The role of hydrogen sulfide as a potential therapeutic agent and as inducer of a hibernation-like state to prevent Alzheimer's disease.

Bachelor thesis

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Introduction

Hibernation learnt us some interesting facts about the protective role of hydrogen sulfide (H_2S). For a long time H_2S was associated with its toxic character. However, hibernation research showed us that it contributes to remodeling of the lungs, acts as anti-inflammatory agent (1) and that it protects the kidneys against organ damage during hibernation (2). It is also known that H_2S protects cells of hibernators against oxidative damage when their metabolism is suppressed (3). Although, there is not known much about the function of H_2S in the hibernating brain. Interestingly is that it's also possible to induce a hibernation-like state in non-hibernators through inhalation of H_2S, which will not lead to permanent damage (4). However, a more recent study showed that pharmacological induction of hibernation with 5'AMP is not H_2S dependent, and therefore H_2S is not essential to mimic a hibernation-like state (2). H_2S Induced hibernation enables mice to survive in environments with 3% oxygen. Those mice had no behavioral disorders, which suggest that inhalation of H_2S will

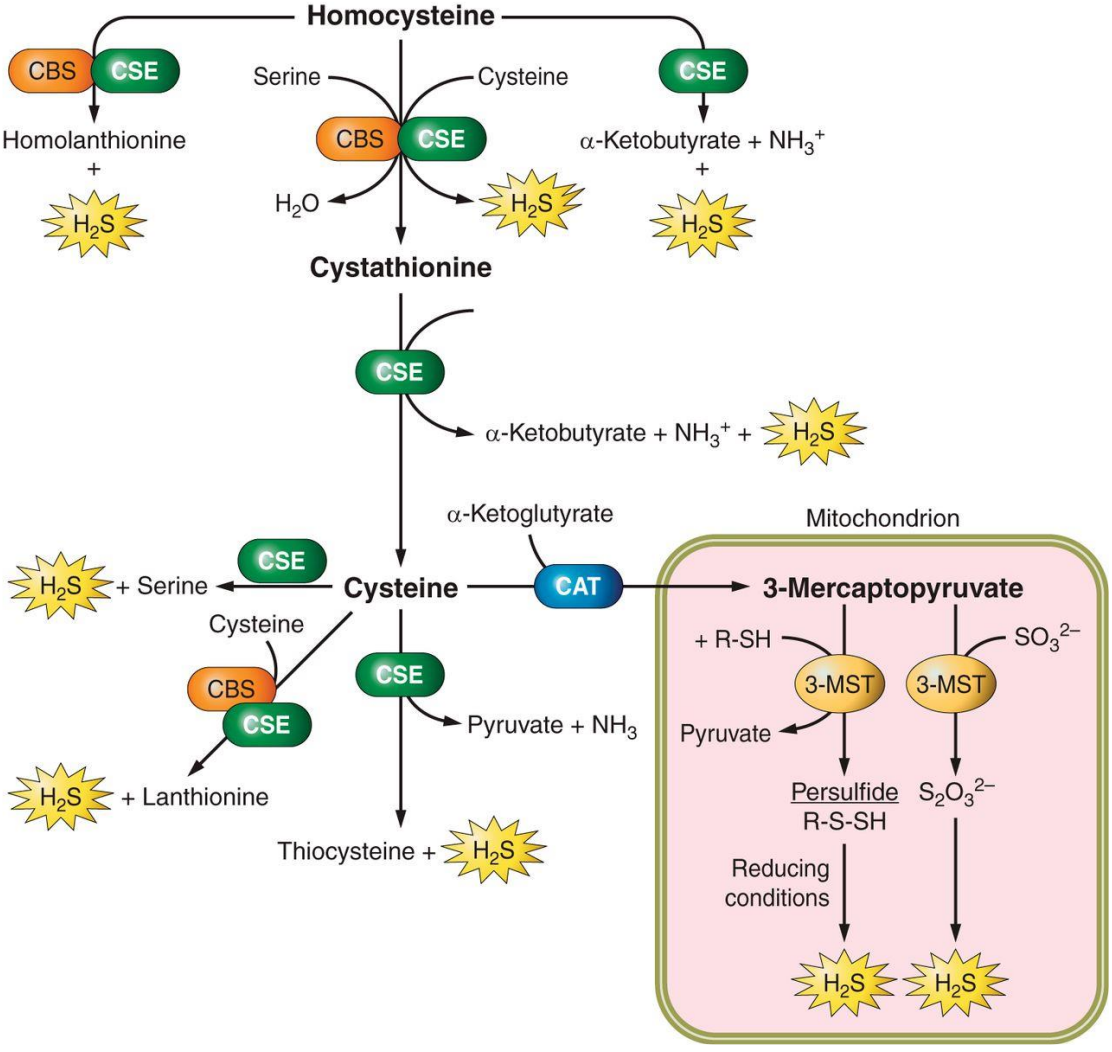

Figure 1 H_2S pathway. *cystathionine-β-synthase (CBS), cystathinonine-γ-lysase (CSE) and 3-mecaptopyruvate sulfurtransferase (3-MTS) are the main enzymes which catalysis the production of H_2S. CBS and CSE generate H_2S through transsulfuration of homocysteine. Further CBS and CSE are able to generate homolanthionine with H_2S from homocysteine and to generate lanthionine and H_2S from cysteine. CSE alone generates H_2S from homocysteine, cystathionine and cysteine. And CAT in combination with 3-MST generate H_2S within the mitochondria* (12).

not affect the brain (5). Squirrels as natural hibernators on the other hand, lose their synapses during hibernation (6) and the microtubule-associated protein tau becomes hyperfosforylated (7) (8). The remarkable thing of hibernation is that during arousal the squirrels repair their damage. Synapses of hibernating squirrels fully recover during arousal (9) and tau tangles will be dephosphorylated (7). Also 5'AMP induced hibernating mice undergo reversible tau phosphorylation during their

hibernation-like state (10). Therefore it is suggested that hibernation research can contribute to find therapeutic agents for Alzheimer's diseases (AD), as hallmarks of AD are similar to brain damage during hibernation. Although H_2S is not essential to induce this hibernation-like state, without endogenous H_2S production there will be a lot of organ damage (2). In this review I will discuss the protective role of H_2S to prevent AD and how hibernation can help us to understand this mechanism.

Endogenous H_2S production

First, H_2S is produced by enzymes in the cytosol called cystathionine-β-synthase (CBS) and cystathinonine-γ-lysase (CSE) and more recently it is found that H_2S is produced through cysteine aminotransferase (CAT) and 3-mecaptopyruvate sulfurtransferase (3-MTS), which is a mitochondrial enzyme (11). CBS is needed to catalyze homocysteine to cysteine and CSE to catalyze cysteine to H_2S. Furthermore, cysteine is deanimanted to mercaptopyruvate by CAT and subsequently, H_2S is produced within the mitochondria (fig. 1) (12). Different cell types are responsible for H_2S production. In the brain astrocytes are the main H_2S producing cells (13) but also by neurons and endothelial cells. Endothelial cells probably use H_2S for relaxation of the blood vessels in the brain. Astrocytes together with endothelial cells use H_2S to control the local blood flow in active brain arias. And neurons use H_2S for anti-oxidant and anti-inflammatory activity (14). AD patients, however, have decreased endogenous H_2S levels in their brain (15) which may play a role in the pathogenesis of AD.

Alzheimer's disease

The neuronal damage Alzheimer patients undergo is similar to the brain damage hibernating animals develop during their torpor phase. Considering that hibernators can repair their neuronal damage during arousal, there should be a mechanism that can repair the same damage in AD patients as well. Intracellular tau aggregation, which also occurs in hibernators, caused by extracellular β-amyloid aggregation leads to AD whereby neurons degrade and subsequently undergo apoptosis. Tau is a microtubule-associated protein which becomes hyperphosphorylated, insoluble and filamentous in cases of AD (16). Furthermore, AD patients have increased levels of β- and γ-secretases, which leads to β-amyloid plaque formation and intracellular APP domains (AICDs) (17)(fig. 2). When there is more α-secretase compared to β and γ, there will be less Aβ formed, as α-secretase cleaves APP into sAPPα and membrane-bound 83 amino acid fragment (C83) (18). The loss of layer-II pyramidal neurons, due to inflammation, oxidative stress and apoptosis (14), starts in the specific brain area called entorhinal cortex and subsequently, neurons in the CA1 region of the hippocampus are affected by this disease (19). The more the disease progresses, the temporal, partial and frontal association lobes undergo neurodegeneration because these areas have highly myelinated neurons (20) (21) (22). The limbic neurons in the hippocampus and association cortex, which are poorly myelinated and which are needed for memory and learning, are damaged in the first phase of AD (22). In this review, I will discuss the protective role of H_2S as a therapeutic compound concerning apoptosis, inflammation and oxidative stress in AD, as H_2S levels are increased in hibernators (1) but they have mechanisms to protect themselves against those 3 symptoms (23).

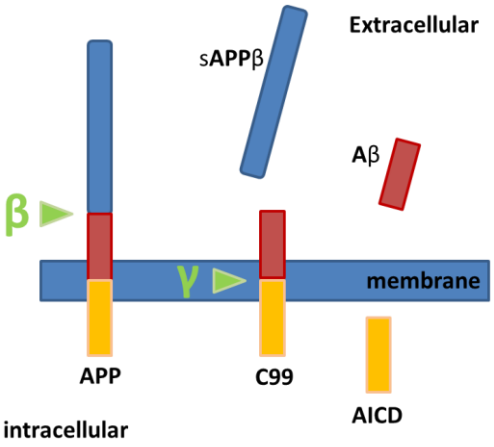

Figure 2 Formation of toxic Aβ through cleavage of the amyloid precursor protein (APP). *β-secretase 'cuts' the APP protein whereby sAPPβ is formed and the 99 amino acid fragment (C99) is still membrane-bound. γ-secretase subsequently cleaves C99 whereby Aβ and AICD are generated*. High levels of extracellular Aβ will lead to β-amyloid plaques in the brain **(18)**.

Protective role of H_2S in Alzheimer's disease

In 2013 researcher already showed that the apoptosis promoter factors BAX and caspase-3 were highly expressed in AD mice and Bcl-2, an apoptosis suppressor factor, reduced. After treatment with Tabiano's spa-water, which contains a high dose of H_2S, those factors showed similar levels as the wild-type mice. The same applies for the inflammation factor TNF-α which was also overexpressed in AD mice but reduced after spa-water treatment. Furthermore, this research showed that H_2S is able to reduce the levels of malondialdehyde and nitrite in the cortex of AD mice, which indicates that H_2S protect the brain against free radicals. Taken together, this research exhibited the 3 of the protective features of H_2S namely anti-apoptosis, anti-inflammation and anti-oxidation (24) (25). Therefore H_2S is interesting to discuss as a potential therapeutic compound for AD, as the neurons of AD patients become inflamed and undergo oxidative stress due to Aβ plaque formation and tau hyperfosforylation. Subsequently, the inflammation and oxidative damage leads to neurodegeneration and finally the neurons undergo apoptosis (26).

Inflammation

First the anti-inflammation property of H_2S. The study of McGeer used immunohistochemistry to illustrate the immune response in AD. They stained the Aβ-plaques present in the brain and they also stained complement factors and microglia which are both part of the immune system in the brain. They found that Aβ-plaques activates the complement system which subsequently activates microglia and thereby inflammation occurs which in turn results in neuronal damage (27). By knowing this, the following study showed that NaHS, an H2S donor, reduces TNF-α, IL-1β, and IL-6 which are released as pro-inflammatory cytokines in the hippocampus of rats injected with Aβ. Furthermore, this study showed that NaHS also inhibits the Aβ-upregulated inflammation enzyme COX-2 expression in the hippocampus. NF-kB plays an important role in the production of cytokines and COX-2 and is also overstimulated in the Aβ injected mice. However, NaHS treatment brings NF-kB levels back to normal (28). An earlier study confirms that inhibition of the NF-κB activity through NaHS results in a decreased production of the cytokines TNF-α and IL-1β (29).

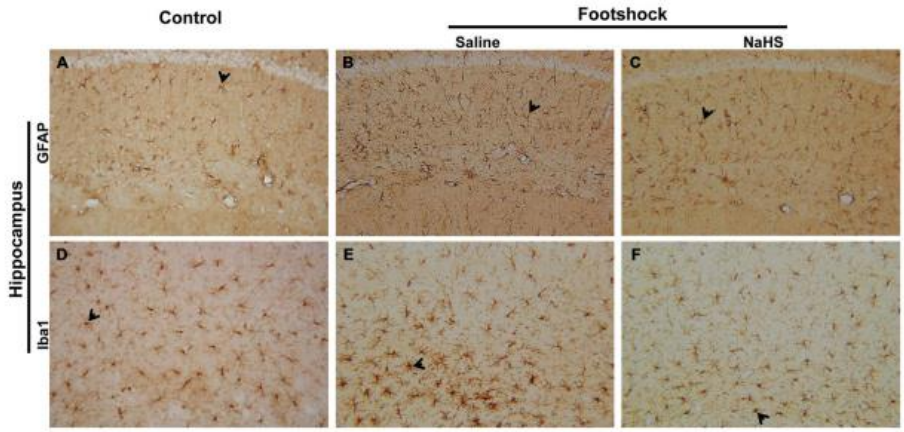

Figure 3 Anti-inflammatory role of H2S in the brain of mice with high Aβ level. *Activated astrocytes(A,B,C) and microglia (D,E,F), indicated with arrows, are reduced to normal after NaHS treatment compared with the saline, untreated, group in the CA1 region of the hippocampus of the mice.*

A more recent study gives AD mice inescapable footshocks to increase the toxicity of oligomeric Aβ and uses this model for NaHS treatment. They also found increased IL-6 levels in the plasma of those mice which were decreased after NaHS treatment. Also activated astrocytes and microglia were decreased after NaHS injections (fig. 3). However, their results suggest that the increased Aβ40 toxicity will not affect the levels of COX-2 and NF-kB. Taken together, mild stress through inescapable footshocks stimulates the pathology of AD but can be reversed by fighting the inflammation with NaHS (30).

Yang et al. suggested that there is a possibility that the inflammation inhibitory effect of NaHS stimulates the expression of GluN2B-containg NMDA receptors in the hippocampus and thereby increases the synaptic plasticity. Long-term potentiation (LTP) contributes learning and memory and is regulated by synaptic plasticity. The ability of hippocampal-depended learning and memory decreases when AD progresses (31). TBS was used to get a NMDA receptor dependent LTP in WT and APP/PS1 mice, with or without NaHS treatment. They found no big difference between the NaHS treated groups and the WT. But they demonstrated a decrease in hippocampus NMDA receptor dependent LTP of the untreated APP/PS1 mice, indicating that NaHS is needed to restore LTP levels in AD mice to the normal levels in WT mice. The following western blot (fig 4.) showed that NaHS only interacts with the GluN2B subunit, as the expression of GluN2B increases to normal levels after APP/PS1 mice were injected with NaHS (32). Taken together, increased expression of GluN2B, due to H_2S, contributes to the anti-inflammation effect in AD. Furthermore, this overexpression compensates for the decreased levels of GluN1 and GluN2A.

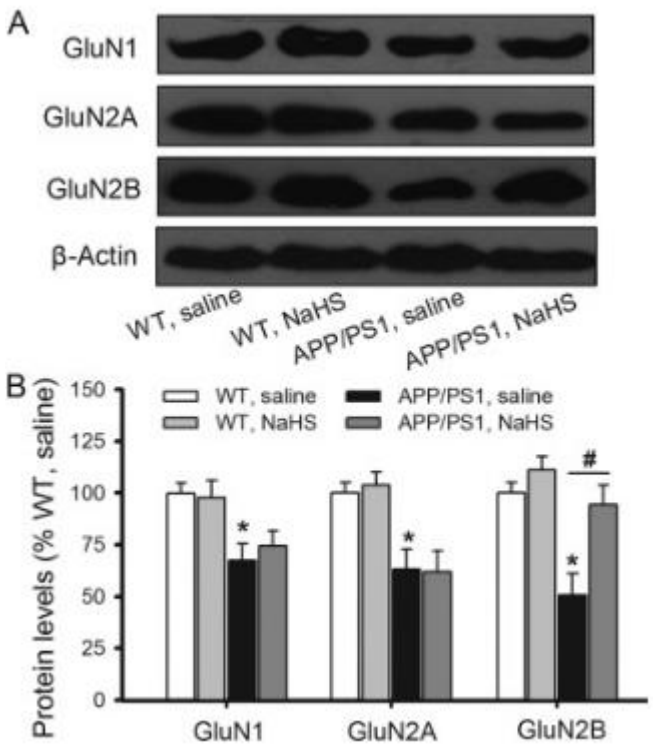

Figure 4 GLuN2B epression decreases after NaHS treatment, whereas GluN1 and GLuN2A were not affected by NaHS. *A: Protein band from hippocampal tissue of WT and APP/PS1 mice. B: Protein levels the hippocampus of compared between WT and APP/PS1 mice.GLuN1, GLuN2A and GLuN2B levels were decreased in AD mice but GluN2B became increased and were similar to normal levels after NaHS treatment* **(32)**.

Oxidative stress

Prior to β-amyloid plaque formation, oxidative stress occurs in the neurons of AD patients (33). There are 2 theories about increased oxidative stress in the pathology of AD. 1; β-amyloid itself stimulates the oxidative stress and 2; AD patients have a decrease of antioxidants in their brain (34). AD patients do not only have oxidative stress in their neurons but also in their peripheral blood mononuclear cells. Therefore the following study (35) used PBMCs of AD patients to test the anti-oxidative feature of H_2S, as PBMCs have similar physiological and biochemical characteristics as neurons. They found that DNA oxidative damage was increased in PBMCs of AD patients compared to control PBMCs. They used pro-oxidant molecules to induce oxidative stress into PBMCs, which results in oxidative DNA damage and loss of cell viability. Thereafter they expose the PBMCs to sulfurous mineral-medical water (SW), which contains H_2S, and found that this treatment results in less oxidative DNA damage. In addition, SW protects the cell viability after administrations of the pro-oxidants (35). It is known that aging results in fewer anti-oxidants (36), so it is thought that SW is only capable of restoring the reduced anti-oxidants and has no effect on the underlying processes in AD.

Another research used chronic unpredictable stress (CUMS) rats as a model to induce oxidative stress in the hippocampus, whereby hippocampal neurons undergo apoptosis. It is also known that CUMS rats have deviating endogenous H_2S level in their hippocampus. Therefore they used NaHS to protect CUMS rats for oxidative hippocampal damage. They found that BDNF expression was reduced after NaHS treatment and that inhibition of the BDNF-TrkB pathway inhibits the anti-oxidant effect of H_2S (37). However, TrkB knock-out mice showed no effect on β-amyloid plaques but only reduction of

BDNF (38). Thus reduction of oxidative stress through inhibition of the BDNF-TrkB pathway with H_2S has no effect on the underlying causes of AD, like reduction of β-amyloid plaques.

Apoptosis

It is known that the neurons of AD patients undergo apoptosis as a final result of Aβ plaques. APP/PS1 mice were also used as an AD mice model in the study of He et al. APP/PS1 mice have an increased production of β-amyloid and behavior abnormalities correspond with AD. They treated 6 and 12-month-old mice with NaHS and stained their brain sections with antibodies directed to capase-3, an apoptosis marker, and found that fewer neurons undergo apoptosis after treatment (fig. 5). Therefore they suggest that NaHS protects neurons from apoptosis and thus needed to restore the reduce H2S concentrations in AD (39)

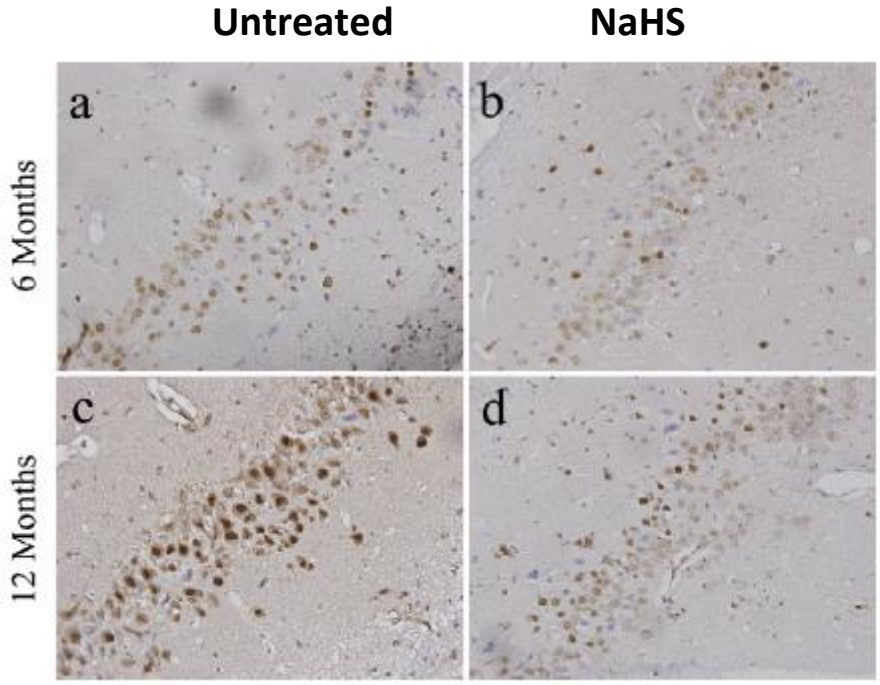

Figure 5 NaHS treatment results in less neuronal apoptosis in both 6 and 12-month-old APP/PS1 mice. *Brain tissue of AD mice was stained for the apoptosis marker caspase-3 (brown). A: Untreated 6 months old mouse demonstrated higher levels of caspase-3 then B: a 6 months old NaHS treated mouse. The same applies for 12 months old mice (C-D)* **(39)**.

More recent they found that NaHS activates the PI3K/Akt pathway and thereby decreases BACE1 and PS1 levels in the brain. Furthermore, they showed that ADAM17 levels became increased after NaHS treatment. These findings suggest that mice with decreased levels of β- and γ-secretase (BACE1 and PS1), enzymes which cut the APP protein into toxic β-amyloid, develop less Aβ plaques in their brain (40). When there is no plaque formation, neurons will not degenerate and finally undergo apoptosis (41) (42). In the same study they found less caspase-3 expression in mice with decreased levels of β- and γ-secretase, which support this suggestion (40). In addition another study also showed that H2S protect microglia cells against Aβ plaque formation (43). To prove that NaHS indeed decreases those APP secretases of the amyloidogenic pathway via the PI3K/Akt, they inhibit this pathway. After inhibition of the PI3K/Akt pathway, NaHS had no decreasing effect on BACE1 and PS1 anymore. Contrary, blocking the PI3K/Akt pathway result in increased levels of the β- and γ-secretases after NaHS treatment (40).

Spatial memory

Furthermore, H_2S treatment improves spatial memory in mice AD models, which is highly affect trough the loss of neurons in the hippocampus of AD patients (22). However, as described before H_2S functions as an anti-oxidation, anti-inflammation and anti-apoptosis agent and thereby it slows down he progression of AD. Through performing spatial tests it is possible to investigate if the hippocampus of H_2S treated AD mice is functional again, as spatial learning is hippocampal dependent (44). The described studies stimulates the idea that fighting inflammation, oxidative stress and apoptosis in neurons of AD mice results in improved spatial memory. First, protecting neurons to become inflamed in 'AD mice' under footshock stimuli results in improvement of spatial learning. By using the Moris Water Mase (MWM) test, it is found that untreated AD mice had many difficulties to find an invisible platform in the water, which confirmed that AD indeed affects the hippocampus. NaHS treated mice, however, were able to learn where the platform was hidden, so H_2S is able to reverse the damage in AD. Furthermore, the non-footshock group learned quicker where the platform was then NaHS treated mice and therefore this study did not prove that H_2S reverses all the hippocampal damage (30). Two other studies support the idea that the anti-inflammation effect of H2S is responsible for improved spatial learning. They demonstrated a correlation between IL-6 reduction and improved cognition and behavior in AD mice and rats (45) (46).

Further, the same study demonstrated that the anti-oxidation feature of H2S improves spatial memory in rats through reduction of asymmetric dimethylarginine (ADMA). It is known that ADMA inhibits nitric oxide synthase (NOS) whereby nitric oxide (NO) cannot be produced (46). NO is a pro-oxidant molecule and dysregulation of NO generation will lead to neurodegeneration (14). This research showed that high levels of ADMA are linked with oxidative stress and also neuro-inflammation. Moreover, low levels of ADMA are linked with better cognitive performance (46). These results indicate that reduction of oxidative stress through H_2S treatment reduces the hippocampal damage in AD and improves thereby spatial learning and memory.

Furthermore, He et al. demonstrated that H_2S removes β-amyloid plaques by decreasing β- and γ-secretase so the neurons of AD mice will not undergo apoptosis. In the same mice, the found that their spatial memory increases after H_2S treatment (40), which links the anti-apoptosis feature of H_2S with spatial memory improvement.

Taken together, H_2S inhibits inflammation, oxidative stress and apoptosis whereby β-amyloid plaques will be removed and the AD pathology will be halted. However, another hypothesis is that spatial memory will be improved by using mitochondrially targeted H_2S donor compounds. In addition, this treatment method reverses the progression of AD, whereas other H_2S compounds only end the progression of AD in its current phase. Therefore mitochondrially targeted H_2S compounds are promising therapeutics to cure AD.

Mitochondrially targeted H_2S donor compounds

Zhao et al. used AP39, a compound which contains the mitochondria-targeting compound triphenylphosphonium (TPP+) and which also donates H_2S, and found that this compound increases H_2S in neurons but also in mitochondria of APP/PS1 mice. Further, they found that AP39 improves spatial memory in those AD mice through using the MWM test (fig. 6). In addition, AP39 inhibits brain atrophy and reduces the levels of Aβ. However, after AP39 treatment there were still β-amyloid plaques visible in AD mice. Therefore, the doses of AP39 should be increases in testing if higher doses can reverse the β-amyloid plaque formation. They already found that 100 nM reverses the spatial memory impairment and that lower concentration of AP39 showed similar MWM test results as untreated AD mice, but they did not try a higher concentration of AP39. Nonetheless, AP39 can inhibit β-amyloid plaque formation and reduction of brain volume in an early phase of AD whereby the pathology will not further develop. It is known that Aβ will accumulate in mitochondria whereby the production of energy is decreased. Reactive oxygen species (ROS) however are increases in mitochondria. By targeting the mitochondrial in the brain with AP39, neuronal ATP was increased and ROS levels were decreased, which suggest that stimulating mitochondria with AP39 results in

reparation of oxidative damage (47). This study made not completely clear if all those therapeutic effects are due to the mitochondrially targeted H_2S donor compound AP39, or H_2S itself. Therefore, AP39 should be compared for example to NaHS.

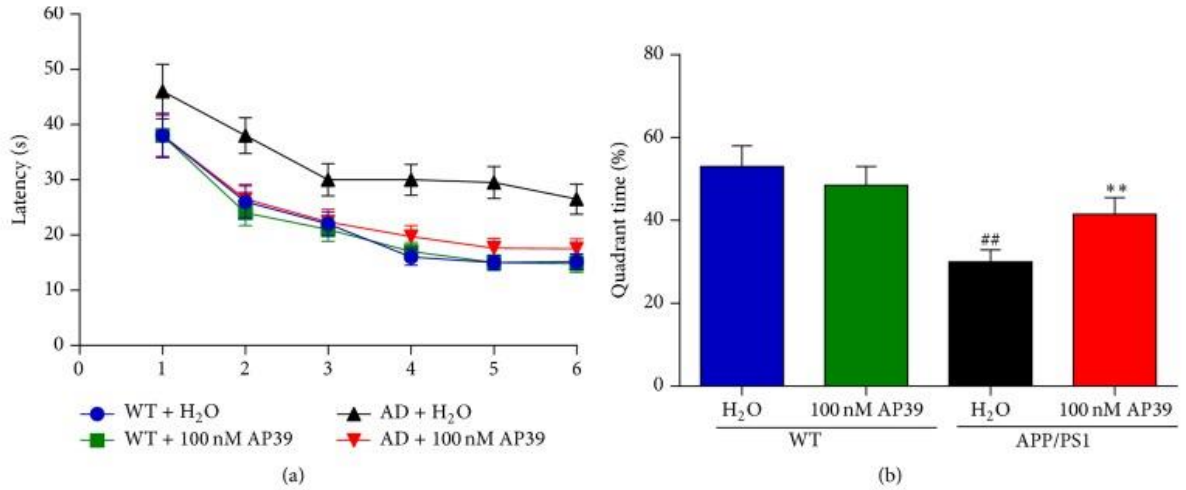

Figure 6 AD mice, treated with AP39, showed better MWM test results compared to untreated AD mice.
A: AP39 treatment in AD mice decreases the latency time, time which was needed to find the hidden platform, compared with untreated AD mice, which suggest that AP39 increases their spatial memory.
B: WT mice and the AP39 treated AD mice spend the same time in the target quadrant. Untreated AD mice search more random and spend thereby less time in the target quadrant. Therefore this study proved that treatment with the mitochondrially targeted H_2S donor compound AP39 reverses the spatial memory impairment.

How hibernation research helps us to improve the therapeutic effect of H2S

Finally, 5'AMP induced hibernation in mice showed reversible tau-phosphorylation (10). And as there is observed more organ damage in pharmacological induced hibernating hamsters, where endogenous H_2S production is blocked (2), we can assume that H2S plays an important role in the reversibility of hyperphosphorylated tau. Also reduction in hyperphosphorylated tau was found in AD mice treated with H_2S (24) (30), although the phosphorylation was not reversed. Therefore, probably more mechanisms are involved in 5'AMP hibernation to dephosphorylate tau and to protect neurons against oxidative stress, inflammation, and apoptosis. Of course, the metabolism of 5'AMP induced hibernating mice is enormous depressed, as demonstrated in the study of Boerema et al. (10), which probably correlates with reversed tau phosphorylation. There is no decreased metabolism reported in H_2S treated AD mice which were able to reduce tau hyperphosphorylation (24) (30) but not to reverse it. Therefore it will be helpful to investigate the metabolism depressing role of H_2S in those mice and if H_2S act via the A1 adenosine receptors (A1AR) in the brain. Olson et al. demonstrated that 5'AMP at least does. They showed that signals via A1AR lead to metabolic depression and induction of torpor in squirrels (48) (49). However, AP39 was able to completely reverse spatial memory in AD mice, and therefore it will be more interestingly to investigate mitochondrially targeted H_2S donor compounds in combination with suppressing the metabolism. This combination may lead to reversible tau phosphorylation and stronger anti-oxidation, anti-inflammation and anti-apoptosis properties. Therefore, research about putting the brain into a hibernation-like state would be helpful for further Alzheimer research and to investigate the role of H_2S in this process.

More helpful is to unravel the mechanism of 5'AMP induced hibernation which leads to reversible brain damage in AD. Furthermore, 5'AMP is less toxic then H_2S, which is more useful to create new therapeutics for AD. However, it is not tested if 5'AMP induced mice reverse their spatial memory after they aroused, which still needs to be done. Natural hibernators already showed that the did not lose their hippocampal-dependent memory during hibernation (50), and therefore we perfume that pharmacologically induced hibernators also retain their spatial memory.

Conclusion

This review described the anti-oxidation, anti-inflammation and anti-apoptosis feature of H_2S and how H_2S increases spatial memory in AD. Therefore H_2S is a potential therapeutic compound for AD. Mitochondrially targeted H_2S donor compounds, however, showed even more promising feature to cure AD. In addition, this research described the collaboration between H2S and hibernation and how future hibernation research can lead to improvement of the understanding of H_2S as a therapeutic compound. Hibernation demonstrates an even better reversibility of the AD symptoms then H_2S donor compound does in non-hibernators. Future Alzheimer research should be focused on putting the brain into a hibernation-like state. Therefore, pharmacological induction of hibernation in non-hibernating AD animals, whit H_2S as an important key role, will help us to improve H_2S dependent therapeutic compounds.

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