Research Article

Pharmacology

Pharmacology 2019;103:50–60
DOI: 10.1159/000494113

Received: July 27, 2018
Accepted after revision: September 26, 2018
Published online: November 16, 2018

Mechanisms of Hydrogen Sulfide against the Progression of Severe Alzheimer's Disease in Transgenic Mice at Different Ages

Eleonora Vandini[a] Alessandra Ottani[a] Davide Zaffe[b] Anita Calevro[a] Fabrizio Canalini[a] Gian Maria Cavallini[c] Rosario Rossi[d] Salvatore Guarini[a] Daniela Giuliani[a]

[a]Department of Biomedical, Metabolic and Neural Sciences, Section of Pharmacology and Molecular Medicine, University of Modena and Reggio Emilia, Modena, Italy; [b]Department of Biomedical, Metabolic and Neural Sciences, Section of Anatomy, University of Modena and Reggio Emilia, Modena, Italy; [c]Department of Ophthalmology, University of Modena and Reggio Emilia, Modena, Italy; [d]Department of Cardiology, University of Modena and Reggio Emilia, Modena, Italy

Keywords
Hydrogen sulfide · Severe Alzheimer's disease · Learning · Memory · 3x-Tg-Alzheimer's disease · Neuroprotection

Abstract
Backgroud: Alzheimer disease is an age-related severe neurodegenerative pathology. The level of the third endogenous gas, hydrogen sulfide (H_2S), is decreased in the brain of Alzheimer's disease (AD) patients compared with the brain of the age-matched normal individuals; also, plasma H_2S levels are negatively correlated with the severity of AD. Recently, we have demonstrated that systemic H_2S injections are neuroprotective in an early phase of preclinical AD. ***Objectives:*** This study focuses on the possible neuroprotection of a chronic treatment with an H_2S donor and sulfurous water (rich of H_2S) in a severe transgenic 3×Tg-AD mice model. ***Method:*** 3×Tg-AD mice at 2 different ages (6 and 12 months) were daily treated intraperitoneally with an H_2S donor and sulfurous water (rich of H_2S) for 3 months consecutively. We investigated the cognitive ability, brain morphological alterations, amyloid/tau cascade, excitotoxic, inflammatory and apoptotic responses. ***Results:*** Three months of treatments with H_2S significantly protected against impairment in learning and memory in a severe 3×Tg-AD mice model, at both ages studied, and reduced the size of Amyloid β plaques with preservation of the morphological picture. This neuroprotection appeared mainly in the cortex and hippocampus, associated with reduction in activity of c-jun N-terminal kinases, extracellular signal-regulated kinases and p38, which have an established role not only in the phosphorylation of tau protein but also in the inflammatory and excitotoxic response. ***Conclusion:*** Our findings indicate that appropriate treatments with various sources of H_2S, might represent an innovative approach to counteract early and severe AD progression in humans.

KARGER

E-Mail karger@karger.com
www.karger.com/pha

Daniela Giuliani
Department of Biomedical, Metabolic and Neural Sciences
Section of Pharmacology and Molecular Medicine
University of Modena and Reggio Emilia, via G. Campi 287, IT–41125 Modena (Italy)
E-Mail daniela.giuliani@unimore.it

Introduction

Alzheimer's disease (AD) is a severe, progressive and age-related neurodegenerative disorder that can be divided into a 3-stage model: early stage (characterized by mild AD: general forgetfulness) middle stage (moderate AD: forgetfulness, language and communication difficulty), and late stage (severe AD: inability to recognise familiar people and objects) [1]. Amyloid β (Aβ) accumulation in different areas of the brain, (as hippocampus and cortex, which are mainly involved in learning and memory) and tau hyperphosphorylation are the 2 main etiopathological factors of AD, leading to an excitotoxic and inflammatory response, with synaptic dysfunction, neurodegeneration, apoptotic death associated with neuronal loss and severe cognitive decline: this is the typical neurodegeneration process of AD [2–8]. The number of AD patients is constantly increasing. Indeed, according to estimates, 40 million people worldwide have AD, and this number is expected to redouble by 2050 [9]. So, to find new effective therapies is the urgent need of the hour.

Hydrogen sulfide (H_2S) is best known as a highly toxic, colourless and flammable gas, but recently it has been demonstrated that it is also the third endogenous gas, after nitric oxide and carbon monoxide, and all these gases play important roles both in health and pathologic conditions [10]. The endogenous micromolar levels of H_2S have been measured in the brain, blood and peripheral organs of rats, humans, and bovine and production differs considerably between the various mammalian species [11]; moreover, levels of H_2S decrease with ageing [12]. Endogenous H_2S is generated from cysteine metabolism through 2 enzymes: cystathionine-β-synthase and cystathionine-γ-lyase whose productions are mainly in the brain for the former and largely in the cardiovascular system for the latter [13, 14]. Growing evidence shows that H_2S is an important signalling molecule in various body systems, and it gives beneficial effects in animal models of inflammation, hypertension, and ischemia/reperfusion injury inducing anti-inflammatory, cytoprotective, immunomodulating and trophic effects [10, 13–18]. The relatively high endogenous concentrations of H_2S in the brain suggest a neuromodulator role [19]; yet, to date, the detailed mechanisms involved have remained unknown. Furthermore, it has been reported that brain H_2S synthesis is severely decreased in AD patients (up to 55%) [20], and plasma H_2S levels are negatively correlated with the severity of AD [21]. Recently, it has been demonstrated that H_2S donor sodium hydrosulfide reduces Aβ generation in cultured cells and subsequently, we have demonstrated this reduction also in vivo experiments with improvement of cognitive performance in rats and transgenic mice with mild (early stage) AD [22].

This study focuses on the possible ability of a chronic treatment (3 months) with an H_2S donor and a sulfurous water, with high content in H_2S [23], to counteract the progression also in a severe (late stage) AD, at different ages (6 and 12 months). In particular, in a severe 3×Tg animal model of AD, we investigated cognitive ability, brain morphological alterations, amyloid/tau cascade, excitotoxic, inflammatory and apoptotic responses.

Materials and Methods

Animals

For this study, male 6 and 12 month-old (at the start of the study) 3×Tg-AD mice and their wild-type littermates (The Jackson Laboratories, Bar Harbor, ME, USA) were used. These mice harbour human transgenes APP_{Swe}, $PS1_{M146V}$ and tau_{P301L} overexpress mutant human APP, PS1 and tau protein respectively [22, 24]. Animals were kept in air-conditioned colony rooms (temperature 21 ± 1 °C, humidity 60%) on a natural light/dark cycle (lights on at 08:00 am) with free access to food and water. Throughout the study, body weight was recorded. Animal sacrifice at the end of the study was performed under general anaesthesia with sodium pentobarbital intraperitoneally (i.p.; Sigma-Aldrich, Milan, Italy). All experiments were carried out in strict accordance with the European Community guidelines governing animal welfare and protection for scientific purposes (CEE Council 89/609; Italian D.L. No. 26, 2014) and approved by the Committee on Animal Health and Care of Modena and Reggio Emilia University. All efforts were made to minimize animal suffering and to reduce the number of animals used in this study.

Drugs and Treatment Schedules

In this experimental model of severe AD, investigations with sodium hydrosulfide (NaHS), a H_2S donor (Sigma-Aldrich, St. Louis, MO, USA) and sulfurous water (thermal-water with high H_2S content; kindly provided by Salsomaggiore and Tabiano Thermae, Tabiano, Italy) were carried out. Sulfurous water originates from Tabiano's source Pergoli with the following characteristics: H_2S level 125 mg/L (HS^- 43 mg/L and not ionized H_2S 82 mg/L), pH 6.6, osmolarity 41 mOsm/L [23]. Sulfurous water (12 mL/kg i.p.) and sodium hydrosulfide (0.5 mg/kg, i.p.) were administered once daily for 12 weeks starting at 6 or 12 months of age; therefore, mice were sacrificed at 9 and 15 months. Control animals (3×Tg-AD mice and wild-type mice [WT]) received an equal volume of saline by the same route of administration. The doses of sodium hydrosulfide and sulfurous water, and time of animal sacrifice were chosen on the basis of our previous experiments [22]. The number of animals used for each group is indicated in figure legends.

Assessment of cognitive ability. As it is well known, Alzheimer disease is characterized by a progressive and severe cognitive decline. For this reason, in the present study, the Morris water maze test was used to assess spatial learning and memory, as previously

described [6, 22, 25–27]. The apparatus consisted of a circular white pool at a fixed position (100 cm in diameter and 55 cm in height), filled to a depth of 15 cm with tap water (26 ± 1 °C) made opaque with milk. A white, circular-shaped refuge platform of 7 cm in diameter was submerged 1 cm below the water surface in one of the dials (target quadrant). The pool and the location of the platform were constant during all trials.

Assess spatial learning. The spatial acquisition test was conducted for 4 consecutive days and each day included one block of 4 trials. Each trial began when mice were randomly placed into the pool with face at the wall of pool from one of the 4 starting point of a quadrant, and latency to find the hidden platform was recorded (escape latency). Each animal had a period of 60 s to locate the hidden platform, and when they detected the platform, they could stay on for 15 s, to allow the formation of a spatial map. Those mice not reaching the platform within 60 s were gently guided to the platform and allowed to rest for 15 s on it.

Probe test: To examine spatial reference memory, a probe test was administered 24 h after ending spatial acquisition. During the test, the platform was removed from the pool and the mouse started the test from the opposite dial with the face facing the pool wall and swims freely for 1 min [28]. The time spent and the number of crossings, in the quadrant where the platform was first placed (target zone), were recorded as spatial memory retention.

Visible platform task. In order to evaluate the Visual-Motor coordination of mice 1 h after the probe test, a visual cue test was conducted [28]. For this test, the platform was set 1 cm above the water level and marked with black tape so that the mice could locate the platform for musing a local visual stimulus rather than relying on spatial orientation to extra-maze cues. The platform was placed in the opposite of the target quadrant and the mouse was tested once beginning from the opposite quadrant of the platform. The time spent to reach the platform was recorded.

After the last behavioural session, animals were sacrificed and then histological, biochemical and biomolecular studies were performed on their brain.

Histology

These studies were carried out in the mice experimental models. At the end of the last behaviuoral test, transcardial perfusion with ice-cold 4% paraformaldehyde (phosphate-buffered) was performed; then animal brains were removed and processed for histologic examination, as previously described [25–27]. Hippocampus and cortex morphology were studied in 7-μm thick paraffin-embedded sections, hematoxylin-eosin or Nissl method stained; the extent of Aβ plaques was assessed after ethanol-Congo red/Harris hematoxylin staining.

Morphological analyses were performed by using an Axiophot photomicroscope (Carl Zeiss, Jena, Germany), under ordinary and polarized light. Histometry was performed by using an image system (analySIS, Soft Imaging System GmbH, Münster, Germany). Viable neurons in the CA1 subfield and cortex (neurons having granular cytoplasm and euchromatic nucleus with large nucleoli) and Aβ plaques were estimated in 5 randomly selected fields per slide and on 5 sections for each hippocampal sample. Aβ deposit extent was evaluated according to the following score scale of values: 0 = no detection, 1 = 0–100 μm^2, 2 = 100–5,000 μm^2, 3 = 5,000–25,000 μm^2, 4 = >25,000 μm^2.

Isolation of Cytoplasmic Proteins and Western Blot Analysis

At the end of the last behaviuoral test, the whole hippocampus and cortex were dissected from the mouse brain and immediately frozen into liquid nitrogen and stored at –80 °C. After the extraction of cytoplasmatic proteins (for analysis of phosphorylation state/level of APP, Tau, $A\beta_{1-42}$, p-c-jun N-terminal kinases [JNK], p-extracellular signal-regulated kinases [ERK1/2] and p38) as previously described [6, 22, 25–27], proteins (40 μg for each sample) were denatured, electrophoretically separated and transferred onto nitrocellulose membranes. The staining of the blots with Ponceau's solution showed that total protein amount was equal in each lane. The blots were then blocked and incubated overnight at 4 °C with the following primary antibodies: p-APP, phospho (p)-tau, p-tau Ser396, Aβ (1–42 specific), JNK 54/46, ERK1/2 44/42 and p-p38 (Cell Signaling, Charlottesville, VA, USA). The day after, the membranes were incubated with a specific secondary antibody peroxidase-conjugated for 1 h at room temperature. To prove equal loading, the blots were analysed for β-tubulin expression (house-keeping gene) using an anti- β-tubulin antibody (Cell Signaling). The membranes were analysed by the enhanced chemiluminescence system according to the manufacturer's protocol (Millipore). Protein signals were quantified by scanning densitometry using a bio-image analysis system (Bio-Profil, Celbio, Italy) and expressed as relative integrated intensity in comparison with those of normal (naïve) mice. The level of all bands was expressed as relative integrated intensity normalized versus β-tubulin (= 100) [6, 22, 25–27].

Statistical Analysis

All data were collected blinded to the treatment and are shown as mean ± SEM. Parametric data was analysed using SigmaPlot and Adobe Illustrator and SPSS17.0 software. The 2-way repeated measures ANOVA (behavioural data), or 1-way ANOVA (all other data), followed by the Student-Newman-Keuls' test were used to detect the significance from different groups. Aβ deposit data was analysed by means of the Kruskal-Wallis test followed by the Mann-Whitney U-test. A value of $p < 0.05$ was considered significant.

Results

Sulfurous Water Improves Learning and Memory Performance in a Severe Transgenic Mouse Model of AD

WT, both 9 and 15 months, treated with saline, gave latencies to find the hidden platform continuously decreased during the 4 days of training (Fig. 1a, b).

3×Tg-AD mice, both 9 and 15 months, treated with saline (controls) showed impaired ability (as compared with WT) in platform finding during all 4 days of behavioural test (Fig. 1a, b). Conversely, the 2 groups (9 and 15 months old) of transgenic mice treated with sulfurous water (12 mL/kg i.p.) showed an improvement in spatial learning performance on the 4th day of training as compared with controls (Fig. 1a, b). Treatment with H_2S donor, sodium hydrosulfide (0.5 mg/kg i.p.) did not significantly modify

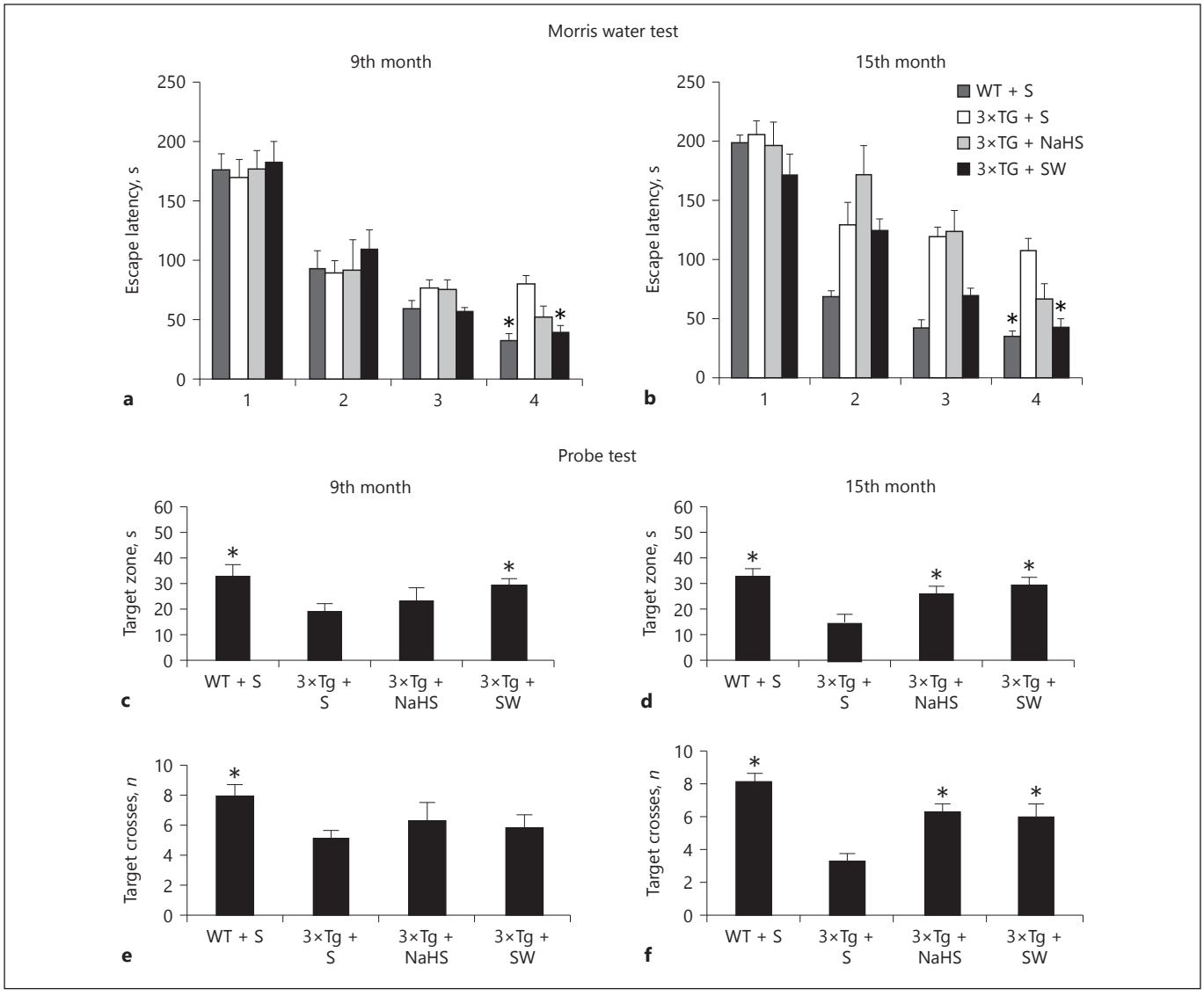


Fig. 1. Donor of Sodium hydrosulfide and sulfurous water improve cognitive performance in 9- (**a**) and (**b**) 15-month old 3×Tg-AD. MWM was used to studied the learning performance, and was performed in 9- and 15-month old mice ($n = 12$ and 8 mice per group, respectively). The MWM (4-day training) started at the beginning of the last week of treatment. The probe test was used to study the memory performance and was performed 24 h after the MWM test. Histograms' height indicates mean values ± SEM of latency to escape onto the hidden platform (**a**, **b**), of time spent into the target zone (original quadrant; **c**, **d**) and number of crossings of the target zone (**e**, **f**). Sulfurous water (12 mL/kg i.p.) and donor of sodium hydrosulfide (0.5 mg/kg, i.p.) were administered once daily for 12 weeks starting at 6 or 12 months of age. * $p < 0.05$, at least, versus the corresponding value of 3×Tg mice treated with saline. WT, wild type; 3×Tg, 3×Tg-AD; S, saline; NaHS, donor of Sodium hydrosulfide; SW, sulfurous water.

the escape latency in 3×Tg-AD versus controls but showed only a trend to improve cognitive ability (Fig. 1a, b).

Twenty four hours after the Morris water test, animals were subjected to probe test. WT mice of both ages spent more time in quadrant where the platform was during the Morris test (original quadrant) respect to the 3×Tg-AD mice treated with saline (Fig. 1c, d), and also the number of crossings of this quadrant has been higher in WT mice respect to controls (Fig. 1e, f).

Animals of 15 months treated with sulfurous water and with H_2S donor sodium hydrosulfide showed both a significant enhancement of time spent in the original quadrant and a significant higher number of crossings with respect to control mice (Fig. 1d, f).

Motor activity. All mice after 1 h of probe test were placed in the pool with the platform visible to evaluate they ability to swim. All animals showed a similar time of escape latency and identical swimming speed in the Morris water maze compared with control mice (data not shown).

Toxicity. No signs of toxicity were recorded throughout the 3 months of treatment with sodium hydrosulfide or sulfurous water (e.g., ruffled fur, diarrhea, lethargy, aggressiveness, hypothermia, alterations of spontaneous locomotor exploration and grooming), and body weight variations throughout the study were similar in all experimental groups (data not shown).

Sulfurous Water Preserves Hippocampus and Cortex Morphological Alterations in a Mice Model of AD at both Ages

To investigate the brain morphological features of AD in 9- and 15-months-old mice, we processed the hippocampus (data not shown) and cerebral cortex (Fig. 2; 2 vulnerable regions of the AD brain) for histology. In both these areas and in both ages studied, in saline-treated 3×Tg-AD mice we found a number of neurons showing irreversible (pyknosis, nuclear dust) or reversible (swollen perikaryon, cellular shrinkage) alterations (Fig. 2c, i), and this led to a significant decrease of viable neurons (Fig. 2a). Saline-treated 3xTg-AD mice showed, in both ages and areas, a substantial amyloid deposit compared with WT mice (Fig. 2b–d, h–l).

On the contrary, treatments with sulfurous water significantly preserved morphological picture, with a higher number of viable neurons (Fig. 2a, g), and a significant reduction in amyloid deposit in cortex (Fig. 2b, e, f, h, m, n) compared with 3×Tg-AD saline-treated mice.

Treatments with sodium hydrosulfide did not significantly preserve hippocampus and cortex morphological alterations (not shown).

Sulfurous Water and/or Donor Sodium Hydrosulfide Reduce APP, Aβ. Tau and MAPK Expression/Activity Level in 3×Tg-AD Mice

In the hippocampus and cortex, we also investigated, by western blot analysis, the main proteins of the amyloid cascade and tau proteins. In both examined areas (hippocampus and cortex), at 9 and 15 months, 3×Tg-AD mice treated with saline showed a high phosphorylation level of APP activity (Fig. 3a, b) and high tau expression and tau activity phosphorylated at Ser396 (Fig. 3c, d), and treatment with sulfurous water and Na HS decreased significantly these markers (Fig. 3a–e).

In 3×Tg-AD mice treated with saline, the expression of Aβ1–42 (Fig. 4a–c) was significantly higher, compared with WT, both hippocampus and cortex. Treatments with sulfurous water or NaHS, significant decreased the expression of Aβ1–42 (Fig. 4a–c).

In neurodegenerative diseases, including AD, the MAPK play an important role in phosphorylation of tau protein [29]. It is well known that MAPK family members (JNK, p38 and ERK) are involved in the transcription regulation of several genes encoding for pro-inflammatory cytokines [29–30]. Our results showed, in the cortex and in the hippocampus of the saline-treated 3xTg-AD mice, at both age, an increase of activity of ERK (Fig. 5a, b), JNK (Fig. 5c, d) and P38 (Fig. 5f–g), as compared with WT mice. Treatment with sulfurous water or donor sodium hydrosulfide decreased significantly all these single MAPK (Fig. 5a–g).

Discussion

Alzheimer's disease is a multi-factor pathology because, not only neurology but also cardiology, ophthalmology and other medical specialties are involved in the study of its etiopathology and therapeutic approach [31–33]. Indeed, in recent years, this broad spectrum of studies has led to demonstrate that endogenous H_2S levels are decreased in the brains of patients and mice with AD [34, 20]. This suggests that the restoration of H_2S level could be a treatment for the Alzheimer's disease. Indeed, our previous data showed that sub-chronic and chronic treatments with H_2S counteract the progression of early AD in rat and mouse models [22]. Herein, our present study demonstrates, for the first time, that sodium hydrosulfide (a donor of H_2S) and sulfurous water (thermal-water rich of H_2S content), administered for 3 months consecutively, preserve or slow down brain alterations and consequent cognitive deficit, in a severe 3×Tg-AD mouse model. In these triplex transgenic 6-month-old mice, senile plagues began to appear, and after 9 months of age, the plagues of beta-amyloid are prominent with notable learning and memory impairments, inflammation, oxidative stress and neuronal loss [22, 24]. All together, these symptoms represent a severe level of AD progression in 3×Tg-AD mice. Our present results showed that the preservation or improvement in cognitive performance induced by H_2S's treatments is associated with a trend of hippocampus and cortex reduction of Aβ deposits, lowered phosphorylation of APP and tau protein, and diminished morphological damage including a decreased neu-

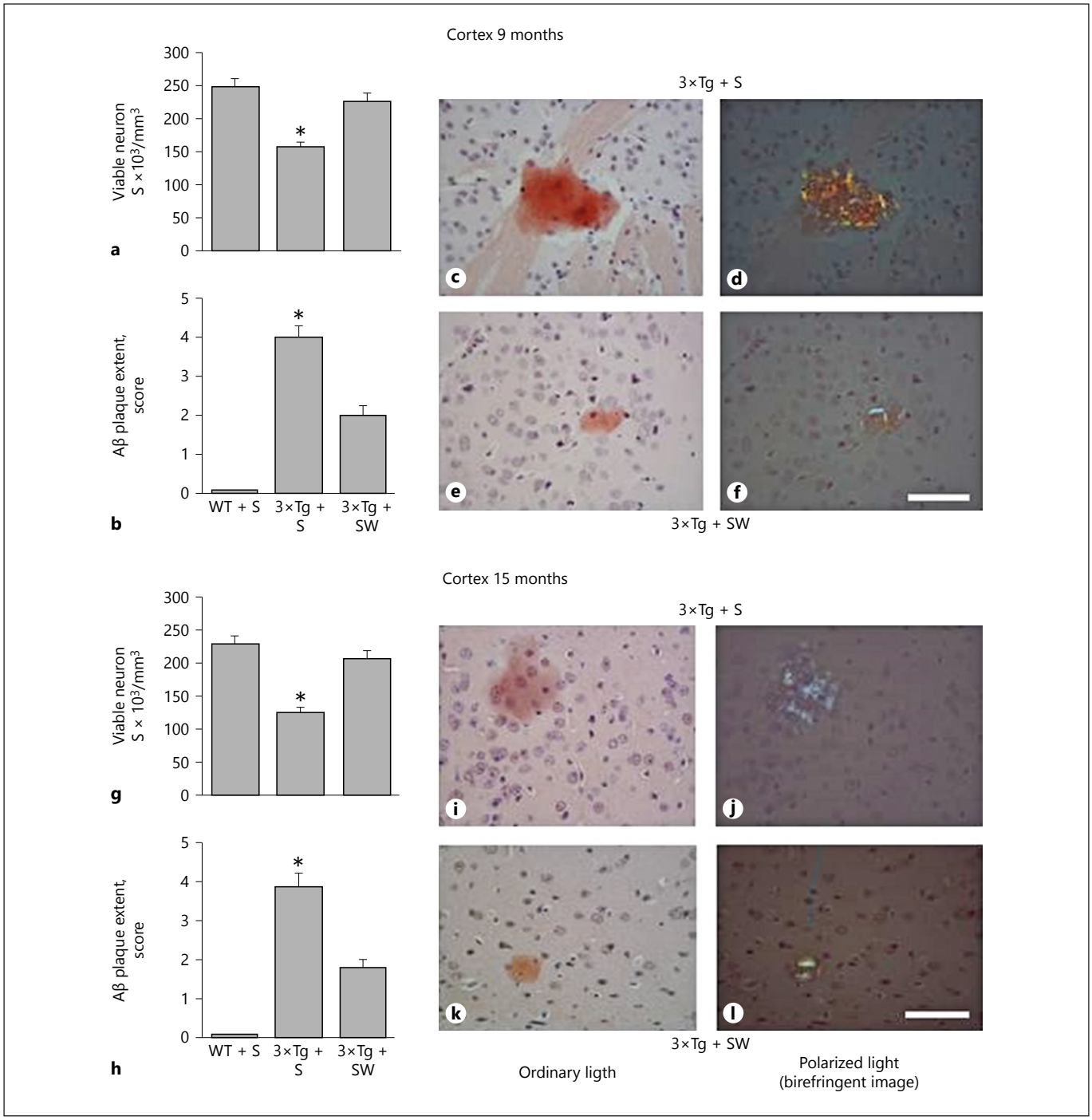


Fig. 2. Sulfurous water reduces histological damage and amyloid deposit in the cortex of 3×Tg mice at (**a**–**f**) 9- and (**g**–**n**) 15-month old. Histograms' height indicates mean values ± SEM of the number of viable neurons (**a**, **g**) and of Aβ plaques extent (**b**, **h**). Groups of 9-month-old mice $n = 12$, and groups of 15-month-old mice $n = 8$. **c**–**f** Representative histological pictures at 9 months of age: Aβ deposit in the sulfurous water-treated mouse both at ordinary (**e**) and polarized (F: birefringent image) light, compared with saline-treated one (**c**, **d**). **i**–**l** Representative histological pictures at 15 months of age: Aβ deposit in the sulfurous water-treated mouse both at ordinary (**k**) and polarized (N: birefringent image) light, compared with saline-treated one (**i**, **j**). Treatment is specified in the Figure 1. * $p < 0.05$, at least, versus the corresponding value of 3×Tg mice treated with saline. Scale bar = 100 μm. WT, wild type; 3xTg, 3xTg-AD; S, saline; SW, sulfurous water.

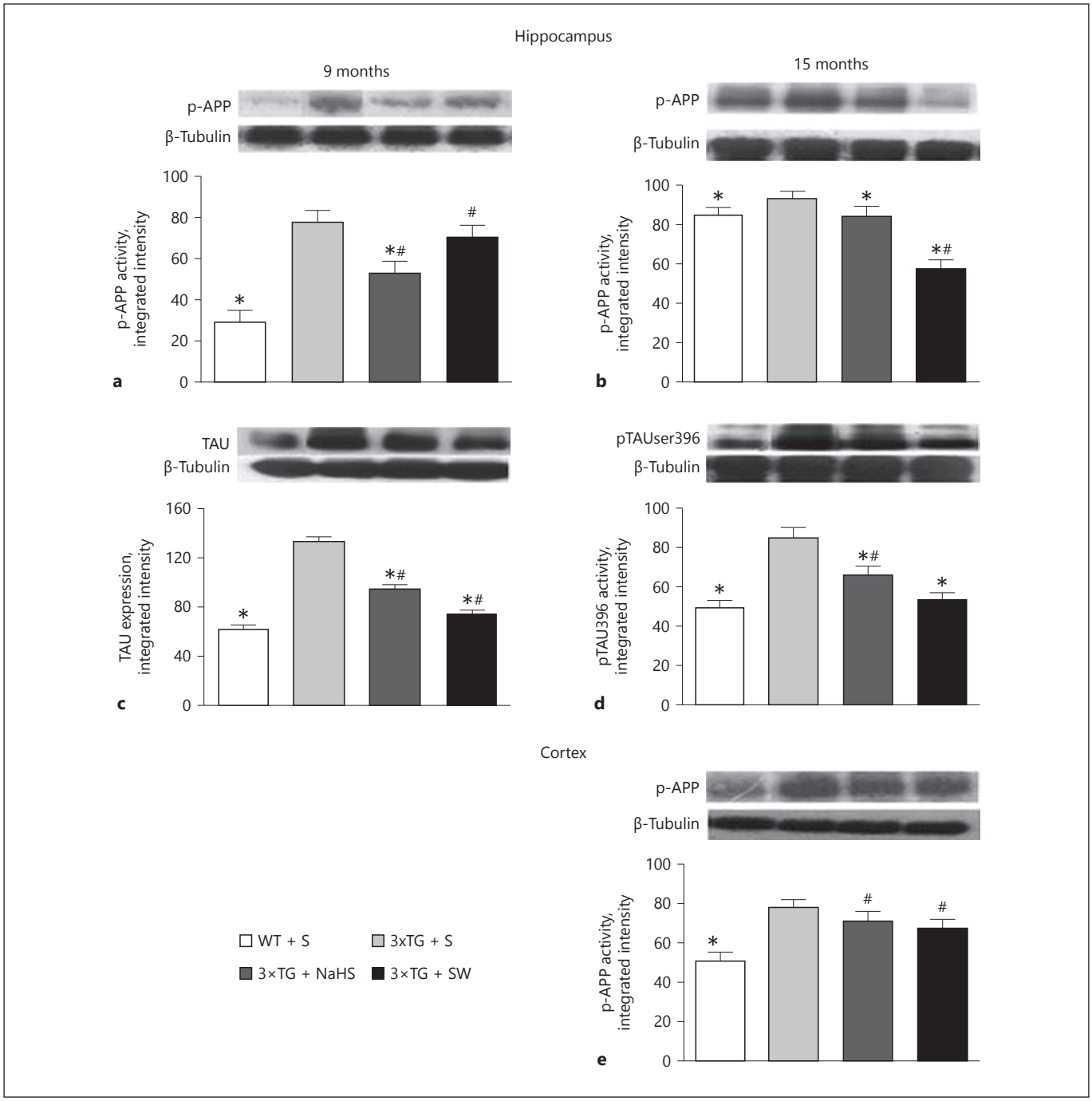


Fig. 3. Sulfurous water and NaHS reduce APP and Tau expression/activity in the hippocampus at 9 and 15 months (**a**–**d**) and in the cortex at 15 months (**e**) of 3×Tg-AD mice. Histograms' height indicates mean values ± SEM of p-APP and Tau. Groups of 9-month old mice $n = 12$, and groups of 15-month old mice $n = 8$. Treatment is specified in the Figure 1. The top of each panel shows representative immunoblots highlighting expression/activity of p-APP and of house-keeping gene product β-tubulin. * $p < 0.05$, at least, versus the corresponding value of 3xTg mice treated with saline. # $p < 0.05$, at least, versus the corresponding value of wild type animals treated with saline. WT, wild-type mice WT; 3×Tg, 3×Tg-AD mice; S, saline; NaHS, donor of Sodium hydrosulfide; SW, sulfurous water.

Pharmacology 2019;103:50–60
DOI: 10.1159/000494113

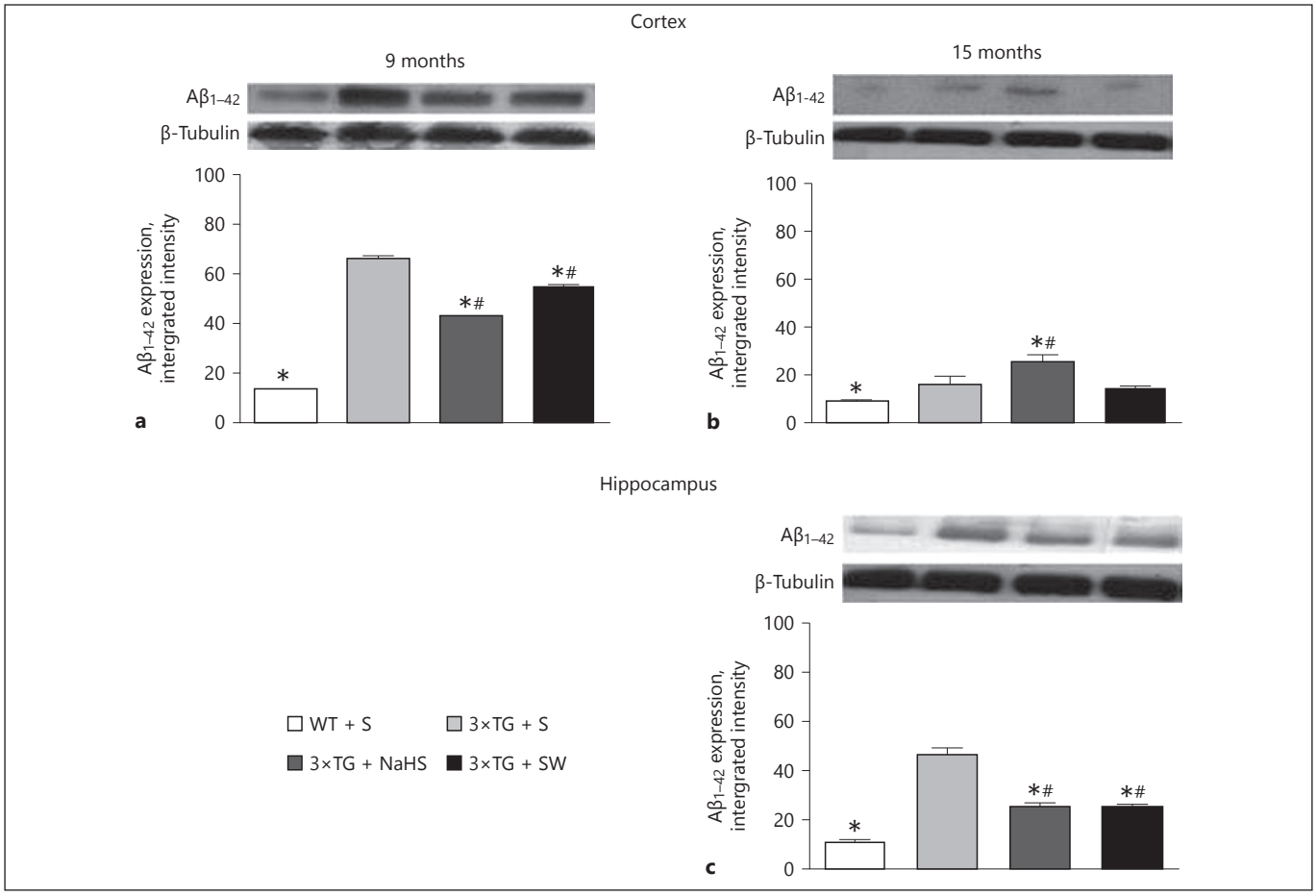
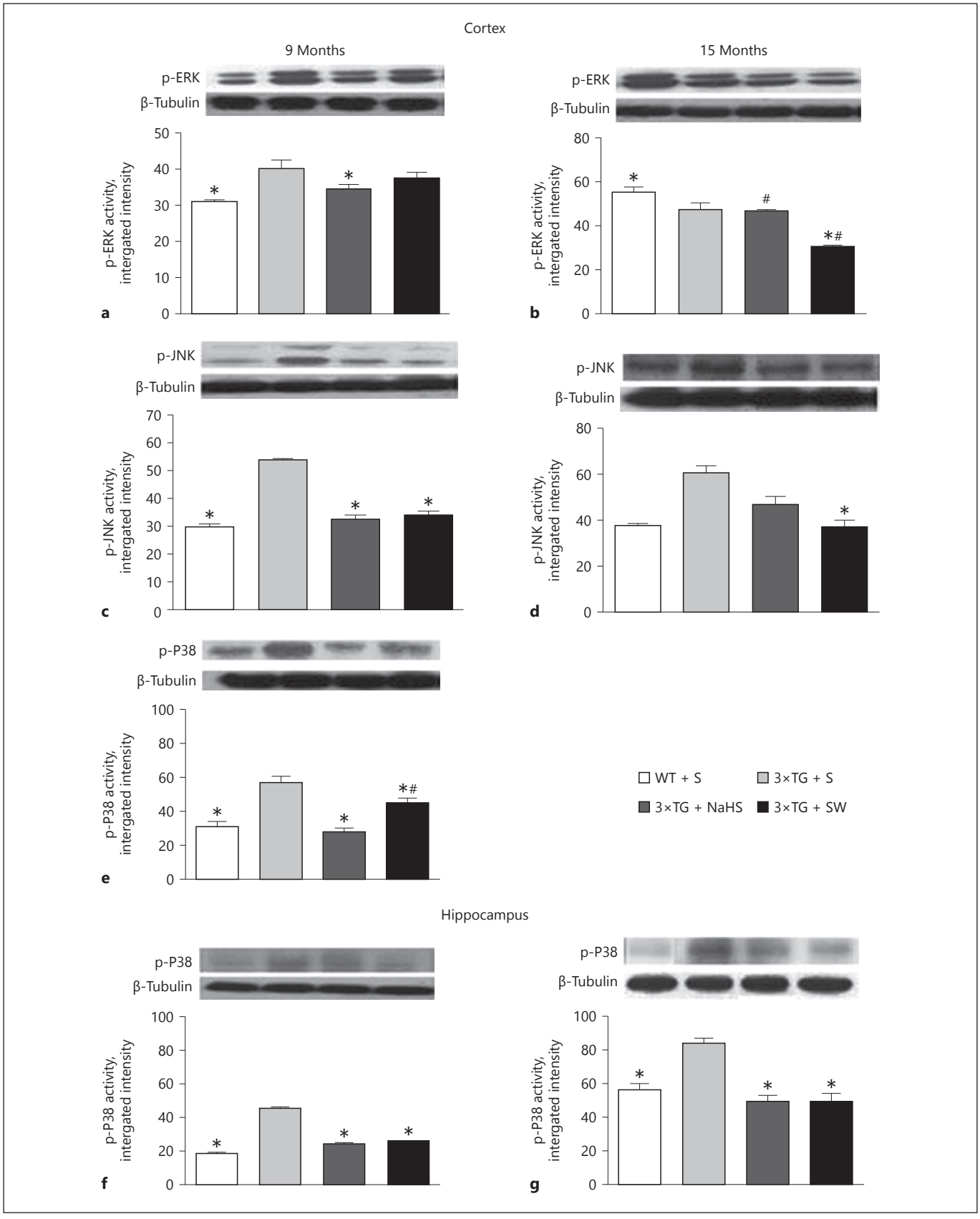


Fig. 4. Sulfurous water and NaHS reduce the Aβ1–42 expression in the hippocampus (**a**) and cortex (**a**, **b**) of 3×Tg-AD mice. Histograms' height indicates mean values ± SEM of Aβ1–42 expression (n = 12 and 8 mice per group respectively). Groups of 9-month-old mice n = 12, and groups of 15-month-old mice n = 8. Treatment is specified in the Figure 1. The top of each panel shows representative immunoblots highlighting the expression of Aβ1–42 and of house-keeping gene product β-tubulin. * $p < 0.05$, at least, versus the corresponding value of 3×Tg mice treated with saline. # $p < 0.05$, at least, versus the corresponding value of wild type animals treated with saline. WT, wild-type mice; 3xTg, 3xTg-AD mice; S, saline; NaHS, donor of Sodium hydrosulfide; SW, sulfurous water.

ronal death in the CA1 subfield. It well known that the 2 main events in AD pathophysiology are Aβ deposits and tau hyperphosphorylation and it has been demonstrated that Aβ and tau exert toxicity both with separate or merge mechanisms [22, 29, 30]. Indeed, in vitro and in vivo studies suggest that Aβ may lead to tau pathology, tau may mediate Aβ toxicity and together Aβ and tau may synergistically target different component of the same system [2, 29, 34]. In addition to the Aβ and tau-dependent mechanisms, there are other several etiopathogenic

Fig. 5. Sulfurous water and donor of sodium hydrosulfide reduce MAP-K (p-ERK, JNK and p-38) in the cortex (**a**–**e**) and in the hippocampus (**f**, **g**) of 3×Tg-AD mice. Histograms' height indicates mean values ± SEM of p-ERK, p-JNK and p-P38 activity. (n = 12 and 8 mice per group, respectively). Groups of 9-month old mice n = 12, and groups of 15-month old mice n = 8. Treatment is specified in the Figure 1. The top of each panel shows representative immunoblots highlighting the activity of p-ERK, p-JNK, p-P38 and of house-keeping gene product β-tubulin.* $p < 0.05$, at least, versus the corresponding value of 3xTg mice treated with saline. # $p < 0.05$, at least, versus the corresponding value of wild type animals treated with saline. WT, wild-type mice; 3×Tg, 3×Tg-AD mice; S, saline; NaHS, donor of Sodium hydrosulfide; SW, sulfurous water.

(*For figure 5a–g see next page.*)



5

Pharmacology 2019;103:50–60
DOI: 10.1159/000494113

mechanisms that could lead to AD, as apoptosis and inflammation [2, 6, 7, 22, 30]. Indeed, in this preclinical, transgenic AD study, we demonstrated, that, in a severe AD model, systemic chronic treatments with sulfurous-water rich in H_2S or NaHS induced an anti-inflammatory response, thereby reducing the activity of the MAPK members JNK, p38 and ERK, in the hippocampus and cortex at both ages considered. It is well known that MAPK members play an important role not only in inflammation but also in phosphorylation of tau protein. These biochemical and biomolecular results confirm important neuroprotective aspects of H_2S, and demonstrate the ability to counteract the main established AD-related mechanisms of brain damage as inflammation. This anti-inflammatory action of H_2S on brain strengthens the well-known systemic anti-inflammatory response of H_2S [35].

The identification of the sequence of events in the pathophysiology of AD is important for the novel identification of appropriate therapeutic approaches, leading to a series of potential therapies in AD because currently this sequence is still not well defined.

Several potential approaches for AD treatment such as cell and gene therapies are currently under investigation; however, a great number of compounds for AD failed to reach the goal [36].

One of the main aspects responsible for these failures of preclinical AD studies is that the compound under study affects only one of the pathophysiological responsible for neurodegeneration of AD, or shows toxic side effects [3, 29, 37, 38]. For this reason AD remains a major cause of disability and mortality without an effective treatment.

Therefore, a winning strategy could be to identify substances able to affect simultaneously more pathophysiological mechanisms of AD. Our present data shows that an H_2S donor and/or sulfurous water with high H_2S content, in a severe model of AD, improve cognitive activity by reducing Aβ deposits, hyperphosphorylation of APP and tau isoform at Ser396, as well as inflammatory responses. Noteworthy, the chronic treatment of 3 months did not show any evident signs of toxicity (perhaps a sign of toxicity could be glimpsed in the cortex of 15-month-old animals treated with NaHS where beta-levels are higher than those of transgenic control animals). Again, our data are in agreement with a large number of evidence confirming that gaseous signalling molecule H_2S exerts a cytoprotection in mammalian cells via multiple mechanisms (modulation of ion channels, enzymes, transcription factors and receptors) [10, 15, 16, 39–41]. For example, there is evidence demonstrating that H_2S is a phosphodiesterases inhibitor: in fact, levels of second messengers cAMP and cGMP increase [41]. Phosphodiesterases inhibition could be an early step leading to neuroprotective mechanisms induced by H_2S also in our experimental condition, and our next goal is to exam specifically this aspect.

A restraint of this work is that we administered the substances by i.p. injection to standardize doses; our next aim will be to study these substances administered by oral and inhaled route, which are routes commonly used in humans for sulfurous waters. Furthermore, levels of H_2S in plasma and brain are under investigation in our laboratory, and will be the focus of our next paper.

In conclusion, our data supports the hypothesis that endogenous H_2S might be physiologically involved in neuroprotection against AD and these positive results, obtained in a severe model of AD, should encourage further studies to better define the potential therapeutic value of H_2S alone or sulfurous waters with high H_2S content.

Acknowledgements

This work was supported by grants received from Fondazione per la Ricerca Scientifica Termale (FoRST), Roma, Italy.

Disclosure Statement

The authors state that they have no conflicts of interests to disclose.

References

1 Xiong C, Weng H, Bennett DA, Boyle PA, Shah RC, Fague S, Hall CB, Lipton RB, Morris JC: Subsets of a large cognitive battery better power clinical trials on early stage Alzheimer's disease. Neuroepidemiology 2014;43:131–139.

2 Ittner LM, Götz J: Amyloid-β and tau – a toxic pas de deux in Alzheimer's disease. Nat Rev Neurosci 2011;12:65–72.

3 Galimberti D, Ghezzi L, Scarpini E: Immunotherapy against amyloid pathology in Alzheimer's disease. J Neurol Sci 2013;333:50–54.

4 Citron M: Alzheimer's disease: strategies for disease modification. Nat Rev Drug Discov 2010;9:387–398.

5 De Strooper B, Vassar R, Golde T: The secretases: enzymes with therapeutic potential in Alzheimer disease. Nat Rev Neurol 2010;6:99–107.

6 Giuliani D, Bitto A, Galantucci M, Zaffe D, Ottani A, Irrera N, Neri L, Cavallini G M, Altavilla D, Botticelli AR, Squadrito F, Guarini S: Melanocortins protect against progression of Alzheimer's disease in triple-transgenic mice by targeting multiple pathophysiological pathways. Neurobiol Aging 2014;35:537–547.
7 Friedlander RM: Apoptosis and caspases in neurodegenerative diseases. N Engl J Med 2003;348:1365–1375.
8 Selkoe DJ, Hardy J: The amyloid hypothesis of Alzheimer's disease at 25 years. EMBO Mol Med 2016;8:595–608.
9 Gustavsson A, Svensson M, Jacobi F, Allgulander C, Alonso J, Beghi E, et al: Cost of disorders of the brain in Europe 2010. Eur Neuropsychopharmacol 2011;21:718–779.
10 Lowicka E, Beltowski J: Hydrogen sulfide (H2S) - the third gas of interest for pharmacologists. Pharmacol Rep 2007;59:4–24.
11 Kamoun P: Endogenous production of hydrogen sulfide in mammals. Amino Acids 2004;26:243–254.
12 Wu W, Hou CL, Mu XP, Sun C, Zhu YC, Wang MJ, Lv QZ: H2S donor NaHS changes the production of endogenous H2S and NO in D-galactose-induced accelerated ageing. Oxid Med Cell Longev 2017.
13 Eto K, Kimura H: A novel enhancing mechanism for hydrogen sulfide-producing activity of cystathionine beta-synthase. J Biol Chem 2002;277:42680–42685.
14 Moore PK, Bhatia M, Moochhala S: Hydrogen sulfide: from the smell of the past to the mediator of the future? Trends Pharmacol Sci 2003;24:609–611.
15 Martelli A, Testai L, Breschi MC, Blandizzi C, Virdis A, Taddei S, et al: Hydrogen sulphide: novel opportunity for drug discovery. Med Res Rev 2012;32:1093–1130.
16 Nicholson CK, Calvert JW: Hydrogen sulfide and ischemia-reperfusion injury. Pharmacol Res 2010;62:289–297.
17 Predmore BL, Lefer DJ, Gojon G: Hydrogen sulfide in biochemistry and medicine. Antioxid Redox Signal 2012;17:119–140.
18 Sowmya S, Swathi Y, Yeo AL, Shoon ML, Moore PK, Bhatia M: Hydrogen sulfide: regulatory role on blood pressure in hyperhomocysteinemia. Vascul Pharmacol 2010;53:138–143.
19 Kimura H: Hydrogen sulfide as a neuromodulator. Mol Neurobiol 2002;26:13–19.
20 Eto K, Asada T, Arima K, Makifuchi T, Kimura H: Brain hydrogen sulfide is severely decreased in Alzheimer's disease. Biochem Biophys Res Commun 2002;293:1485–1488.
21 Liu XQ, Liu XQ, Jiang P, Huang H, Yan Y: [Plasma levels of endogenous hydrogen sulfide and homocysteine in patients with Alzheimer's disease and vascular dementia and the significance thereof]. Zhonghua Yi Xue Za Zhi 2008;88:2246–2249.
22 Giuliani D, Ottani A, Zaffe D, Galantucci M, Strinati F, Lodi R, Guarini S: Hydrogen sulfide slows down progression of experimental Alzheimer's disease by targeting multiple pathophysiological mechanisms. Neurobiol Learn Mem 2013;104:82–91.
23 Artusi GC, Marenghi I, Pisaneschi M: [The sulfhydrate-sulfate-calcium waters of Tabiano (PR): recent analytical controls and therapeutic indications]. G Clin Med 1982;63:482–490.
24 Oddo S, Caccamo A, Shepherd JD, Murphy MP, Golde TE, Kayed R, et al: Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. Neuron 2003;39:409–421.
25 Giuliani D, Mioni C, Altavilla D, Leone S, Bazzani C, Minutoli L, et al: Both early and delayed treatment with melanocortin 4 receptor-stimulating melanocortins produces neuroprotection in cerebral ischemia. Endocrinology 2006;147:1126–1135.
26 Giuliani D, Ottani A, Mioni C, Bazzani C, Galantucci M, Minutoli L, et al: Neuroprotection in focal cerebral ischemia owing to delayed treatment with melanocortins. Eur J Pharmacol 2007;570:57–65.
27 Giuliani D, Zaffe D, Ottani A, Spaccapelo L, Galantucci M, Minutoli L, et al: Treatment of cerebral ischemia with melanocortins acting at MC4 receptors induces marked neurogenesis and long-lasting functional recovery. Acta Neuropathol 2011;122:443–453.
28 Vorhees CV, Williams MT: Morris water maze: procedures for assessing spatial and related forms of learning and memory. Nat Protoc 2006;1:848–858.
29 Yu Y, Run X, Liang Z, Li Y, Liu F, Liu Y, et al: Developmental regulation of tau phosphorylation, tau kinases, and tau phosphatases. J Neurochem 2009;108:1480–1494.
30 Munoz L, Ammit AJ: Targeting p38 MAPK pathway for the treatment of Alzheimer's disease. Neuropharmacology 2010;58:561–568.
31 Cermakova P, Eriksdotter M, Lund LH, Winblad B, Religa P, Religa D: Heart failure and Alzheimer's disease. J Intern Med 2015;277:406–425.
32 Ho WL, Leung Y, Tsang AW, So KF, Chiu K, Chang RC: Review: tauopathy in the retina and optic nerve: does it shadow pathological changes in the brain? Mol Vis 2012;18:2700–2710.
33 Sivak JM: The aging eye: common degenerative mechanisms between the Alzheimer's brain and retinal disease. Invest Ophthalmol Vis Sci 2013;54:871–880.
34 Zipp F, Aktas O: The brain as a target of inflammation: common pathways link inflammatory and neurodegenerative diseases. Trends Neurosci 2006;29:518–527.
35 Gemici B, Elsheikh W, Feitosa KB, Costa SK, Muscara MN, Wallace JL: H2S-releasing drugs: anti-inflammatory, cytoprotective and chemopreventative potential. Nitric Oxide 2015;46:25–31.
36 Glat MJ, Offen D: Cell and gene therapy in Alzheimer's disease. Stem Cells Dev 2013;22:1490–1496.
37 Blennow K: Biomarkers in Alzheimer's disease drug development. Nat Med 2010;16:1218–1222.
38 Sperling RA, Karlawish J, Johnson KA: Preclinical Alzheimer disease – the challenges ahead. Nat Rev Neurol 2013;9:54–58.
39 Szabó C: Hydrogen sulphide and its therapeutic potential. Nat Rev Drug Discov 2007;6:917–935.
40 Xuan A, Long D, Li J, Ji W, Zhang M, Hong L, et al: Hydrogen sulfide attenuates spatial memory impairment and hippocampal neuroinflammation in β-amyloid rat model of Alzheimer's disease. J Neuroinflammation 2012;9:202–213.
41 Bibli SI, Yang G, Zhou Z, Wang R, Topouzis S, Papapetropoulos A: Role of cGMP in hydrogen sulfide signaling. Nitric Oxide 2015;46:7–13.