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Hippocampal cystathionine beta synthase in young and aged mice

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HIGHLIGHTS

- CBS is located in the cytosol of neurons and their axons and dendrites.
- Hippocampal CBS expression is maintained throughout aging.
- Unusually old animals show an increase in hippocampal CBS expression.

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ABSTRACT

Cystathionine beta synthase (CBS) is the main contributor to the production of hydrogen sulfide (H_2S) in the brain. Exogenously administered H_2S has been reported to protect neurons against hypoxic injury, ischemia and LPS-induced neuro-inflammation and in the facilitating of long term potentiation (LTP). Dysregulation of CBS leads to different diseases, which all have mental retardation in common. Although multiple studies have implicated a link between the CBS/H_2S pathway and neurodegeneration, no studies have been performed examining the pathway in healthy aging animals. We hypothesize that CBS/H_2S pathway plays an important role in the protection of learning and memory functions in the brain at the level of the hippocampus. Thus, we studied a set of 8 young (4 months) and 14 aged (24 months ($n=6$) and 28 months ($n=8$)) C57Bl6 mice. The 24-month-old mice displayed a significant decrease of CBS immunoreactivity in the MoDG only, compared to 4-month-old mice. In 28-month-old mice, we observed a significant increase of CBS immunoreactivity in the MoDG, compared to 4-month-old mice. When comparing 28-month-old mice to 24-month-old mice, all areas showed a significant increase of CBS immunoreactivity. Thus, throughout aging, CBS expression is maintained in the hippocampus, and many other forebrain regions as well. Mice at the unusual age of 28 months even have a higher hippocampal CBS expression than young mice. Maintenance (and increase) of CBS levels may sustain memory and learning by precluding neuronal loss in areas of the hippocampus.

1. Introduction

CBS is the main contributor to the production of H_2S in the brain. CBS catalyzes H_2S production by the β-replacement reaction of homocysteine to cystathionine. CBS deficiency in humans leads to homocystinuria, an autosomal recessive metabolic disorder caused by a mutation in the gene that encodes CBS. Homocystinuria is mainly characterized by mental retardation, seizures, ectopia lentis, skeletal deformities and occlusive vascular disease. The severity of this disease, caused by a dysregulation of CBS expression, gives an idea of the importance of the CBS/H_2S pathway in the brain [1].

Several reports demonstrate that exogenously administered H_2S protects neurons against hypoxic injury and ischemia [2–4]. Exogenously administered H_2S also protects against LPS-induced neuro-inflammation [5]. The antioxidant effect of H_2S is thought to represent the main mechanism of protection, either directly or indirectly by increasing the production of reduced glutathione.

In addition to the attenuation of cell damage, H_2S modulates physiological brain function, including facilitation of LTP and the regulation of neurotransmission and calcium homeostasis. The facilitation of LTP in the hippocampus is accomplished by selectively stimulating NMDA receptor-mediated currents, although the underlying mechanism remains unknown [6]. Further, H_2S has been found to upregulate the γ-aminobutyric acid (GABA) B receptor, implicated in inhibitory neurotransmission. Thus, H_2S may play a role in the maintenance of the excitatory/inhibitory neurotransmission balance in the brain. Finally H_2S acts as a regulator

Abbreviations: CBS, cystathionine beta synthase; H_2S, hydrogen sulfide; LTP, long-term potentiation; DAB, diaminobenzidine.

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of calcium levels in astrocytes, possibly modulating neuronal and vascular function, and in microglial cells, which are implicated in the progression of Alzheimer's disease (AD) [5].

Dysregulation of CBS is linked to different diseases, which all have mental retardation as a similar symptom. As described above, CBS deficiency leads to homocystinuria, first detected in 1962 by screening patients with mental retardation [7,8]. Another disease which links mental retardation to a disturbance in CBS regulation is Down syndrome (DS). DS is mainly caused by a trisomy of chromosome 21, the chromosome on which the CBS gene is located. Almost all adults with DS develop Alzheimer's disease (AD), and this development is usually at a much younger age than in non-DS adults. Levels of CBS in brains of DS patients are approximately three times higher than those in healthy individuals [9]. Thus, strangely, both diseases featuring mental retardation appear to root in opposite dysregulation of CBS, which likely underscores the importance of a CBS balance in the brain. However, the exact role of CBS in the mental retardation in homocystinuria and DS and in the development of AD in DS patients remains unclear.

To date, results of research on expression patterns of CBS in the brain are conflicting. CBS has been reported to be preferentially expressed in both the glia/astrocyte lineage and in neurons [10,11]. Further, although CBS expression seems confined to specific areas of the brain, detailed information on areas and cell types is lacking [12]. Even though there are multiple studies that implicate a link between the CBS/H_2S pathway and neurodegenerative diseases, no studies have been performed examining the pathway in healthy aging animals. In the present study, we examined the expression pattern of CBS in young and aged mice by using (semi) quantitive immunocytochemistry, with emphasis on the hippocampus.

2. Materials and methods

2.1. Animals

Male C57Bl6 mice were purchased from Harlan (Horst, The Netherlands) at the age of two months. The mice were individually housed on sawdust bedding with ad libitum water, food and nesting material. All animals were housed in the same animal facility, in the same room, dedicated for aging studies. The young mice were sacrificed at an age of 4 months, the aged mice at an age of 24 ($n=6$) and 28 months ($n=8$). The mice were sacrificed by an overdose of 6% sodium pentobarbital. Six animals of each group were transcardially perfused with 0.1 M PBS, followed by 4% PFA in 0.1 M PBS. After dissection, brains were post-fixed in PFA for 22 h at 4 °C and stored in 0.1 M PBS, containing sodium-azide, at 4 °C. For cutting slices the brains were cryoprotected with a 30% sucrose solution and cut by cryostat (25 μM) to be stored in PBSA at 4 °C.

2.2. Immunohistochemistry

The immunostaining was performed on free-floating brain slices, wherein endogenous peroxidase was blocked by incubating with 0.3% H_2O_2 for 30 min at room temperature, followed by rinsing with 0.01 M phosphate buffer (PBS). Slices were then incubated with the primary CBS antibody (mouse monoclonal, sc-271886, Santa Cruz Biotechnology, Dallas, TX, USA), diluted 1:200 in a 0.1% Triton X, 3% Bovine Serum Albumin (BSA) 0.01 M PBS solution, for 96 h at 4 °C. Subsequently, slices were rinsed in 0.01 M PBS and incubated with the secondary Biotin-SP-conjugated AffiniPure Goat-Anti-Mouse IgG antibody (Jackson ImmunoResearch, Suffolk, UK) diluted 1:500 in 0.01 M PBS, for 2 h at room temperature. Following incubation with the secondary antibody, slices were rinsed in 0.01 M PBS and incubated with avidin–biotin complex (Vector ABC kit; Vector Laboratories, Burlingame, CA, USA), diluted 1:500 in 0.01 M PBS, for 2 h at room temperature. The staining was visualized with 1 mg/ml diaminobenzidine (DAB) and 0.1% H_2O_2, followed by rinsing, mounting the slices on slides, dehydration and coverslipping for microscopic analysis. All of the sections were processed in the same batch. A cresyl violet counterstaining on CBS-stained brain sections was performed to allow discrimination between neuronal and non-neuronal cell types (e.g. glial cells).

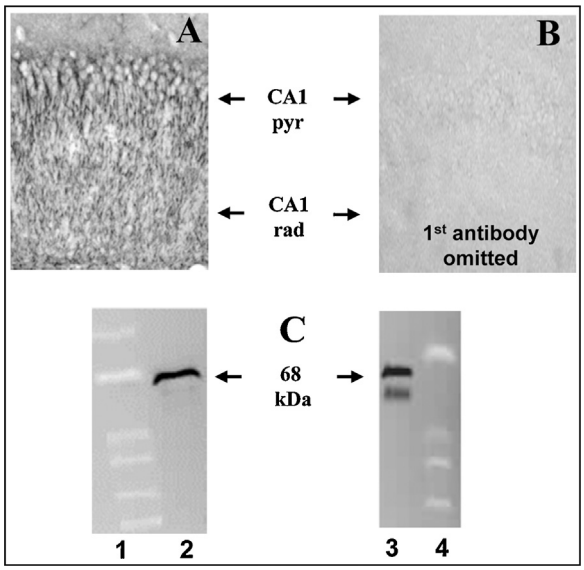

Fig. 1. Confirmation of antibody specificity. Immunostaining on mouse brain slices without the use of the primary antibody show immunonegative results (panel B). When performing a Western blot on mouse brain (panel C, lane 2) and liver (panel C, lane 4) tissue. A band can be found at 63 kDa (full-length) and 45 kDa (truncated form).

To exclude the possibility of cross-reactivity resulting from nonspecific binding, negative controls were included by incubating several brain slices and performing immunostainings without the primary antibody. These reactions yielded immunonegative results, confirming that the obtained immunostainings with our primary antibody were solely due to the immunodetection of these primary antibodies (see Fig. 1A and B).

To confirm that the used mouse monoclonal CBS antibody (sc-271886, Santa Cruz Biotechnology) specifically recognizes the CBS protein a western blot procedure was performed on brain and liver tissue from 4-month-old C57Bl6 mice. These mice were purchased at Harlan and were similar in age and housing to the mice used for immunohistochemistry. A clear band can be seen at 63 kDa in both liver and brain, confirming that the used CBS antibody recognizes the full-length CBS protein. In liver, another band is clearly visible at 45 kDa, confirming that the used antibody recognizes the truncated form of the CBS protein. The second band is nearly absent in brain, which indicates that the full-length form is relatively more present (see Fig. 1C, lane 2 and 3).

2.3. (Semi) quantification of immunohistochemical reactivity

The immunoreactivity of CBS was quantified by measuring optical density (OD) of each staining. Images were taken using a Leica charged-coupled device digital camera mounted on a microscope (DMRIB; Leica, Cambridge, United Kingdom) at ×20. The OD of CBS

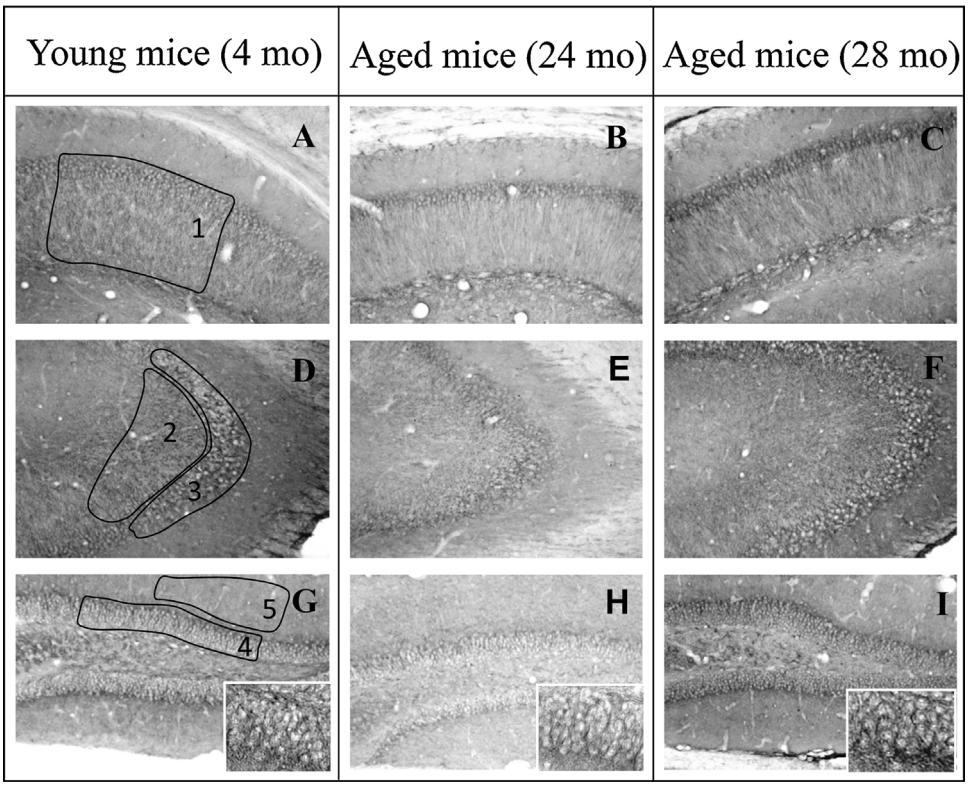

Fig. 2. CBS immunoreactivity in the hippocampal formation of mice. The immunoreactivity signals are present in the neurons of the CA1 and CA3 region, including the stratum lucidum, and the dentate gyrus. The CBS immunoreactivity appears more intense in the CA3 region, including the stratum lucidum (D and E), the granular and the molecular layer of the DG (G and H) of a 4-month-old mouse, compared to an 24-month-old mouse. The immunoreactivity in the CA1 region is similar between a 4-month-old mouse and 24-month-old mouse in (A and B). The CBS immunoreactivity in the CA1 (A and C) and CA3 region, including stratum lucidum (D and F) of a 28-month-old mice is similar compared to a 4-month-old mice. Using the same comparison, a small increase in intensity is observed in the granular and molecular layer of the DG (G and I). The immunoreactivity appears increased in all hippocampal areas of the 28 old mouse, compared to the 24-month-old mouse (B, C, E, F, H and I). The boxed areas, indicated in (A, D and G), represent the areas used to measure the optical density in; 1: CA1, 2: Slu, 3: CA3, 4: GrDG, 5: MoDG. Inserts in (G, H and I) represent higher magnification images to illustrate the dot-like appearance of CBS immunoreactivity.

was quantified in multiple hippocampal regions of interest (ROI) and corrected for nonspecific background labeling, measured in the corpus callosum. The final corrected OD (COD) = the OD measured in the ROI – the OD measured in the corpus callosum.

The measured ROIs were the CA1, CA3, Stratum Lucidum (Slu), the molecular and granular layer of the Dentate Gyrus (MoDG, GrDG), layer 1–6 of the somatosensory cortex, the medial and lateral habenular nuclei (MHb, LHb), the lateral posterior thalamus (LP) and the ventral posteromedial nucleus (VPM). The COD was expressed in arbitrary units corresponding to gray level using a Quantimet 550 image analysis system (Leica).

The semi quantification of CBS immunoreactivity was performed by scoring brain areas using a 0 to +++ scoring system. Scoring was classified as low (−/+), moderate (+), high (++) and very high (+++). A very high score was given when an area had a similar intensity as the dentate gyrus and CA3 region of 28-month-old animals. A high score was given when an area had a similar intensity as the CA1 in 24-month-old animals. A moderate score was given when an area had a lower intensity as the CA1 in 24-month-old animals, but still had a considerate amount of immunoreactivity.

2.4. Statistical analysis

The one-way ANOVA test was used to compare the two groups. SigmaPlot v11 (Systat Software Inc., Chicago, IL, USA) was used to perform the statistical analysis. All p-values were considered statistically significant at a threshold of $p < 0.05$.

3. Results and discussion

We found a specific expression pattern of CBS immunoreactivity in the brain of adult mice, with CBS expression being present throughout the brain. CBS is located in the cytosol of neurons and their axons and dendrites, in all brain regions examined and notably in the hippocampus (Fig. 2), cortex and amygdala. In the white matter, numerous CBS-positive fibers were present. Part of the CBS labeling was typically present in a dot-like fashion, possibly connected to synaptic structures (see inserts in Fig. 2). The cresyl violet counterstaining confirmed the exclusive CBS expression in neurons. The young and aged mice did not differ in their distribution pattern of CBS immunoreactivity. The found pattern of CBS expression is not mouse-specific as a similar pattern with this primary antibody was found by us in the golden hamster.

CBS optical density measurements of the hippocampus showed a significant decrease in CBS expression in the MoDG of 24-month-old mice, compared to 4-month-old mice. A significant increase was observed in the optical density of 28-month-old mice, compared to 4-month-old mice. The 28-month-old mice showed a significant increase in optical density in all areas, compared to the 24-month-old mice, as shown in Fig. 3A. In the somatosensory cortex as significant increase in optical density was found in layer 1, when comparing 24 or 28-month-old mice to 4-month-old mice. A significant increase was also found in layer 2, when comparing 24-month-old mice to 4-month-old mice, as shown in Fig. 3B. The quantification of optical density of the thalamus showed a significant increase in the MHb, when comparing 24 or 28-month-old

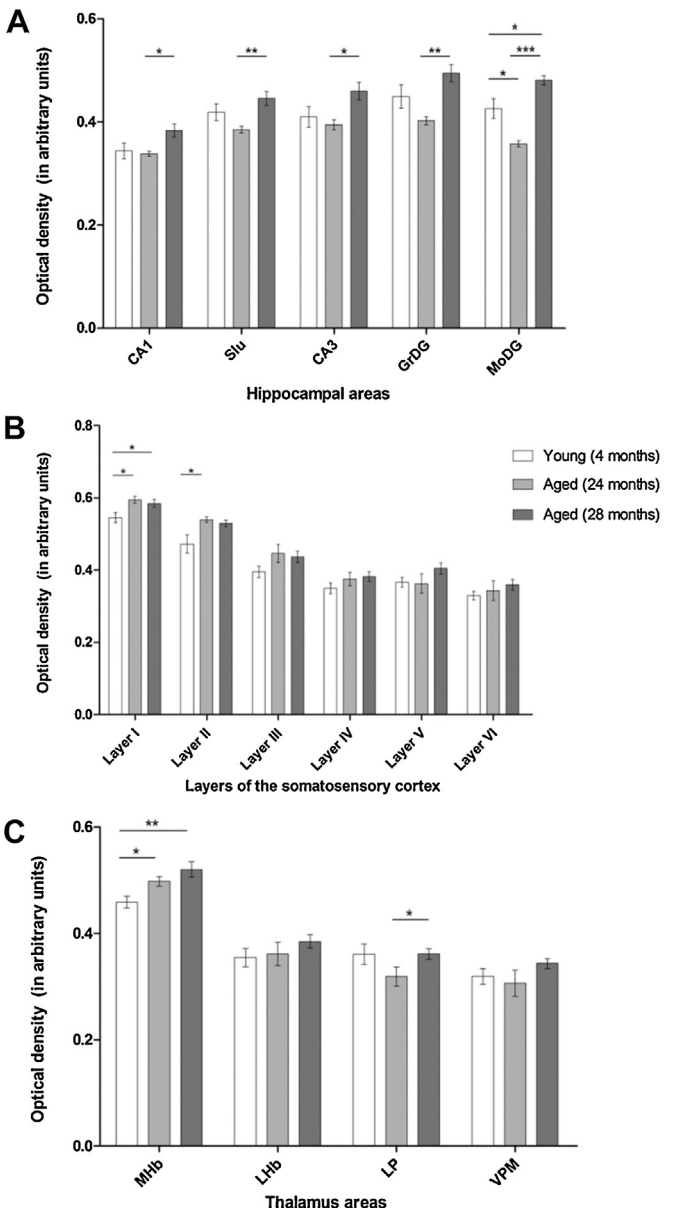

Fig. 3. Comparison of quantified CBS immunoreactivity (optical density (OD)) in different areas of the hippocampus, (panel A), somatosensory cortex (panel B) and thalamus (panel C) between young and aged mice. Panel A: The OD in all areas is higher in 28-month-old mice, compared to 24-month-old mice. The MoDG has a decreased OD in 24-month-old mice and increase OD in 28-month-old mice, compared to 4-month-old mice. Panel B: The OD in layer 1 is higher in 24-month and 28-month-old mice, compared to 4-month-old mice. The OD in layer 2 is significantly higher in 24-month-old mice, compared to 4-month-old mice. Panel C: The OD in the MHb is higher in 24-month and 28-month-old mice, compared to 4-month-old mice. The OD in the LP is higher in 28-month-old mice, compared to 24-month-old mice. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

mice to 4-month-old mice. A significant increase was also found in the LP, when comparing 28-month-old mice to 24-month-old mice, as shown in Fig. 3C.

The semi quantification, as shown in Table 1, showed that C57Bl6 mice maintain CBS expression in all layers of the retrosplenial cortex and white matter, when comparing 24-month-old to 4-month-old mice. Using the same age-comparison, a slight decrease was observed in the layer 2 and 3 of the entorhinal cortex and layer 2 of the piriform cortex. Interestingly, the 28-month-old mice showed an increase of CBS expression in the entire amygdala, compared to the 24-month-old mice. This age-specific increase resembled the increase seen in the hippocampus and parts of the thalamus. Using the same age-comparison, CBS expression was maintained in the striatum, layer 2 of the piriform cortex and all layers of the retrosplenial cortex.

No previous study was performed examining the expression of CBS in young and aged mice, which makes a comparison with the present study impossible. Robert et al. examined the CBS expression during mouse development and found a similar expression pattern of CBS in adult mouse brain [11]. In early brain development they found a strong signal in many areas of the nervous system (ventricular zone, neopallial cortex, medulla oblongata, midbrain and spinal cord). However, they did not examine the CBS expression in the brains of aged mice.

A recent hypothesis that could help explain our findings about the location of CBS expression is the function of H_2S as a gaseous neurotransmitter [6]. H_2S appears to signal by S-sulfhydrating cysteines in its target proteins, in a similar way the gasotransmitter NO causes S-nitrosylation. The S-nitrosylation caused by NO typically inhibits enzymes, whereas H_2S induced S-sulfhydrating activates enzymes. The physiological gasotransmitter-like effect of H_2S is comparable to that of NO and carbon monoxide. The gasotransmitter role of H_2S is relatively new and has to be investigated further. Another established role of H_2S is neurotransmission is the regulation of inhibitory neurotransmission by upregulation of the GABA B receptor, as previously mentioned. H_2S not only help modulates neuronal functions, it also assists astrocyte communication, by its role in the regulation of calcium levels. The possible function of H_2S as a gasotransmitter may help explain the omnipresent expression of CBS in the brain. Both hypotheses about the function of H_2S, protective agent or gaseous neurotransmitter, can be combined. The fact that CBS is omnipresent in the brain, in different conditions, gives the impression that H_2S needs to be continuously produced. Neurotransmitting and protection against hypoxia/reperfusion damage are two processes that are also continuously present in the brain.

The hippocampus is known for its role in learning and memory. The hippocampus is also one of the brain areas where adult neurogenesis occurs, which is also the case in the C57 mice [13]. A reduction in the ability of neurogenesis, as seen in aging, contributes to memory deficits [14]. Previously, we showed that C57Bl6 mice of the age of 24 months have a hippocampus-specific memory deficit. In the novel object recognition task, mice of this age, as compared to 3 months old controls, performed significantly worse in hippocampus-specific spatial recognition version of the task, but equally well in the object recognition version [15]. Hence, the maintenance of CBS expression in the CA1 and CA3 of the hippocampus of the brains of aged mice does not prevent memory performance deficits, but may be important in the survival of aged neurons as no signs of neurodegeneration were present in the analyzed brain sections of any of the aged mice. However, the decrease of CBS expression in the dentate gyrus might be involved in the arising of memory performance deficits.

The difference between the 24-month and 28-month-animals is especially interesting. C57Bl6 mice at the age of 28 months belong to a selective subpopulation of advanced aging. The fact that all of the 28-month-animals had a significant higher CBS expression, compared to the 24-month-old animals, implicates a role for the CBS/H_2S pathway in healthy aging. A comparable observation of an age-selective enhancement in protein expression at advanced age was found in healthy old rabbits for the low subunit (70 kD) of neurofilaments [16]. Such changes may hint at a more general mechanism of enhanced expression of selective proteins accompanying healthy aging at old age [16].

There are two reasons why a maintenance or increased CBS expression may be necessary, ensuring the clearance of its substrate, homocysteine, or the production of its product, H_2S. Elevated

Table 1
Expression of CBS in various compartments of the mouse central nervous system.

Brain region	Young (4 months)	Aged (24 months)	Aged (28 months)
Retrosplenial cortex			
Layer I	++	++	++
Layer II	++	++	++
Layer III/IV	+	+	+
Layer V	++	++	++
Entorhinal cortex			
Layer II/III	+++	++/+++	++/+++
Piriform cortex			
Layer II	+++	++/+++	++/+++
Amygdala			
Central amygdaloid nucleus	+++	++/+++	+++
Medial amygdaloid nucleus	+++	++/+++	+++
Lateral amygdaloid nucleus	+	+	+/++
Striatum	+	+	+
White matter			
Fimbria	++	++	++
Corpus callosum	+/−	+/−	+/−
Optic tract	+/−	+/−	+/−

Scoring was classified as absent (−), low (+/−), moderate (+), high (++) and very high (+++).

levels of homocysteine are related to neurovascular and neurodegenerative diseases, necessitating its clearance in the brain. Given the functions of H_2S in the brain, the second possibility also seems logical. H_2S attenuates LTP, one of the mechanisms underlying synaptic plasticity. Synaptic plasticity is essential for the hippocampal role in learning and memory. In addition to its function in LTP attenuation, H_2S has a high antioxidant potency, plays a role in the calcium regulation in the brain and can protect against hypoxic injury and ischemia. A recent study showed treatment with H_2S attenuated neurodegeneration and neurovascular dysfunction, induced by homocysteine [17]. These findings implicate, once again, the possible importance of the CBS/H_2S pathway in the brain.

4. Conclusion

Taken together, the CBS/H_2S pathway seems to constitute an important pathway in the brain, which possibly plays a role in the protection of the brain from different damage inducing mechanisms (neuro-inflammation, hypoxia/reperfusion and hypothermia rewarming) and the (regulation of) neurotransmission. The maintenance of CBS expression in the CA1, CA3, somatosensory cortex and thalamus of 24 and 28-month-old animals, compared to 4-month-old animals and the increase of CBS expression in the dentate gyrus, layer 1 of the somatosensory cortex and the medial habenular nucleus of 28-month-old animals, compared to 4-month-old animals underscores the possible importance of the CBS/H_2S pathway in the brain. This is further strengthened by the semi-quantification data of the amygdala and retrosplenial cortex, showing a comparable pattern.

The next step, which will substantiate the current interpretation of our findings, will be to study the up- and downstream markers of the CBS/H_2S pathway. When studied more extensively, the CBS/H_2S pathway could not only shed new lights on the pathophysiology of mental retardation and neurodegenerative diseases, but could also be a target to improve damage in the otherwise non-pathological aging brain.

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