Disturbance of hippocampal H$_2$S generation contributes to CUMS-induced depression-like behavior: involvement in endoplasmic reticulum stress of hippocampus

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Abstract

The chronic unpredictable mild stress (CUMS) model is a widely used experimental model of depression. Exogenous stress-induced neuronal cell death in the hippocampus is closely associated with the pathogenesis of depression. Excessive and prolonged endoplasmic reticulum (ER) stress triggers cell death. Hydrogen sulfide (H$_2$S), the third endogenous signaling gasotransmitter, plays an important role in brain functions as a neuromodulator and a neuroprotectant. We hypothesized that the disturbance of endogenous H$_2$S generation and ER stress in the hippocampus might be involved in CUMS-induced depression-like behaviors. Thus, the present study focused on whether CUMS disturbs the generation of endogenous H$_2$S and up-regulates ER stress in the hippocampus and whether exogenous H$_2$S prevents CUMS-induced depressive-like behaviors. Results showed that CUMS-treated rats exhibit depression-like behavior and hippocampal ER stress responses including up-regulated levels of glucose-regulated protein 78, CCAAT/enhancer binding protein homologous protein, and cleaved caspase-12 expression, while the endogenous generation of H$_2$S in the hippocampus is suppressed in CUMS-treated rats. Furthermore, exogenous H$_2$S prevents CUMS-induced depression-like behavior. These data indicated that CUMS-induced depression-like behaviors are related to the disturbance of endogenous H$_2$S generation and ER stress in the hippocampus and suggested that endogenous H$_2$S and ER stress are novel treatment targets of depression.

Key words: chronic unpredictable mild stress, depression, hydrogen sulfide, endoplasmic reticulum stress

Introduction

Depression is a serious mental disorder that affects ~16% of the population and is one of the top three most widespread and debilitating illnesses worldwide [1]. Despite considerable advances in the knowledge related to brain development and function, the underlying mechanisms for the pathogenesis of depression are still not well understood. Chronic stress acts as a predisposing and participating factor in the onset of depression in humans [2,3]. Rats or mice exposed to chronic unpredictable mild stress (CUMS) develop many behavioral and
accompanied by the decrease in the volume of the hippocampus [7,8] further understand the pathogenesis and treatment of depression. It has been demonstrated that major and prolonged depression is accompanied by the decrease in the volume of the hippocampus [7,8] and that exogenous stress-induced neuronal cell death, and the decrease of neurogenesis in the hippocampus is associated with the pathogenesis of depression [9]. However, the mechanisms underlying exogenous stress-induced neuronal cell death in hippocampus remain poorly understood.

Endoplasmic reticulum (ER), a principal site for protein synthesis, folding, and calcium signaling, is highly sensitive to alterations in calcium homeostasis and perturbations [10]. ER stress, a condition that impairs the function of the ER, can lead to an accumulation of unfolded proteins in the ER lumen [11]. Glucose-regulated protein 78 (GRP78), CCAAT/enhancer binding protein homologous protein (CHOP), and cleaved caspase-12 are molecular markers of ER stress [12]. Excessive and prolonged ER stress can trigger cell death [13]. Some reports have indicated an association between depression and ER stress [14]. In postmortem brain studies, increased levels of ER stress have been found in the temporal cortex of subjects with major depressive disorder who died of suicide [15]. In corticosterone-treated animal depression models, the levels of GRP78 and other ER stress-related proteins are up-regulated in the dentate gyrus (DG) and other regions [16,17]. Chronic social defeat stress increases the expression of GRP78 and CHOP in the brains of adolescent mice [18]. Therefore, it is necessary to investigate whether CUMS-induced depression-like behavior is related to hippocampal ER stress.

Hydrogen sulfide (H$_2$S) is the third endogenous signaling gasotransmitter, besides nitric oxide and carbon monoxide [19,20]. H$_2$S plays an important role in brain functions, probably through acting as a neuromodulator as well as a neuroprotectant [21–23]. In the mammalian brain, H$_2$S is formed from the amino acid cysteine by the action of cystathionine beta-synthase (CBS) [19,20,24]. Deficiency in H$_2$S synthesis was observed in Down’s syndrome [25], stroke [26], and possibly Alzheimer’s disease [27–29]. We have demonstrated that the disturbance of H$_2$S synthesis contributes to 1-methyl-4-phenylpyridinium ion-, formaldehyde (FA)-, and homocysteine (Hcy)-induced neurotoxicity [30–32], as well as to FA- and Hcy-induced deficits in learning and memory [33,34]. This raises the questions whether CUMS disturbs H$_2$S synthesis and whether CUMS-induced depression involves the imbalance of proportion to endogenous H$_2$S.

In this study, it was found that CUMS induces depression-like behavior and causes hippocampal ER stress including the up-regulation of GRP78, CHOP, and cleaved caspase-12. It was also demonstrated that CUMS inhibits the endogenous generation of H$_2$S in the hippocampus and that exogenous H$_2$S prevents CUMS-induced depression-like behavior. These findings suggested that the disturbance of hippocampal H$_2$S generation mediates CUMS-induced depression-like behavior and that the hippocampal ER stress is a potential mechanism underlying CUMS-induced depression-like behavior.

**Materials and Methods**

**Reagents**

Sodium hydrosulphide (NaHS, a donor of H$_2$S) was obtained from Sigma (St Louis, USA). NaHS was dissolved in 0.9% non-pyrogenic NaCl and injected intraperitoneally (i.p.) at a dose of 1.68 or 5.60 mg/kg. Specific monoclonal anti-CBS was purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Specific monoclonal anti-GRP78 and anti-CHOP antibodies were purchased from Epitomic (Burlingame, UK). Specific monoclonal anti-caspase-12 antibody was obtained from Sigma.

**Animals**

Adult male Sprague-Dawley rats (250–280 g) were obtained from the SJA Lab Animal Center of Changsha (Changsha, China). All protocols were validated by the Science and Technology Department of Hunan Province (number 2009-0012). Rats were individually housed with free access to food and water under a 12/12 h light/dark cycle. After being housed, rats were acclimated to the surroundings for 1 week to habituate the experimenter. The animal experiment was performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the Animal Use and Protection Committee of University of South China.

**CUMS procedure**

Rats were divided into two groups: control and CUMS animals. Except for the control rats, the other animals were kept in individual cages. CUMS procedure involves a variety of mild stressors: (i) 24 h of food deprivation, (ii) 24 h water deprivation, (iii) 1 h of exposure to an empty bottle, (iv) 7 h cage tilt (45°), (v) overnight illumination, (vi) 24 h of soiled cage (200 ml of water in 100 g of sawdust bedding), (vii) 30 min of forced swimming at 8°C, (viii) 3 h of physical restraint, and (ix) 24 h of exposure to a foreign object (e.g., a piece of plastic) [35]. These stressors were randomly scheduled over a 1-week period and repeated throughout the 4-week experiment. In all of the experiments, the first two drug-free weeks of CUMS were followed by 2 weeks of UCMS application during which the rats were treated with NaHS (1.68 and 5.60 mg/kg) or saline (Fig. 1) by intraperitoneal injection. The control animals were left undisturbed in their home cages with the exception of general handling and saline treatment (Fig. 1).

**Sucrose preference test**

The sucrose preference test was used to assess anhedonia induced by the CUMS protocol. Prior to the start of the experiment, rats were trained for adaption to 1% sucrose solution (w/v) for 48 h, in which two bottles of 1% sucrose solution were placed in each cage. After adaptation, rats were deprived of food and water for 4 h and then

![Figure 1. Schematic plan of the experimental procedure for CUMS and treatments in rats](image-url)
Roles of H$_2$S generation and ER stress in CUMS-induced depression

exposed to water or a 1% sucrose solution in pre-weighed plastic bottles for 1 h. The sucrose and water consumption were measured by measuring the change in weight of fluid, and sucrose preference was calculated as: sucrose consumption/(sucrose consumption + water consumption) × 100%.

**Western blot analysis**

Hippocampal tissue was homogenized in ice-cold homogenizing buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% sodium dodecyl sulfate, 1% NP-40, 1% deoxycholate, 1% Triton X-100, 10 mM PMSF, 50 mM sodium vanadate, and 0.1% protease inhibitors cocktail) (Roche, Basel, Switzerland). After centrifugation at 14,000 g for 30 min at 4°C, the supernatant was collected and the protein content was subsequently assayed by using a BCA protein assay kit (Beyotime, Shanghai, China). Total protein (25 μg/lane) was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. The membranes were then blocked with 5% bovine serum albumin in TBS-T buffer containing 0.1% Tween-20, pH 7.6) for at least 2 h at room temperature. The primary antibody was diluted in TBS-T buffer containing 5% bovine serum albumin. Membranes were incubated with the primary antibody (anti-CBS, 1:2000; anti-CHOP, 1:500; anti-GRP78, 1:2000; and anti-cleaved caspase-12, 1:2000) at room temperature for 2 h or at 4°C overnight. The membranes were washed three times for 20 min each with TBS-T, and incubated with secondary antibody for 2 h. After three times wash with TBS-T for 20 min and once with TBS for 5 min, the electrogenerated chemiluminescence reaction solutions were added (solution 1: 0.1 M Tris–HCl, luminol, p-coumaric acid; solution 2: 0.1 M Tris–HCl, hydrogen peroxide) and incubated for 2 min. The signal of the immunoblot was visualized using an image analysis system equipped with a software BIO-ID (Vilber Lourmat, Marne La Vallee, France) and the integrated optical density for the protein band was calculated by Image-J software.

**Assay of H$_2$S generation**

Hippocampus was homogenized in 50 mM ice-cold potassium phosphate buffer (pH 6.8). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.4), 10 mM l-cysteine (20 μl), 2 mM pyridoxal 5’-phosphate (20 μl), saline (30 μl), and 11% (w/v) tissue homogenate (430 μl). The reaction was performed in tightly stoppered cryovial test tubes and initiated by transferring the tubes from ice to a shaking water bath at 37°C. After incubation for 30 min, 1% (w/v) zinc acetate (250 μl) was added to trap evolved H$_2$S, followed by addition of 10% (v/v) trichloroacetic acid (250 μl) to denature the protein and stop the reaction. Subsequently, N,N-dimethyl-p-phenylenediamine sulfate (NNDPD) (20 μM; 133 μl) in 7.2 M HCl was added, followed immediately by addition of FeCl$_3$ (30 μM; 133 μl) in 1.2 M HCl. The absorbance of the resulting solution at 670 nm was measured by spectrophotometry. The H$_2$S concentration was calculated against a calibration curve of NaHS. H$_2$S synthesizing activity is expressed as the amount (nmol) of H$_2$S formed per milligram protein (determined using a BCA™ protein assay kit) per minute (nmol/min/mg protein).

**Statistical analysis**

Data were expressed as the mean ± SEM. The significance of intergroup differences was evaluated by one-way analyses of variance followed by post hoc Dunnett’s test for multiple comparisons. Differences were considered significant when $P < 0.05$.

**Results**

CUMS induces a decrease in the sucrose preference of rats

To confirm the role of CUMS in depression-like behavior, the difference of sucrose preference between CUMS-treated rats and control rats was tested. As shown in Fig. 2A, the sucrose preference was markedly decreased in the rats exposed to CUMS for 2 or 4 weeks. However, the total fluid intake was not affected by CUMS (Fig. 2B). These data confirmed the depression-like behavior in CUMS-treated rats.

CUMS induces up-regulation of GRP78 expression in hippocampus of rats

To investigate whether CUMS induces ER stress in the hippocampus of rats, the expression of GRP78, an important marker for ER stress, in the hippocampus of rats was measured. As shown in Fig. 3, CUMS (for 4 weeks) significantly increased GRP78 expression in the hippocampus of rats, which indicated the effects of CUMS on ER stress in the hippocampus.

CUMS up-regulates the expression of CHOP in hippocampus of rats

To further explore whether CUMS exposure induces ER stress, the CHOP protein level in the hippocampus of rats was monitored. CHOP expression in the hippocampus of rats treated with CUMS for 4 weeks was significantly increased (Fig. 4). This result also indicated that CUMS could induce hippocampal ER stress.

**Figure 2. Effect of CUMS on the sucrose preference of rats** After treatment with CUMS for 2 or 4 weeks, the sucrose preference (A) and the total fluid intake (B) of rats were examined. Results are expressed as the mean ± SEM ($n = 10–15$). *$P < 0.05$ vs. control group.
CUMS increases the expression of cleaved caspase-12 in hippocampus of rats
Cleaved caspase-12 participates in ER stress. Therefore, the expression of cleaved caspase-12 in the hippocampus of rats was further investigated. As shown in Fig. 5, after CUMS exposure for 4 weeks, the expression of cleaved caspase-12 was significantly up-regulated in the hippocampus of rats, which further revealed that CUMS treatment produces the hippocampal ER stress.

CUMS reduces the expression of CBS and the generation of H₂S in the hippocampus of rats
To explore whether the distribution of H₂S generation involves in CUMS-induced depression-like behavior, the expression of CBS and the generation of H₂S in the hippocampus of rats were investigated. As shown in Fig. 6A, after 4-week exposure of CUMS, the expression of CBS in the hippocampus was significantly decreased. Simultaneously, the generation of H₂S in the hippocampus was significantly inhibited by 4 weeks of treatment with CUMS (Fig. 6B). These data demonstrated that CUMS could disturb the synthesis of endogenous H₂S in the hippocampus of rats.

Figure 3. Effect of CUMS on expression of GRP78 in the hippocampus of rats
Rats were treated with CUMS for 4 weeks and the expression of GRP78 in the hippocampus was detected by western blot analysis using an anti-GRP78 antibody, and β-actin was used as a loading control. Results are expressed as the mean ± SEM (n=3–5). **P<0.01 vs. control group.

Figure 4. Effect of CUMS on expression of CHOP in the hippocampus of rats
Rats were treated with CUMS for 4 weeks and the expression of CHOP in the hippocampus was detected by western blot analysis using an anti-CHOP antibody, and β-actin was used as a loading control. Results are expressed as the mean ± SEM (n=3–5). **P<0.01 vs. control group.

Figure 5. Effect of CUMS on expression of cleaved caspase-12 in the hippocampus of rats
Rats were treated with CUMS for 4 weeks and the expression of cleaved caspase-12 in the hippocampus was detected by western blot analysis using an anti-cleaved caspase-12 antibody, and β-actin was used as a control. Results are expressed as the mean ± SEM (n=3–5). **P<0.01 vs. control group.

Figure 6. Effect of CUMS on the expression of CBS and the generation of H₂S in the hippocampus of rats
After 4-week CUMS treatment, the hippocampus of rats was homogenized. (A) The level of CBS expression was detected by western blot analysis using an anti-CBS antibody. β-Actin was used as a control. (B) The generation of H₂S was measured by the NNPD method as described in ‘Materials and Methods’ section. Results are expressed as the mean ± SEM (n=4). **P<0.01 vs. control group.
H2S blocks CUMS-induced decrease in the sucrose preference of rats

To confirm the contribution of distribution in H2S generation to CUMS-induced depression-like behavior, whether exogenous H2S attenuates CUMS-induced depression-like behavior was investigated. CUMS caused the predicted decrease in sucrose preference (Fig. 7), and this effect was blocked by treatment with NaHS (a donor of H2S, 1.68 and 5.6 mg/kg, i.p.) for 2 weeks, which indicated the inhibitory action of H2S in CUMS-induced depression-like behavior.

Discussion

The present study explored the contribution of endogenous H2S generation disturbance and ER stress in the hippocampus to CUMS-induced depression-like behavior. It was demonstrated that in CUMS-treated rats (i) the sucrose preference is decreased, (ii) ER stress is increased in the hippocampus, and (iii) the endogenous H2S generation in the hippocampus is disturbed. Furthermore, exogenous H2S application suppressed CUMS-induced decrease in the sucrose preference of rats. Our results suggested that the disturbance of H2S generation and ER stress in the hippocampus plays important roles in CUMS-induced depression-like behavior.

In rodents, CUMS decreases the normal preference for sucrose-containing water vs. regular drinking water, paralleling a state of anhedonia, which is a core symptom of depressed patients [36]. In the present study, rats subjected to this CUMS paradigm exhibited a consistent decrease in sucrose preference, which demonstrated that CUMS could induce depression-like behavior in rat model. The paradigm of CUMS evokes a number of behavioral and neurobiological changes. Thus, the CUMS model may be a suitable tool to explore novel systems that could be disturbed in depression and be helpful to the identification of novel targets for the treatment of depression [4].

The ER lumen, a unique oxidative environment, is critical for protein folding and formation of disulfide bonds and susceptible to various type of injury [37]. Various destructive stimuli may impair the ER function and lead to the activation of unfolded protein response (UPR)–ER stress [10,38,39]. In response to ER stress, cells develop a self-protective signal pathway termed the UPR, leading to induction of molecular chaperones such as GRP78, translational attenuation, and ER degradation [40]. However, if the damage is excessive, the UPR ultimately activates apoptotic pathways such as activations of CHOP and caspase-12 [40]. Increasing evidence suggested that significant relationships between ER stress response and neuropsychiatric disorders such as major depressive disorder [15] and bipolar disorder [41,42]. Thus, we hypothesized that ER stress could be one of the pathological mechanisms related to the depressive disorder in CUMS-treated rats. To explore the underlying molecular mechanisms for depression-related behavioral responses after CUMS, potential candidates such as GRP78, CHOP, and cleaved caspase-12 were explored in this study. GRP78 plays a crucial role in the regulation of ER dynamic homeostasis, and is a marker for ER stress [43–45]. CHOP, an apoptotic transcription factor, reflects the status of ER stress [46]. Pro-caspase-12 is localized on the cytoplasmic side of ER and is proteolytically activated during excessive ER stress. In the present study,
we demonstrated that CUMS leads to the up-regulation of GRP78, CHOP, and cleaved caspase-12 expressions in the hippocampus of rats. This is the first report on the up-regulatory actions of CUMS in the hippocampal ER stress. A significant increase in ER stress has been reported in the brain after restraint and sleep deprivation stress [47,48]. Furthermore, the levels of GRP78 and other ER stress-related proteins were also increased in the brains of corticosterone- and chronic social defeat stress-treated animal depression models [16–18]. These findings indicated a possible association among exogenous stress, depression, and ER stress and provided a reasonable explanation for our results. Taken together, these findings indicated that ER stress in the hippocampus may play important roles in the pathogenesis of depression and suggested that a more full understanding of the role of ER stress pathways in major depression could lead to some novel and much needed therapeutic targets.

It is now known that deficits in synaptic plasticity, resulting from chronic stress, can set the stage for the onset of depression [49,50]. Increasing evidence demonstrated that stressors can increase the risk of onset of mood disorders in susceptible individuals and stressful conditions in animals can disrupt synaptic plasticity in the hippocampus [49,51]. Chronic restraint stress impairs long-term potentiation (LTP) in region III of hippocampus proper (CA3) [52], while CUMS impairs LTP in DG and CA1 subregions [53,54]. These stress-induced alterations in synaptic plasticity can be reversed by chronic treatment with antidepressant drugs [55,56]. These findings strongly indicated an involvement of deficits in synaptic plasticity in the pathophysiology of stress and depression. It has been demonstrated that physiological concentrations of H2S specifically potentiate the activity of N-methyl-D-aspartate receptor and facilitate the induction of LTP in the hippocampus [57] and that LTP is altered in the absence of H2S [58]. These observations suggested the involvement of H2S in synaptic activity [59]. Thus, it is logical to speculate that the disturbance in hippocampal H2S generation may play a pivotal role in the pathophysiology of stress and depression.

The results of the present study showed that 4 weeks of CUMS leads to suppression in the expression of hippocampal CBS and decrease in the generation of hippocampal H2S, which is accompanied with depression-like behavior. These findings suggested that the underlying mechanisms of CUMS-induced depressive-like behavior may involve the deficit in endogenous H2S generation in the hippocampus. Furthermore, the fact that treatment with exogenous H2S significantlyameliorated depressive-like behavior in the CUMS-exposed rats also provided strong evidence that endogenous H2S is a novel target for the treatment of depression. Notably, serotonin, dopamine, and norepinephrine are regarded as the crucial neurotransmitters in the etiology of depression and the decreased levels of neurotransmitters occur in the synaptic cleft or in the depressive brain [60]. Furthermore, dopamine and serotonin can increase H2S production by the endogenous enzyme CBS and protect cells against hyperthermia/re-warming damage [61]. These results indicated that it is worthy to investigate the relationship between H2S and dopamine or serotonin.

In conclusion, the present study demonstrated that CUMS induces depression-like behaviors, accompanied with up-regulation of ER stress and disturbance of endogenous H2S generation in the hippocampus of rats. We also confirmed the antidepressant-like effect of exogenous H2S. Combined with the previous evidence that H2S possesses the protective role against ER stress induced by FA [62] and 6-hydroxydopamine [63] in neuronal cells, our data suggested that CUMS-induced depression-like behavior in rats is involved in the disturbance of hippocampal H2S generation, which in turn causes up-regulation of ER stress in the hippocampus of rats. The present data provide a comprehensive picture of the disturbance of hippocampal H2S generation under CUMS-induced depressive-like behaviors and validate the hypothesis that modulation of hippocampal H2S generation represents a viable target for antidepressant drug development.

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