

Exogenous sodium hydrosulfide can attenuate naloxone-precipitated withdrawal syndromes and affect cAMP signaling pathway in heroin-dependent rat's nucleus accumbens

L.-H. JIANG¹, J. WANG¹, X.-L. WEI², Q.-Y. LIANG³, T.-T. CHENG²

¹College of Animal Science and Technology, Guangxi University, Nanning, P.R. China.

²Department of Human Anatomy, Guangxi Medical University, Nanning, P.R. China

³School of Pharmacy, Guangxi Medical University, Nanning, P.R. China

Abstract. – **BACKGROUND:** H₂S is a novel type of endogenous neural regulatory factor and gaseous mediator. Exogenous H₂S can increase heroin-induced learning and memory damage in rat and alleviates heroin-induced rat hippocampus damage through antioxidant and anti-apoptosis effects.

OBJECTIVE: Aim of this study was to identify whether hydrogen sulfide (H₂S) protects heroin withdrawal rat is related with adenylate cyclase (AC)-cAMP-protein kinase A (PKA)-cAMP response element-binding protein (CREB) signaling pathway in heroin-dependent rat's nucleus accumbens or not.

METHODS: Male Spragne-Dawley rats were randomly divided into Saline + Saline group, Saline + sodium hydrosulfide (NaHS) group, Saline + Heroin group, NaHS + Heroin group according to the principle of increasing heroin dosage day by day, with the establishment of heroin-naloxone-induced withdrawal symptoms determined at day 10. Then the levels of H₂S and cAMP and AC and PKA activities were assayed, and the level of phosphorylated CREB (p-CREB), the levels of phosphorylated N-methyl-D-aspartate receptor 1 subunit (p-NR1), phosphorylated N-methyl-D-aspartate receptor 2a subunit (p-NR2A) and phosphorylated N-methyl-D-aspartate receptor 2b subunit (p-NR2B) were assayed in nucleus accumbens.

RESULTS: Exogenous H₂S can alleviate heroin withdrawal symptoms by increasing the level of H₂S level in nucleus accumbens. Exogenous H₂S can decrease the high activities of AC, PKA and the high levels of cAMP, p-CREB caused by heroin. Furthermore, exogenous H₂S can decrease the high level of p-NR1 and can increase the low levels of p-NR2A and p-NR2B caused by heroin. It is surprising that exogenous H₂S treatment alone was able to raise the activities of AC and PKA as well as the levels of cAMP, p-CREB, p-NR1, p-NR2A and p-NR2B.

CONCLUSIONS: Exogenous H₂S decreases naloxone-precipitated withdrawal signs, maybe

through decreasing AC/cAMP/PKA/CREB/NMDR signaling pathway in heroin-dependent rats' nucleus accumbens.

Key Words:

Hydrogen sulfide, Heroin withdrawal symptom, cAMP pathway, Phosphorylated NMDA receptor, Nucleus accumbens, Rat.

Introduction

Heroin addiction leads to serious social, medical and economic problems, but effective treatment for heroin addiction is still limited. Heroin abuse has provoked many investigations to study its mechanism of action. The heroin-induced changes in brain have been associated with neurotoxicity or another different mechanism¹.

Clinical data shows that heroin addiction could cause obvious damage to the central nervous system (CNS), which contains a large amount of endogenous hydrogen sulfide (H₂S). H₂S was suggested as a new neuromodulator playing a physiological role in regulating brain functions². Endogenous H₂S is the final metabolite of sulfur-containing amino acids and exists in both the gaseous form and the NaHS dissolved form *in vivo*. NaHS can be hydrolyzed into Na⁺ and HS⁻, the latter being able to bind H⁺ in the body to produce H₂S, which forms a dynamic equilibrium with NaHS. The H₂S concentration in a NaHS solution is stable, so the NaHS solution has been widely used as a H₂S donor³.

Previous study has shown that heroin addiction can damage learning and memory in rats. Exogenous H₂S donor NaHS increases heroin-induced learning and memory damage in rat⁴ and alleviates

heroin-induced rat hippocampus damage through antioxidant and anti-apoptosis effects³. Chronic exposure to heroin results in a number of cellular and molecular adaptations. Among these adaptations, an up-regulation of the cyclic adenosine 3', 5'-monophosphate (cAMP)-dependent signaling pathway has been observed repeatedly. Heroin treatment acutely augments cAMP production and its repeated administration alter the cAMP-related signaling pathway, i.e. increase adenylate cyclase (AC) and cAMP-dependent (or related) protein kinase activity⁵⁻⁶. Based on these considerations, we investigated the effect of exogenous H₂S on cAMP content and cAMP-dependent protein kinase (PKA) activity.

Many studies have showed that H₂S stimulates NMDA receptor-mediated response via the cAMP pathway. H₂S enhances cAMP production, cAMP activates PKA, and then the activated PKA phosphorylate N-methyl-D-aspartate (NMDA) receptor subunits NR1, NR2A and NR2B in turn at specific site⁷. However, this result has never been confirmed clearly in the field of drug addiction. In the present study, we administer NaHS as the exogenous H₂S donor to detect the change of H₂S content, the effect of exogenous H₂S on cAMP signaling pathway and phosphorylate NMDA receptor in heroin-dependent rats' nucleus accumbens.

Materials and Methods

Animals

Male Sprague-Dawley rats (250 ± 20 g) obtained from Guangxi Province Laboratory Animal Center in China were housed under natural light/dark cycle (12 to 12 h) with food and water available *ad libitum*. All procedures were performed in accordance with institutional guidelines. The animals were treated according to the guidelines of Laboratory Animals and the Animal Ethics Committee of Guangxi University.

Animal Group and Treatment

After a week of adaptation, rats were randomly divided into four experimental groups: Saline + Saline group (n=20), Saline + NaHS group (n=20), Saline + Heroin group (n=20), NaHS + Heroin group (n=20). NaHS (St Louis, MO, USA) dissolved in saline was administered intraperitoneally 30 min prior to heroin. An equal volume of saline was intraperitoneally injected in the saline group.

The following procedures were conducted in order to establish a chronic heroin dependence model (Heroin was provided by Police Bureau of Guangxi Province, China, containing 85.48% of diacetylmorphine, 6.21% of acetylmorphine and 8.31% of acetylcodeine). Heroin was dissolved in 0.9% NaCl solution immediately before injection, and was injected at the volume of 1.0 ml/kg BW (body weight). Rats were subcutaneously injected with heroin twice a day for 10 days. The dose for each injection was 3 mg/kg BW on day 1, and was increased by 3 mg/kg each day. On day 10, 4 h after injection of heroin 30 mg/kg, heroin withdrawal syndrome was precipitated by administration of naloxone (5 mg/kg, i.p.) (Beijing Four-ring, Pharmaceutical Co. Ltd., China). The animals were placed in a plexiglass cylinder for habituation to the test environment one hour prior to the naloxone injection. Naloxone was injected intraperitoneally to precipitate withdrawal symptoms. Signs of withdrawal were scored for a total of 60 min at 15 min intervals. Ten previously identified behavioral characteristics of the rat heroin abstinence syndrome were assessed. The absolute frequency of five episodic behaviors was recorded and scored based on multiples of five incidents (0 = no incidents; 1 = 1-5 incidents; 2 = 6-10 incidents and 3 > 10 incidents). Behaviors scored in this manner included: jumping, teeth chatter, writhing, wet-dog shakes, and rearing. Five withdrawal behaviors could not be defined in discrete episodes (ptosis, lacrimation, piloerection, irritability and diarrhea), and the severity of these behaviors was assessed using a four-point scale: 0 = absent; 1 = mild; 2 = moderate; 3 = severe. The amount of body weight loss was measured at the end of the rating period (i.e., 1 h after the administration of naltrexone) and a score was calculated based on multiples of 5 g (0 = no loss; 1 = 1-5 g; 2 = 6-10 g; 3=11-15 g, etc.). The scores for each time period were then added together⁸⁻⁹. The people measuring behavior were blinded to the experimental groups.

Sample Preparation

Brains were cut in a Reichert-Jung 2800 Frigocut E cryostat at -20°C. Coronal sections (1 mm thick) were cut with their rostral faces at +2.0 mm (for nucleus accumbens) from Bregma¹⁰. Tissue samples were punched from the nucleus accumbens sections with a 14-gauge needle.

Measurement of H_2S Level in Nucleus Accumbens

Nucleus accumbens were removed and flash-frozen with liquid nitrogen, then homogenized in ice-cold 50 mM/L potassium phosphate buffer, pH 8.0 (12% wt/vol), with a Polytron homogenizer. The homogenate was centrifuged (47,000 g; 10 min; 4°C) and the supernatant (75 μ L) was mixed with 0.25 mL Zn acetate (1%) and 0.45 mL water for 10 min at room temperature. Then, trichloroacetic acid (TCA) (10%; 0.25 mL) was added and centrifuged (14,000 g; 10 min; 4°C). The clear supernatant was collected and mixed with N, N-dimethyl-p-phenylenediamine sulfate (20 mM/L; 133 μ L) in 7.2 M/L HCl and FeCl₃ (30 mM/L; 133 μ L) in 1.2 M/L HCl. The absorbance at 670 nm was measured with a microplate reader after 20 min. The calibration curve was linear from 0 to 320 μ M/L NaHS or 96 μ M/L H₂S. Results are presented as nmol/min·g prot.

Measurement of AC Activity Assay Level in Nucleus Accumbens

Nucleus accumbens punches were homogenized in 800 μ L solubilization buffer (50 mM Tris-HCl pH 7.4; 1 mM EGTA; ethylene glycol intraacetic acid) using a potter. Protein about 20 μ g was incubated in assay buffer (50 mM Tris-HCl pH 7.4; 100 mM NaCl; 3 mM MgCl₂; 1 mM EGTA; 50 μ M GTP; 1 mM ATP; 0.2 mM IBMX; 3-isobutyl-1-methylxanthine) with or without 1 μ M forskolin in a total volume of 1 mL at 25°C for 10 min. Samples were heated at 95°C for 5 min to stop the reaction and centrifuged at 13,000 $\times$ g for 5 min. The amount of cAMP formed during the reaction was quantified using a method based on binding to PKA (Protein Kinase A), as described by Munirathinam and Yoburn¹¹. Nucleus accumbens supernatant or a standard amount of cAMP (0. 1. 2. 4. 8. or 16 pmol) about 10 μ L was incubated in a buffer containing 50 mM Tris-HCl pH 7.4, 4 mM EDTA, 25 nCi [8-3H] cAMP and 4 μ g PKA in a total volume of 200 μ L at 4°C for several hours. 100 μ L 3.5% charcoal slurry containing 50 mM Tris-HCl pH 7.4, 4 mM EDTA with 2% BSA (bovine serum albumin) was added to each sample and centrifuged at 13,000 $\times$ g for 7 min at 4°C. Radioactivity contained in 200 μ L supernatant was counted using liquid scintillation analysis (Beckman Instruments, Mountain View, CA, USA). Blank value was determined in the absence of PKA and subtracted from all other values. Results are presented as pmol/ μ g cAMP protein¹².

Measurement of cAMP Level in Nucleus Accumbens

Nucleus accumbens tissue micropunches were placed into Ependorff tubes containing 300 μ L of ice cold 6% trichloroacetic acid, and were then homogenized and sonicated. Samples were then centrifuged and the supernatant was washed four times with 5 vol. of water-saturated diethyl ether. The extract was lyophilized and the pellet was dissolved in radioimmunoassay (RIA) buffer. The concentration of sample cAMP was measured in triplicates using [¹²⁵I] cAMP acetylation RIA kits (Amersham, Little Chalfont, Buckinghamshire, UK) according to the protocol recommended by the company. Standard cyclic nucleotide concentrations were repeatedly measured throughout each assay procedure in order to ensure the stable performance of the assay. Results are presented as pmol/mg protein.

Measurement of PKA Activity Assay Level in Nucleus Accumbens

PKA activity was determined essentially according to the method described by Lou and Pei¹³. The animals were acutely decapitated, and the nucleus accumbens tissue was dissected rapidly and homogenized on ice in homogenization buffer (25 mM Tris-HCl, 1 mM EDTA, 1 mM DTT (Dithiothreitol), and 100 M leupeptin). The homogenate was centrifuged at 20,000 g for 5 min at 4°C. The resulting supernatant was assayed for PKA activity using a PepTag nonradioactive PKA assay kit (Promega, Madison, WI, USA). All reaction components were added on ice in a final volume of 25 μ L of the following mixture: 5 μ L of PepTag PKA reaction buffer, 5 μ L of PepTag A1 Peptide (0.4 g/l), 5 μ L of cAMP (5 M), and 5 μ L of sample homogenate. The mixture was incubated at 30°C for 30 min. Then, the reaction was stopped by placing the tube in a boiling water bath for 10 min, and the samples were loaded onto the gel for electrophoresis. Before loading samples, 1 μ L of 80% glycerol was added to the sample to ensure that it remained in the well. PKA-specific peptide substrate used in this experiment was PepTag A1 Peptide, L-R-R-A-S-L-G (kemptide). The assay was based on the changes in the net charge of the fluorescent PKA substrates after phosphorylation. This change allowed the phosphorylated and unphosphorylated versions of the substrate to be rapidly separated on an agarose gel at neutral pH. The phosphorylated substrate migrated toward the positive electrode, whereas the non-phosphorylated substrate migrated toward the negative electrode. The intensity of fluorescence of

phosphorylated peptides, which reflected the activity of PKA, was quantified using a bio-imaging system (Syngene, Cambridge, UK). Results are presented as pmol/(min /mg) protein.

Western Blots Assay p-CREB, p-NR1, p-NR2A, p-NR2B Proteins Level in Nucleus Accumbens

Proteins expression in tissue was quantified using Western blot. Briefly, samples were homogenized in 20% (wt/vol) ice-cold buffer containing 10 mmol/L Tris-HCl, pH 7.4; 1% sodium dodecyl sulfate (SDS); 1 mmol/L sodium vanadate; 10 µg/mL aprotinin; 10 µg/mL leupeptin and 1 mmol/L phenylmethylsulfonyl fluoride. The tissue homogenate was centrifuged at 13,400 g for 20 min at 4°C. The supernatant was aspirated and stored at -80°C until use. Protein concentration in the supernatant was detected with the Brad board method. SDS-PAGE (polyacrylamide gel electrophoresis) was performed on a 10 % gel, on which 30 µg of total protein per well was loaded. After SDS-PAGE, the proteins were transferred from the gels onto polyvinylidene difluoride membrane. After being blocked in 5% non-fat milk in 1×PBS (phosphate buffered saline) containing 0.1% Tween-20 at room temperature for 60 min, membranes were blocked in 5% nonfat milk and probed sequentially with antibodies against phosphorylated rabbit anti-pNR1 (phosphorylated N-methyl-aspartate receptor 1 submit) antibody (Ser897) (1:1,000, Upstate Biotechnology Inc., Lake Placid, NY, USA), phosphorylated NR2A (phosphorylated N-methyl-aspartate receptor 2a submit) (Ser1232) (1:1,000, Tocris Bioscience, Bristol, BS11 OKL, UK), phosphorylated NR2B (phosphorylated N-methyl-aspartate receptor 2b submit) (Tyr1472) (1:1,000, Sigma Chemical Co, St Louis, MO, USA), phosphorylated cAMP response element-binding protein (CREB) (1:500; Cell Signaling Technology, Danvers, MA, USA) and antibody against -actin (1:500, Millipore Co, Billerica, MA, USA) respectively. Actin was used for the normalisation of protein loaded. The blots were incubated with horse-radish peroxidase-conjugated antibody (1:2,000, Santa Cruz, Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature and visualized with ECL solution (5 min) followed by film exposure. Protein bands were visualized through an ECL detection system (Pierce Ltd., Rockford, IL, USA). The X-ray films were scanned and the optical densities of Western blots were analyzed by densitometry using quantity-one software (Bio-Rad Laboratories, Hercules, CA, USA).

Assay for Protein Content

Protein content in tissue was measured by Lowry et al method with bovine serum albumin as a standard sample¹³.

Statistical Analysis

The results are presented as mean average ± SEM. Statistical significance of the changes was determined using Graph Prism version 5.0 (Graph Pad Software, San Diego, CA, USA). Student's *t*-test was used to compare the difference between two experimental groups, while one-way analysis of variance (ANOVA) was used to compare the differences among three or more groups followed by Dunnett's *t*-test for individual group comparisons. *p* < 0.05 values for all tests was considered as statistically significant.

Results

Exogenous H₂S Attenuates Heroin Withdrawal Symptoms

The result was showed in Figure 1. Heroin-administered rat exhibited significant withdrawal signs, thus successfully establishing a heroin-dependent model in rat. There were no withdrawal symptoms in Saline group. The results showed that exogenous H₂S significantly attenuated naloxone-precipitated withdrawal symptoms. The results still showed that exogenous H₂S has no effect on naloxone-precipitated withdrawal symptoms in Saline + Saline group.

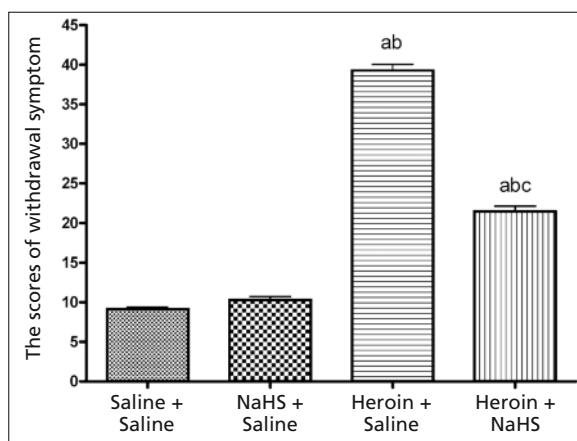


Figure 1. Exogenous H₂S reduced the scores of heroin withdrawal symptom NaHS can alleviate the heroin withdrawal symptom; moreover, can not lead to withdrawal symptom. ^a*p* < 0.05 compared with the Saline + Saline group; ^b*p* < 0.05 compared with the Saline + NaHS group; ^c*p* < 0.05 compared with the Saline + Heroin group. Data is presented as mean ± SEM n=10 rats per group.

Effect of Exogenous H_2S on the Levels of H_2S in Nucleus Accumbens

The result was showed in Figure 2. The result showed that exogenous H_2S increased the level of H_2S in nucleus accumbens in every group, while heroin can decrease the level of H_2S .

Effect of Exogenous H_2S on the Activities of AC in Nucleus Accumbens

The result was showed in Figure 3. The result showed that exogenous H_2S decreased the activity of AC caused by heroin. It is interesting that there is a significant increase of the activity of AC in Saline + NaHS group compared with Saline + Saline group.

Effect of Exogenous H_2S on the Levels of H_2S cAMP Level in Nucleus Accumbens

As showed in Figure 4, there was significantly increased cAMP content between Saline + Heroin group and Saline+ Saline group. When Heroin + Saline group was compared with NaHS + Heroin group, cAMP content in nucleus accumbens was significantly decreased in NaHS + Heroin group. However, when Saline + Saline group was compared with Saline + NaHS group, the level of cAMP content was still increased in NaHS + Saline group.

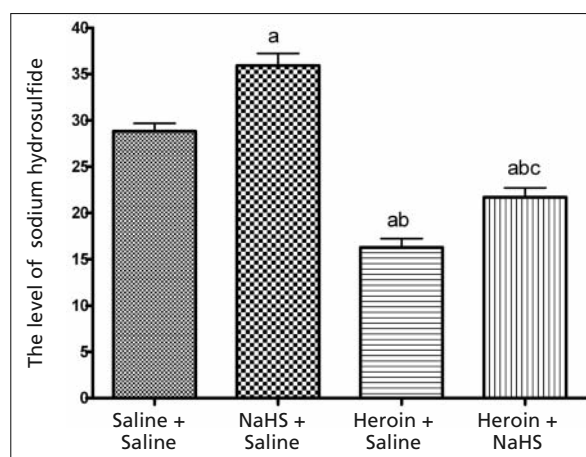


Figure 2. The effect of exogenous H_2S on H_2S level in nucleus accumbens of heroin-induced addiction rat. There are significantly different levels between any two groups. Compared with Saline group, NaHS significantly increased the level of H_2S in NAC. The heroin significantly decreased the level of H_2S in NAC. When comparing NaHS + Heroin group and Saline + Heroin group, the result showed that the level of H_2S in NAC was significantly increased in NaHS + Heroin group. *Note:* The unit of H_2S content was expressed as nmol/min.gprot. ^a $p < 0.05$ compared with the Saline + Saline group; ^b $p < 0.05$ compared with the Saline + NaHS group; ^c $p < 0.05$ compared with the Saline + Heroin group. Data is presented as mean $\pm$ SEM n=10 rats per group.

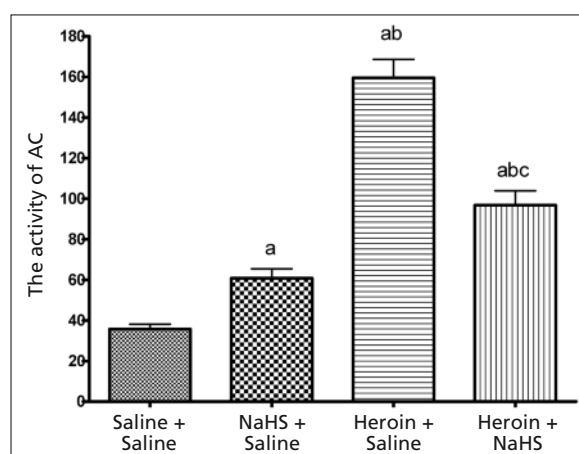


Figure 3. The effect of exogenous H_2S on the activity of AC in nucleus accumbens of heroin-induced addiction rat. There are significantly different levels between two groups expect Saline+ Saline and Saline + NaHS. Compared with Saline group, NaHS significantly increased the activity of AC in NAC. The heroin significantly decreased the activity of AC in nucleus accumbens. When comparing NaHS + Heroin group and Heroin + Saline group, the result showed that the activity of AC in NAC was significantly increased in NaHS + Heroin group. *Note:* The unit of AC activity was expressed as pmol cAMP per μg protein. ^a $p < 0.05$ compared with the Saline + Saline group; ^b $p < 0.05$ compared with the Saline + NaHS group; ^c $p < 0.05$ compared with the Saline + Heroin group. Data is presented as mean $\pm$ SEM n=10 rats per group.

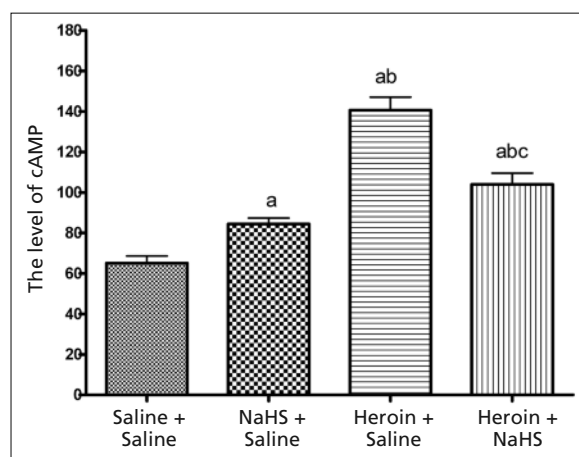


Figure 4. The effect of exogenous H_2S on the level of cAMP in nucleus accumbens of heroin-induced addiction rat. There are significantly different levels between any two groups. Compared with Saline group, NaHS significantly increased the level of cAMP in nucleus accumbens. The heroin significantly decreased the level of cAMP in NAC. When comparing NaHS + Heroin group and Saline + Heroin group, the result showed that the level of cAMP in NAC was significantly increased in NaHS + Heroin group. *Note:* The unit of cAMP was expressed as pmol/mg protein. ^a $p < 0.05$ compared with the Saline + Saline group; ^b $p < 0.05$ compared with the Saline + NaHS group; ^c $p < 0.05$ compared with the Saline + Heroin group. Data is presented as mean $\pm$ SEM n=10 rats per group.

Effect of Exogenous H₂S on the Activity of PKA in Nucleus Accumbens

As showed in Figure 5, as far as PKA activity is concerned, when Saline + Saline group was compared with Saline + NaHS group, the level of PKA activity was increased in Saline + NaHS group. Furthermore, when Saline + Heroin group was compared with NaHS + Heroin group, the level of PKA was significantly decreased in NaHS + Heroin group.

Effect of Exogenous H₂S on the Level of p-CREB, p-NR1, p-NR2A, p-NR2B Receptor Protein in Nucleus Accumbens

We hypothesized that cAMP/PKA-mediated phosphorylation and activation of CREB are key mediators in the regulation of NMDA expression in the rat nucleus accumbens. To confirm it, Western blot analysis was used to assay phosphorylated CREB, NR1, NR2A and NR2B receptor.

Figure 6 showed that in Saline + Heroin, NaHS + Heroin and Saline + NaHS groups, the level of p-CREB was significantly increased compared with Saline + Saline group. When Saline + Heroin group was compared with NaHS + Heroin group, the level of p-CREB was significantly decreased in the NaHS + Heroin group.

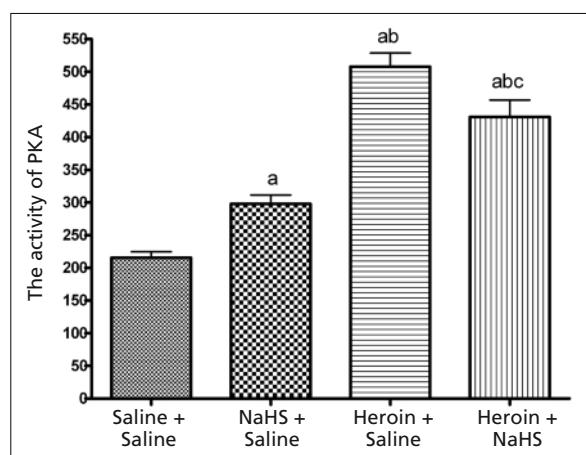


Figure 5. The effect of exogenous H₂S on the activity of PKA in nucleus accumbens of heroin-induced addiction rat. Compared with Saline group, NaHS significantly increased the activity of PKA in NAC. The heroin significantly decreased the activity of AC in nucleus accumbens. When comparing NaHS + Heroin group with Saline + Heroin group, the result showed that the activity of PKA in NAC was significantly increased in NaHS + Heroin. Note: The unit of PKA activity was expressed as pmol/min/mg protein. ^a*p* < 0.05 compared with the Saline + Saline group; ^b*p* < 0.05 compared with the Saline + NaHS group; ^c*p* < 0.05 compared with the Saline + Heroin group. Data is presented as mean ± SEM n = 10 rats per group.

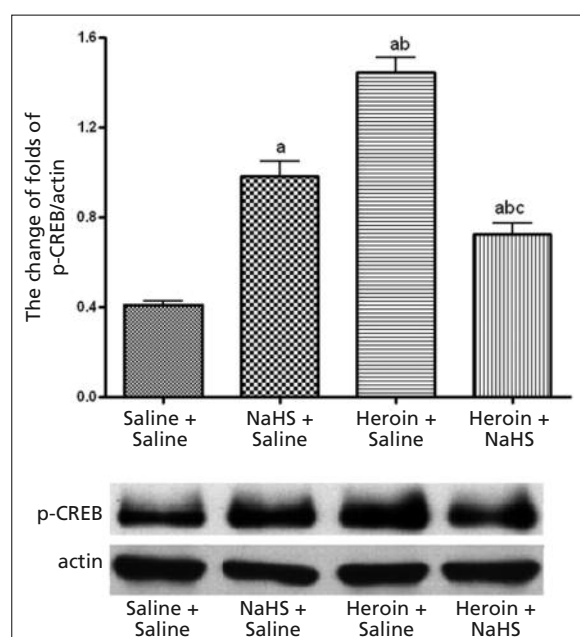


Figure 6. The effect of exogenous H₂S on the level of p-CREB protein in nucleus accumbens of heroin-induced addiction rat. The result showed that exogenous H₂S can decrease the levels of p-CREB protein caused by heroin. Furthermore, Exogenous H₂S treatment alone can increase the levels of p-CREB protein in nucleus accumbens. The Western blot is representative of five independent experiments. Optical density of protein bands was quantified by scanning densitometry and plotted relative to β-actin. ^a*p* < 0.05 compared with the Saline + Saline group; ^b*p* < 0.05 compared with the Saline + NaHS group; ^c*p* < 0.05 compared with the Saline + Heroin group. Data is presented as mean ± SEM n = 5 rats per group.

As showed in Figure 7, 8 and 9, the results showed that the level of p-NR1 proteins in Saline + Heroin group was significantly increased when compared with Saline + Saline group. When NaHS + Heroin group was compared with Saline + Heroin group, the level of p-NR1 was significantly decreased in NaHS + Heroin group.

When NaHS + Heroin group was compared with Saline + Heroin group, the levels of p-NR2A and p-NR2B proteins were significantly increased in NaHS + Heroin group. Moreover, the NaHS + Saline group can increase the levels of p-NR2A and p-NR2B proteins compared with Saline + Saline group.

Discussion

Hydrogen sulfide (H₂S) is a gaseous molecule synthesized both in the peripheral nervous system and in the CNS in mammals², and this molecule has been suggested to influence hippocampus synaptic plasticity¹⁵.

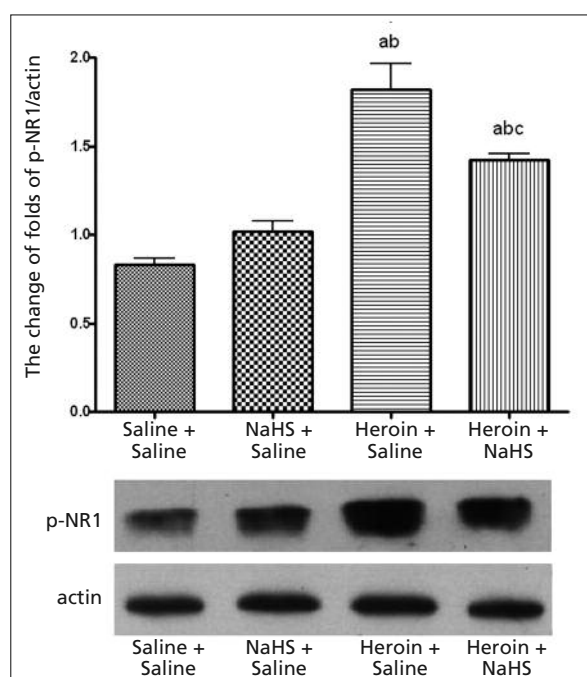


Figure 7. The effect of exogenous H_2S on the level of p-NR1 receptor protein in nucleus accumbens of heroin-induced addiction rat. The result showed that exogenous H_2S treatment alone can increase the level of p-NR1 receptor protein in nucleus accumbens. Furthermore, exogenous H_2S can decrease the high level of p-NR1 receptor protein caused by heroin. However, the level of p-NR1 in each group is insignificantly different. The Western blot is representative of five independent experiments. Optical density of protein bands was quantified by scanning densitometry and plotted relative to β -actin. ^a $p < 0.05$ compared with the Saline + Saline group; ^b $p < 0.05$ compared with the Saline + NaHS group; ^c $p < 0.05$ compared with the Saline + Heroin group. Data is presented as mean $\pm$ SEM $n = 5$ rats per group.

Heroin dependence is a chronic and relapsing disease and characterized by compulsive drug seeking and use, also produces excessive degrees of tolerance and physical dependence, which are also important factors in the emergence of withdrawal syndrome. Heroin withdrawal typically appears if use is reduced abruptly or opioid antagonist (that blocks the action of heroin) is administered. Heroin withdrawal is generally characterized by drug craving and can cause serious physical and emotional trauma including: stroke, heart attack, and even death¹⁶.

The best established molecular adaptations to long-term drug exposure are up-regulation of the cAMP/PKA signal pathway, which plays a key role in the development of opioid tolerance and dependence^{17,18}. Chronic opioid treatment-induced up-regulation of the cAMP pathway occurs in many important brain regions such as the nucleus

accumbens^{19,20}. In cAMP/PKA signal pathway, AC converts ATP to cAMP, which in turn activates cytoplasmic PKA. The catalytic units of PKA cross the nuclear membrane and phosphorylate inactive CREB. Phosphorylation of CREB in the nucleus accumbens up-regulates several genes associating with reward and reinforcement²¹.

In our experiment, Heroin-administered rat exhibited significant withdrawal signs, thus establishing a heroin-dependent model in rat. Exogenous H_2S can alleviate withdrawal syndrome in heroin-administered rat. Moreover, our study showed that exogenous hydrogen sulfide can stimulate the AC-cAMP-PKA-CREB pathway. On the other hand, exogenous hydrogen sulfide still can depress AC-cAMP-PKA-CREB pathway by heroin stimulation.

NMDA receptors (N-methyl D-aspartate R) can induce synaptic plasticity change of neurons by activating the AC-cAMP-PKA-CREB path-

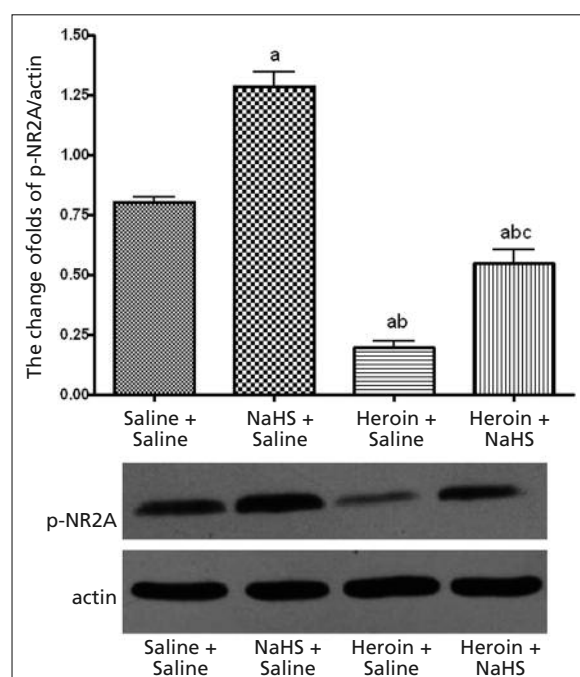


Figure 8. The effect of exogenous H_2S on the level of p-NR2A receptor protein in nucleus accumbens of heroin-induced addiction rat. The result showed that exogenous H_2S treatment alone can increase the level of p-NR2A receptor protein in nucleus accumbens. Furthermore, exogenous H_2S can increase the low level of p-NR2A receptor protein caused by heroin. The Western blot is representative of five independent experiments. Optical density of protein bands was quantified by scanning densitometry and plotted relative to β -actin. ^a $p < 0.05$ compared with the Saline + Saline group; ^b $p < 0.05$ compared with the Saline + NaHS group; ^c $p < 0.05$ compared with the Saline + Heroin group. Data is presented as mean $\pm$ SEM $n = 5$ rats per group.

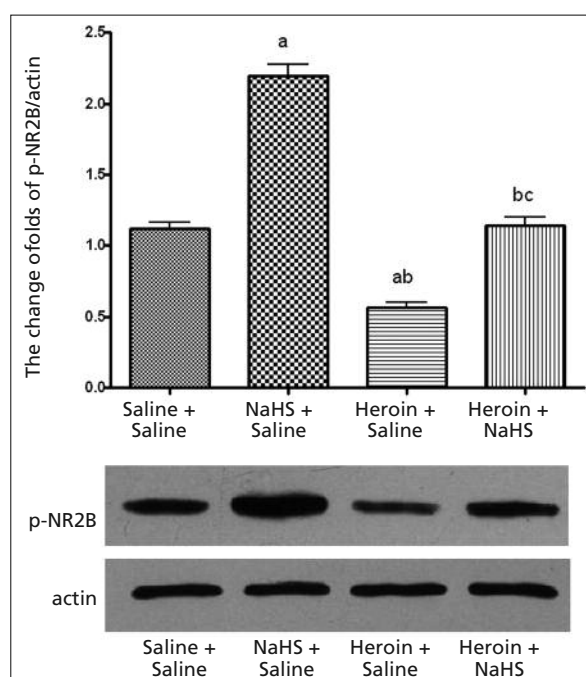


Figure 9. The effect of exogenous H₂S on the level of p-NR2B receptor protein in nucleus accumbens of heroin-induced addiction rat. The result showed that exogenous H₂S treatment alone can increase the level of p-NR2B receptor protein in nucleus accumbens. Furthermore, exogenous H₂S can increase the low level of p-NR2B receptor protein caused by heroin. Moreover, the difference between NaHS + Heroin group and Saline + Saline group is insignificant. The Western blot is representative of five independent experiments. Optical density of protein bands was quantified by scanning densitometry and plotted relative to β -actin. ^a $p < 0.05$ compared with the Saline + Saline group; ^b $p < 0.05$ compared with the Saline + NaHS group; ^c $p < 0.05$ compared with the Saline + Heroin group. Data is presented as mean $\pm$ SEM $n = 5$ rats per group.

way, and involved in opioid dependence and withdrawal²². It was well confirmed that NMDA receptors are widely involved in neural and behavioral plasticity¹⁸. NMDA receptors have been implicated in several different forms of drug-induced neural and behavioral plasticity including the development of tolerance, sensitization and physical dependence on a variety of psychoactive drugs including heroin, amphetamine, cocaine, nicotine and ethanol²³⁻²⁷. The NMDAR are composed of an obligatory NR1 subunit and other subunits from the NR2 and NR3 family. The NR2 family consists of NR2A, NR2B, NR2C and NR2D family members²⁸. NR2A and NR2B subunits have distinct intracellular protein associations and signaling pathways²⁹. NR2A-containing receptors are predominately located synaptically, while NR2B-containing receptors are ex-

pressed both synaptically and extrasynaptically³⁰. The NMDARs are regulated by phosphorylation, and kinases such as tyrosine kinase and serine/threonine kinase have been shown to modulate NMDAR ion channel activity^{31,32}. The phosphorylation of NR1 is by protein kinase C and cAMP-dependent protein kinase (PKA), respectively³³. NR2B subunits are phosphorylated by CaM kinase II, PKA, PKC and protein tyrosine kinase³⁴⁻³⁶. Our study showed that the phosphorylation of NMDA receptors was changed by NaHS. However, for different NMDA receptors, the changes were different. The p-NB2B and p-NR2A were significantly increased by NaHS and decreased by heroin. However, NaHS + Heroin group showed that the level of p-NB1R was significantly decreased when compared with Saline + Heroin group. The changes of p-NB2B and p-NR2A levels may be related to up-regulation of the cAMP/PKA signal pathway.

Conclusions

The present report demonstrates that behavioral and neuro-chemical effects of heroin can be significantly attenuated by administration of exogenous H₂S. Therefore, a low dose of NaHS offering protective effect may act through suppressing AC-cAMP-PKA-CREB pathway. However, there are still many questions to be clarified-especially, why can exogenous H₂S depress AC-cAMP-PKA-CREB pathway by heroin stimulation? And why can simple exogenous H₂S treatment without heroin treatment stimulate AC-cAMP-PKA-CREB pathway? They need to be further studied.

Acknowledgements

This study was supported by Guangxi science funding (NO: 2011GXNSFB018078).

References

- 1) BUTTNER A, MALL G, PENNING R, WEIS S. The neuropathology of heroin abuse. *Forensic Sci Int* 2000; 113: 435-442.
- 2) KIMURA H. Hydrogen sulfide as a neuromodulator. *Mol Neurobiol* 2002; 26: 13-19.
- 3) JIANG LH, LUO X, HE W, HUANG XX, CHENG TT. Effects of exogenous hydrogen sulfide on apoptosis proteins and oxidative stress in the hippocampus of rats undergoing heroin withdrawal. *Arch Pharm Res* 2011; 34: 2155-2162.

- 4) LUO XM, PAN GS, CHEN YS. Effects of exogenous hydrogen sulfide on learning and memory of heroin-addictive rats. *Acta Acad Med Zunyi* 2009; 32: 2108-2110.
- 5) SLOTKIN TA, SEIDLER FJ, YANAI J. Heroin neuroteratogenicity: targeting adenylyl cyclase as an underlying biochemical mechanism. *Brain Res Dev Brain Res* 2001; 132: 69-79.
- 6) SHICHINOHE S, OZAWA H, HASHIMOTO E, TATSCHNER T, RIEDERER P, SAITO T. Changes in the cAMP-related signal transduction mechanism in postmortem human brains of heroin addicts. *J Neural Transm* 2001; 108: 335-347.
- 7) KIMURA H. Hydrogen sulfide induces cyclic AMP and modulates the NMDA receptor. *Biochem Biophys Res Commun* 2000; 267: 129-133.
- 8) WANG R. Two's company, three's a crowd: can H₂S be the third endogenous gaseous transmitter? *FASEB J* 2002; 16: 1792-1798.
- 9) ZHU H, HO IK. NMDA-R1 antisense oligonucleotide attenuates withdrawal signs from morphine. *Eur J Pharmacol* 1998; 352: 151-156.
- 10) PAXINOS G, WATSON C. *The Rat Brain in Stereotaxic Coordinates*, 4th edn. Academic Press, 1998.
- 11) MUNIRATHINAM G, YOBURN BC. A simple procedure for assaying cAMP. *Pharmacol Biochem Behav* 1994; 48: 813-816.
- 12) CONTET C, FILLIOL D, MATIFAS A, KIEFFER BL. Morphine-induced analgesic tolerance, locomotor sensitization and physical dependence do not require modification of mu opioid receptor, cdk5 and adenylyl cyclase activity. *Neuropharmacology* 2008; 54: 475-486.
- 13) LOU LG, PEI G. Modulation of protein kinase C and cAMP-dependent protein kinase by delta-opioid. *Biochem Biophys Res Commun* 1997; 236: 626-629.
- 14) LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193: 265.
- 15) ABE K, KIMURA H. The possible role of hydrogen sulfide as an endogenous neuromodulator. *J Neurosci* 1996; 16: 1066-1071.
- 16) XU B, WANG Z, LI G, LI B, LIN H, ZHENG R, ZHENG Q. Heroin-administered mice involved in oxidative stress and exogenous antioxidant-alleviated withdrawal syndrome. *Basic Clin Pharmacol Toxicol* 2006; 99: 153-161.
- 17) NESTLER EJ. Is there a common molecular pathway for addiction? *Nat Neurosci* 2005; 8: 1445-1449.
- 18) NESTLER EJ, AGHAJANIAN GK. Molecular and cellular basis of addiction. *Science* 1997; 278: 58-63.
- 19) NESTLER EJ, HOPE BT, WIDNELL KL. Drug addiction: a model for the molecular basis of neural plasticity. *Neuron* 1993; 11: 995-1006.
- 20) WIDNELL KL, SELF DW, LANE SB, RUSSELL DS. Regulation of CREB expression in vivo: evidence for a functional role in morphine action in the nucleus accumbens. *J Pharmacol Exp Ther* 1996; 276: 306-315.
- 21) CARLEZON WA JR, DUMAN RS, NESTLER EJ. The many faces of CREB. *Trends Neurosci* 2005; 28: 436-445.
- 22) TRUJILLO KA. Are NMDA receptors involved in opiate-induced neural and behavioral plasticity? A review of preclinical studies. *Psychopharmacology* 2000; 151: 121-141.
- 23) HERMAN BH, VOCCI F, BRIDGE P. The effects of NMDA receptor antagonists and nitric oxide synthase inhibitors on opioid tolerance and withdrawal. Medication development issues for opiate addiction. *Neuropsychopharmacology* 1995; 13: 269-293.
- 24) STEPHENS DN. A glutamatergic hypothesis of drug dependence: extrapolations from benzodiazepine receptor ligands. *Behav Pharmacol* 1995; 6: 425-446.
- 25) INTURRISI CE. Preclinical evidence for a role of glutamatergic systems in opioid tolerance and dependence. *Semin Neurosci* 1997; 9: 110-119.
- 26) WOLF ME. The role of excitatory amino acids in behavioral sensitization to psychomotor stimulants. *Prog Neurobiol* 1998; 54: 679-720.
- 27) JI D, SUI ZY, MA YY, LUO F, CUI CL, HAN JS. NMDA receptor in nucleus accumbens is implicated in morphine withdrawal in rats. *Neurochem Res* 2004; 29: 2113-2120.
- 28) ISHII T, MORIYOSHI K, SUGIHARA H, SAKURADA K, KADOTANI H, YOKOI M, AKAZAWA C, SHIGEMOTO R, MIZUNO N, MASU M, NAKANISHI S. Molecular characterization of the family of the N-methyl-D-aspartate receptor subunits. *J Biol Chem* 1993; 268: 2836-2843.
- 29) KIM MJ, DUNAH AW, WANG YT, SHENG M. Differential roles of NR2A and NR2B containing NMDA receptors in ras-ERK signaling and AMPA receptor trafficking. *Neuron* 2005; 46: 745-760.
- 30) TOVAR KR, WESTBROOK GL. The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses in vitro. *J Neurosci* 1999; 19: 4180-4188.
- 31) KOHR G, SEEBURG PH. Subtype-specific regulation of recombinant NMDA receptor-channels by protein tyrosine kinases of the src family. *J Physiol* 1996; 492: 445-452.
- 32) SWOPE SL, MOSS SI, RAYMOND LA, HUGANIR RL. Regulation of ligand-gated ion channels by protein phosphorylation. *Adv Second Messenger Phosphoprotein Res* 1999; 33: 49-78.
- 33) TINGLEY WG, ROCHE KW, THOMPSON AK, HUGANIR RL. Regulation of NMDA receptor phosphorylation by alternative splicing of the C-terminal domain. *Nature* 1993; 364: 70-73.
- 34) GARDONI F, CAPUTI A, CIMINO M, PASTORINO L, CATTABENI F, DI LUCA M. Calcium/calmodulin-dependent protein kinase II is associated with NR2A/B subunits of NMDA receptor in postsynaptic densities. *J Neurochem* 1998; 71: 1733-1741.
- 35) LEONARD AS, HELL JW. Cyclic AMP-dependent protein kinase and protein kinase C phosphorylate N-methyl-D-aspartate receptors at different sites. *J Biol Chem* 1997; 272: 12107-12115.
- 36) LAU LF, HUGANIR RL. Differential tyrosine phosphorylation of N-methyl-D-aspartate receptor subunits. *J Biol Chem* 1995; 270: 20036-20041.