24 h later to retest in the EPM (n = 7). In the OF test (Fig. 1, upper panel) the rats were randomly divided into the same five groups described above, with the following particularity: the animals were injected with Na2S (4, 8 or 12 mg/kg), and 1 h later submitted to the OF test for 5 min (n = 7).

2.5.2. Protocol 2

The animals' behavior was evaluated for 5 min (test or retest). The rats were injected with the H2S donor for eight consecutive days, submitted to test and retest in the EPM (Fig. 1, bottom panel), and randomly divided into five groups: (i) Naive: animals not treated were submitted to test, and 24 h later to retest in the EPM (n = 7); (ii) Vehicle: animals were injected with saline for eight consecutive days, 1 h later submitted to test, and 24 h later to retest in the EPM (n = 7); Groups (iii), (iv), and (v): animals were injected with Na2S (4, 8 or 12 mg/kg, respectively) for eight consecutive
Fig. 3. Effect of the H₂S donor (Na₂S; 4, 8, 12 mg/kg, i.p.) on behavioral activity of rats submitted to the open field test. Panels A and D: number of grooming. Panels B and E: number of line crossings in the central part. Values are expressed as mean ± SE. * P < 0.05, compared to naïve and vehicle; † P < 0.05, compared to naïve, vehicle and Na₂S 4 mg/kg; ## P < 0.01, compared to naïve, vehicle and Na₂S 4 mg/kg. Number of animals in each group is shown in parenthesis.

2.6. Data expression and statistics

Data are expressed as mean ± SE, and were analyzed by Kruskal-Wallis's non-parametric test followed by Dunn's Multiple Comparison Test. P values lower than 0.05 were considered statistically significant.

3. Results

3.1. Effects of H₂S treatment on behavioral activities of rats submitted to the OF test

3.1.1. Locomotor activity parameters

The effects of Na₂S on locomotor activity of rats submitted to the OF test are shown in Fig. 2. Locomotor activity of rats injected with Na₂S (Protocol 1) at doses of 4, 8 or 12 mg/kg was not significantly different in comparison to controls (naïve and vehicle) as to the
number of line crossings (P > 0.05; Fig. 2A), and rearing (P > 0.05; Fig. 2B). Na2S treatment for eight consecutive days (Protocol 2), at doses of 4, 8 or 12 mg/kg did not alter the number of line crossings (P > 0.05; Fig. 2C) and rearing (P > 0.05; Fig. 2D) in the OF test.

3.1.2. Behavioral activity parameters
Treatment with Na2S at doses of 4, 8 or 12 mg/kg (Protocol 1) did not affect the number of grooming (P > 0.05, Fig. 3A), freezing (P > 0.05; Fig. 3B), and line crossings in the central part of the arena (P > 0.05; Fig. 3C) in comparison to controls (naive and vehicle). Treatment for eight consecutive days (Protocol 2) showed that all doses tested (4, 8 or 12 mg/kg) caused a significant decrease in the number of grooming (P < 0.05; Fig. 3D). Regarding freezing behavior, Na2S at 8 or 12 mg/kg (Protocol 2) caused a pronounced decrease in freezing behavior (dose of 8 mg/kg: P < 0.05; dose of 12 mg/kg: P < 0.01; Fig. 3E). The highest dose (12 mg/kg) caused a significant increase in the number of line crossings in the central part of the arena (P < 0.01; Fig. 3F).

3.2. Effects of H2S treatment on behavioral parameters of rats submitted to test and retest in the EPM

3.2.1. Classic behavioral parameters
Regarding classic behavioral parameters, treatment with Na2S at doses of 4, 8 or 12 mg/kg (Protocol 1) to rats submitted to the EPM test did not significantly alter the number of open arm entries (P > 0.05; Fig. 4A), percentage of entries into open arms (P > 0.05; Fig. 4B), and time spent on open arms (P > 0.05; Fig. 4C). Besides,
in the retest no differences were found as to the number of open arm entries (P > 0.05; Fig. 4D), percentage of entries into open arms (P > 0.05; Fig. 4E), and time spent on open arms (P > 0.05; Fig. 4F).

At the highest dose (12 mg/kg) Na$_2$S treatment for eight consecutive days (Protocol 2) to rats submitted to the EPM test showed a very significant increase in the number of open arm entries (P < 0.01; Fig. 5A). Treatment with Na$_2$S at 12 mg/kg caused a significant increase in the percentage of open arms entries in comparison to the naïve group (P < 0.05; Fig. 5B). Moreover, the animals treated with Na$_2$S at 12 mg/kg showed an increase in time spent on open arms compared to naïve (P < 0.05) and vehicle (P < 0.01; Fig. 5C).

When the rats were submitted to retest in the EPM and treated with Na$_2$S at 12 mg/kg we observed an increase in the number of open arm entries in comparison to the naïve group (P < 0.05; Fig. 5D), and an augmentation of the percentage of open arms entries in comparison to the naïve and vehicle groups (P < 0.01; Fig. 5E). Besides, Na$_2$S at 12 mg/kg caused an increase in time spent on open arms compared to naïve, vehicle, and Na$_2$S 4 mg/kg (P < 0.01; Fig. 5F).

3.2.2. Novel ethological categories

Regarding the effect of Na$_2$S on novel ethological categories in the EPM test and retest, the results showed no statistical differences (Protocol 1) among the groups of rats treated with Na$_2$S at 4, 8 or 12 mg/kg in head dipping (test and retest: P > 0.05; Fig. 6A), end-arm exploration behavior (test and retest: P > 0.05; Fig. 6B), and
stretched-attend postures (test and retest: P > 0.05; Fig. 6C) compared to the naive and vehicle groups. Conversely, rats treated with Na$_2$S at 12 mg/kg for eight consecutive days (Protocol 2) exhibited a significant decrease in stretched-attend postures (test and retest: P < 0.05; Fig. 7A), increase in end-arm exploration behavior (test and retest: P < 0.05; Fig. 7B), and increase in head dipping behavior (test and retest: P < 0.05; Fig. 7C) compared to the naive and vehicle groups.

4. Discussion

The present study provides evidence that H$_2$S exerts anxiolytic-like effects in rats as H$_2$S caused (i) a decrease in conflict approach/avoidance of open arms, and an increase in the number of open arm entries and percentage of time spent on open arms in both test and retest in the EPM test; (ii) a decrease in stretched-attempt posture in test and retest in the EPM; (iii) an increase in head dipping and end-arm exploration, novel ethological categories; and (iv) a decrease in grooming and freezing behaviors, and an increase in the number of line crossings in the central part of the OF test.

The EPM test is an animal model of anxiety [3,14,17,16,6]. In the present study, chronic (eight consecutive days) administration of the H$_2$S donor (i.p.) at the highest dose (12 mg/kg) caused an increase in the number of open arms entries, percentage of open arms entries, and time spent on open arms, suggesting an anxiolytic-like effect. Our findings corroborate the findings by Chen et al. [10]. Indeed, they have shown that chronic treatment with a H$_2$S donor (NaHS; at a lower dose, 5.6 mg/kg) reduces anxiety-like behaviors in the EPM test.

In the EPM retest, our results showed that rats treated with the H$_2$S donor for eight consecutive days exhibited a decrease in “aversive learning”. It has been established that benzodiazepines (BZD) induce anxiolytic-like effects in test (i.e., elevate the percentage of entries and time spent on open arms of the maze), but not in retest in the EPM [13,18,19]. This phenomenon is known as “one-trial tolerance” and appears to be dependent on learning [19]. Studies have shown that during the EPM test and retest two types of behavior patterns are observed: anxiolytic-sensitive in the first trial, and anxiolytic-insensitive in retest due to a shift across the test and retest or a habituation process [20-22]. Albrechet-Souza et al. [11] have proposed that in retest occurs an acquirement of spatial information about the maze leading to a decrease in the animals’ motivation for exploring the open arms (“aversive learning”). Thus, our findings suggest that chronic treatment with H$_2$S seems to modulate the “aversive learning” of rats submitted to retest in the EPM. To demonstrate that H$_2$S modulates behavioral responses to fear Wang et al. [23] showed in experimental protocols of cue fear conditioning that the gas regulates amygdala-dependent emotional memory in rats, since intraamygdala and systemic administration of H$_2$S led to an enhanced cue fear memory.

The conventional analysis of the exploratory behavior in the EPM test has been extended to incorporate the so-called novel ethological categories which have disclosed additional dimensions to plus-maze behavior patterns, for example, risk assessment [15]. Risk assessment measurements have been proved to be extremely
Fig. 7. Effect of the H2S donor (Na2S; 4, 8, 12 mg/kg, i.p.) on the novel ethological categories in rats submitted to test and retest in the EPM (Protocol 2). Values are expressed as mean ± SE. * P<0.05, compared to the respective naïve and control groups. Number of animals in each group is shown in parenthesis.

valuable in identifying anxiolytic-like action of drugs (e.g. 5-HT1A receptors ligands) not detected by conventional scoring methods [15,24,25]. The present study extends the work by Chen et al. [10] since we showed that administration of H2S for eight consecutive days, in test and retest, caused a decrease (anxiolytic effect) in stretched-attend posture, one of the “risk assessment behavior” [26]. Moreover, we showed that H2S exerts an anxiolytic-like effect in rats submitted to retest in the EPM due to an increase in head dipping and end-arm exploration, demonstrating an increase in exploratory behavior. Based on these findings, it is possible to propose that the H2S-induced decrease in stretched-attend posture in retest did not reinstate the information processing initiated in the first trial, leading to an increase in anxiolytic state, thus increasing end-arm exploration and head dipping exploration.

It has been demonstrated a positive relationship between plasma corticosterone and risk assessment behavior in rats and mice exposed to the EPM [11–13,22]. Of particular interest are the studies by Dello Russo et al. [7] and Mancuso et al. [8] which reported that increased H2S bioavailability, in vitro and in vivo, led to a reduction of CRF release from the hypothalamus under stress-induced activation of the HPA axis. Taking these previous studies into account, it seems plausible to suggest that chronic administration of H2S may act in the CRF system altering the levels of corticosterone [7,8,11,13], consequently decreasing the risk assessment behavior in test and retest in the EPM.

Regarding the OF test, our results showed that the chronic treatment with H2S led to an anxiolytic-like effect due to a decrease in grooming and number of immobility. Furthermore, we found an increase in the number of line crossing in the central part of the OF, indicating an anxiolytic effect. An increase in time spent on the central part as well as the ratio central/total locomotion or decrease in the latency to enter the central part are indicative of anxiolysis [27,28]. It has been demonstrated that treatment with clonazepam [6] or doramectin (GABA receptor agonist, s.c.) [28] causes a decrease in the frequency of grooming in rats.

Our results showed that both acute and chronic treatment with Na2S did not cause any significant change in locomotor activity; in other words, H2S did not affect horizontal locomotion (number of crossings of the lines marked on the floor) and frequency of rearing (sometimes termed vertical activity), see ref. [15]. This information is relevant since it rules out the possibility of a H2S-induced alteration in locomotor activity, which would lead to attenuated behavioral activities [29].

The mechanisms by which H2S regulates anxiety is still unknown, but one of them seems to be associated with the modulation of CRF release [8]. The present findings provide promising elements potentially related to a H2S-mediated neuroendocrine regulation and/or modulation of neurotransmission in fear/anxiety.

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Conflict of interest

The authors declare that there is no conflict of interest.

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References
