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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).

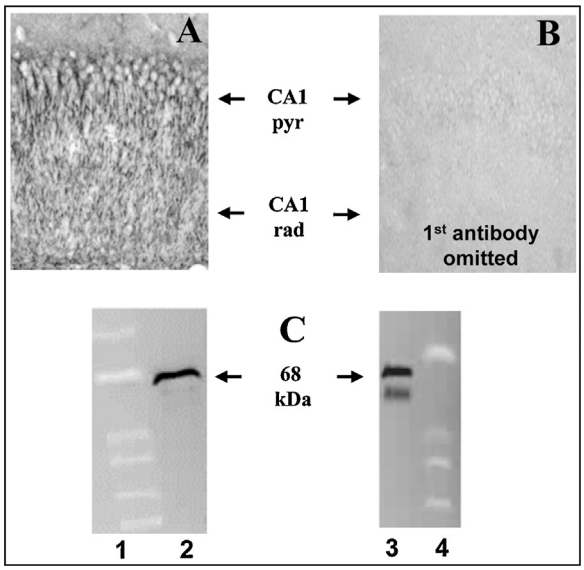

Fig. 1. Confirmation of antibody specificity. Immunostaining on mouse brain slices without the use of the primary antibody show immunonegative results (panel B). When performing a Western blot on mouse brain (panel C, lane 2) and liver (panel C, lane 4) tissue. A band can be found at 63 kDa (full-length)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