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Intranasal delivery of berberine via *in situ* thermoresponsive hydrogel with non-invasive therapy exhibits better antidepressant-like effects

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Abstract

The efficacy of antidepressant therapy is frequently limited by challenges of potential to reach the brain. The development of new strategies to deliver more antidepressant to brain bypass blood-brain barrier (BBB) is beneficial for the treatment of nervous system disease, especially for depression. Here, we report an unconventional strategy by intranasal delivery of berberine with *in situ* thermoresponsive hydrogel as holder in nasal cavity to improve its antidepressant-like activity. Berberine/hydroxylpropyl-β-cyclodextrin (HP-β-CD) inclusion complex was firstly prepared to improve the solubility of berberine, and loaded into thermoresponsive hydrogel system of poloxamers. Radioactive tracer of ^{125}I labeled berberine was used to investigate the brain targeting. Liquid chromatography technique coupled with tandem mass spectrometry (LC-MS/MS) analysis was performed to study the pharmacokinetic change in hippocampus. Monoamine neurotransmitters were analyzed in reserpine-induced depression model, and metabolomic analysis of hippocampus was performed in chronic unpredictable mild stress (CUMS)-induced depression model. The radioactive tracer analysis manifested that thermoresponsive hydrogel by intranasal administration could maintain a high concentration gradient of berberine to the brain, and the relative bioavailability of berberine was enhanced approximately 110 folds than oral berberine/HP-β-CD inclusion complex in the hippocampus. The thermoresponsive hydrogel system results in similar or better antidepressant-like efficacy even with the lower dosage in reserpine and CUMS-induced depressant-like rats. The pharmacometabolomics analysis revealed that in addition to increasing the hippocampal monoamines levels, berberine via intranasal administration exhibited the unique mechanism by restoring mitochondrial dysfunction, phospholipids and sphingolipids abnormalities than intragastric (IG)

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administration. We consider this as a safer and more effective strategy with a lower dosage than traditionally oral drugs for the treatment of depression.

Key Words: Berberine; thermoresponsive hydrogel; intranasal delivery; antidepressant-like effect; pharmacometabolomics analysis

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Introduction

Natural products have been paid more attention as their vast structural diversity with biological relevance, and natural products or secondary metabolites generated from herbal medicines are widely used because of the wide application and least side effects [1]. About 34% of FDA approved small molecule drugs were natural products or their derivatives between 1981 and 2010 [2]. Berberine, one major bioactive compounds from Chinese herb *Coptis chinensis* Franch, has been employed for hundreds of years with a wide range of its antimicrobial and antiprotozoal properties in Chinese and Ayurvedic Medicine [3]. It has been demonstrated that berberine alleviates depression via serotonergic, dopaminergic and noradrenergic interventions [4]. More importantly, no severe side effects were reported even with large doses in clinical application. Berberine was characterized by low solubility, extensive first-pass and reduced intestinal absorption as a substrate of glycoprotein (P-gp) [5], limiting its absorption and transport to brain. How to increase transport of berberine to the brain was the key factor for the treatment of depression.

Intranasal (IN) administration is considered as one of the most attractive and feasible alternative routes for the central nervous system (CNS) targeting drug delivery [6, 7]. Compared with the conventional oral route, IN administration is convenient to avoid hepatic first-pass metabolism and bypass blood-brain barrier (BBB) via the olfactory pathway and trigeminal neural pathway [8]. Recently, a new nasal spray medication of esketamine for the treatment of depression in adults was approved by the U.S. Food and Drug Administration (FDA) [9]. The highly vascularized nasal mucosa was the main drug absorption and deposition site, while the limited surface of the nasal cavity and fast mucociliary clearance hindered the drug delivery. Several pharmaceutical strategies have been investigated for the

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formulation of IN delivery, including mucoadhesive polymers [10], lipid emulsions [11] and surfactants [12]. Among those formulations, *in situ* thermoresponsive hydrogel is currently recognized as a promising one with higher viscosity to release the drug slowly and continuously, maintaining the high concentration gradient of the drug.

In this study, we constructed an *in situ* thermoresponsive hydrogel system by intranasal administration to improve the brain targeting bioavailability of berberine hydrochloride (BBH) for treatment of depression (Figure 1), which manifested faster and more effective antidepressant-like activities with a lower dose than oral administration. The solubility of BBH was improved by the inclusion of 1, 3-benzodioxole groups of BBH into the cavity of HP-β-CD, and the BBH/HP-β-CD inclusion complex was loaded into poloxamers to construct *in situ* thermoresponsive hydrogel system. The radioactive trace of ^{125}I labeled BBH revealed that BBH/HP-β-CD thermoresponsive hydrogel system could prolong nasal residence to sustain drug release for brain-targeting BBH delivery. The relative bioavailability of BBH via BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system (IN administration) was enhanced approximately 110 folds than BBH/HP-β-CD inclusion complex (IG administration) hippocampus. Furthermore, the BBH /HP-β-CD inclusion complex thermoresponsive hydrogel system (IN administration) showed stronger antidepressant-like effect than BBH/HP-β-CD inclusion complex (IG administration) on reserpine and CUMS-induced depression-like rats. Additionally, the pharmcometabolomics analysis showed more dramatic impacts on the restoration of hippocampal abnormalities in rats.

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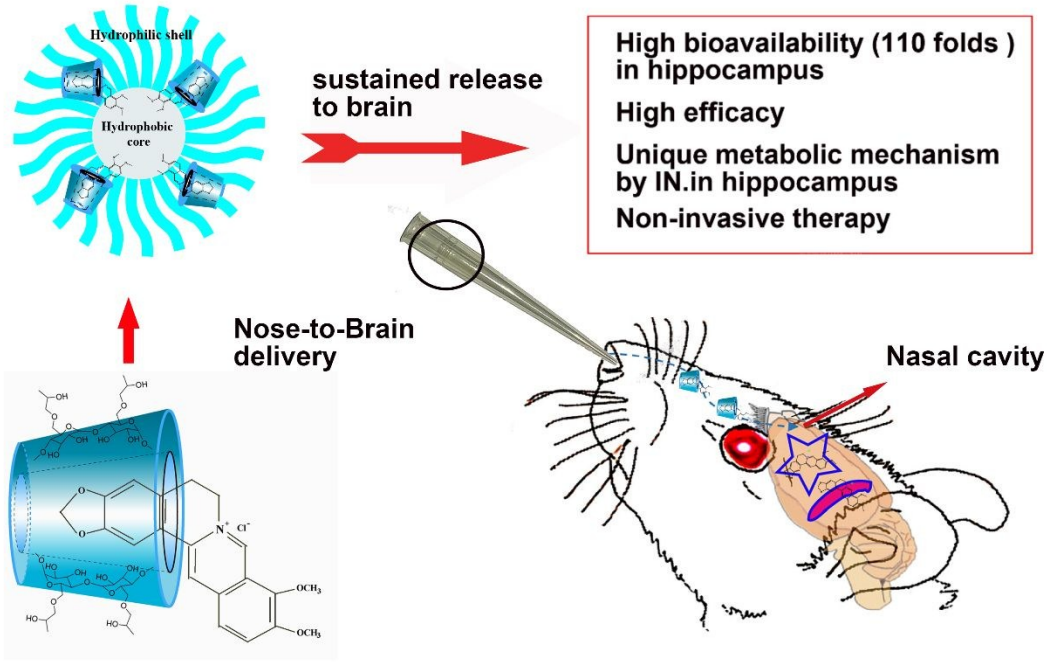

Figure 1. Schematic illustration for the nose-to-brain delivery of Berberine/HP-β-CD inclusion complex thermoresponsive hydrogel with higher bioavailability and unique metabolic mechanism than oral BBH/HP-β-CD inclusion complex for treatment of depression.

Materials and Methods

Materials

Berberine hydrochloride (purity > 98%), poloxamer P407, P188 were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Fluoxetine hydrochloride (FLX) was purchased from Eli Lilly and Company (Indianapolis, IN, USA). Acetonitrile and methanol were of chromatographic grade. All the other chemicals were of analytical grade.

Male Wistar rats (6-7 week-old, 180-220 g) were obtained from Laboratory Animal Center of the Chinese Academy of Medical Science & Peking Union Medical College (Beijing, China) and housed under standard conditions for 7 days before experiments (12:12 light-dark cycle; food and water *ad libitum*; temperature: 22-24 ºC

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and humidity: 40-60%). All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Chinese Academy of Medical Science & Peking Union Medical College, and the protocol was approved by the Animal Ethics Committee of Chinese Academy of Medical Science & Peking Union Medical College.

Preparation of BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system

The BBH/HP-β-CD inclusion complex was prepared via hydrogen binding and hydrophobic interaction according to our previously method [13]. Briefly, BBH (0.25 g) was dissolved in methanol (1:2, w/v) at 45 °C and added to an aqueous solution (100 ml) containing HP-β-CD (1 g), and then stirred continuously for 8 h . Afterwards, the suspension was cooled slowly to room temperature, filtered quickly through 0.45 μm pore size syringe filters, lyophilized in a Savant Modulyo D freeze-drier (Thermo, Holbrook, NY, USA). Inclusion complex of BBH/HP-β-CD was characterized and loading efficiency was investigated. X-ray powder diffractometry (XRD) maps were produced in the range of 2θ = 4 - 50° by step scanning on a Rigaku D/MAX-2500 diffractometer (Rigaku Co., Tokyo, Japan) with Cu Kα radiation operated at 40 kV and 100 mA. ^{1}H-nuclear magnetic resonance spectroscopy (^{1}H-NMR) spectra for BBH, HP-β-CD and the BBH/HP-β-CD inclusion complex were obtained using an Avance DRX 500 spectrometer (Bruker Analytik, Rheinstetten, Germany) at 25 °C in D_2O. Two-dimensional ^{1}H-^{1}H rotating frame Overhauser effect spectroscopy (2D-ROESY) experiments were run on the same instrument. Samples were equilibrated for at least 24 h before measurement.

Subsequently, various proportions of P407, P188 (Table S1) were slowly added into inclusion complex solution with preservative solution (benzalkonium chloride,

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BKC, 0.01%, w/v) in beakers with magnetic stirring at 4 °C. Aqueous polymers mixture was kept overnight at 4 °C to ensure complete dissolution of the polymers. To optimize the *in situ* hydrogel, the viscoelastic behavior of BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system was performed with an AR 2000 ex Rheometer (TA Instrument, New Castle, DE, USA). During the gelation process, the rheological properties of samples were measured with a temperature range of 15-40 °C. The changes of the elastic modulus (G′) and the viscous modulus (G″) were recorded as the function of temperature at a fixed frequency of 1 Hz.

The *in vitro* release of BBH from BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system was determined by a dialysis bag method using a dissolution test apparatus (ZRS-8G, Tianjin, China). After enclosed in dialysis bag, the hydrogel solution (2 mL) was added to PBS (preparation with NaH_2PO_4 and Na_2HPO_4, 200 mL) at 34 ± 0.2 °C with stirring constantly at 50 rpm. At predetermined time points, 1 mL of sample was withdrawn for detection, and replaced with equal volume of dissolution medium which has preheated at 34 °C. The amount of BBH was measured by HPLC method equipped with a Waters 2487 Dual λ Absorbance Detector and Waters 2695 Separations Module (Waters, USA) with a Venusil XBP C18 Column (250×4.6 mm, 5 μm). The mobile phase was a mixture of 0.1% phosphoric acid solution containing 0.05% triethylamine-acetonitrile (60:40, v/v), and the flow rate was 1.0 mL/min. The ultraviolet detection wavelength of 263 nm was selected.

Radioactive tracer studies of BBH from nose to brain

Before the radioactive tracer studies, the BBH was first labeled with $Na^{125}I$ (^{125}I-BBH) according to Chloramine T method [14]. BBH was dissolved in PBS (pH 7.4) and mixed with $Na^{125}I$ solution (160 μCi) in an Eppendorf tube. Reaction was

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initiated by addition of 50 μL of chloramine-T solution (1 mg/mL) and vortexed for 30 min at room temperature. Reaction was terminated by adding 50 μL of disodium disulfite solution (2 mg/mL). Then, the reaction mixture was centrifuged at 5000 rpm for 3 min and whole liquid was removed. Following, a total of 300 μL sodium chloride solution (2%, w/v) was added and suspension was centrifuged at 5000 rpm for 3 min. The above steps were repeated twice to purify ^{125}I labeled BBH (^{125}I-BBH). The radiolabeling yield, purity and stability of ^{125}I-BBH were assessed by AR2000 radioactive thin-layer chromatography scanner (Bioscan, USA). The ^{125}I-BBH/HP-β-CD inclusion complex was characterized by XRD and Differential scanning calorimetry (DSC). ^{125}I-BBH was then used to prepare ^{125}I-BBH/HP-β-CD inclusion complex and ^{125}I-BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system as above mentioned.

Following, rats were randomly divided into two groups (N=18 per group) and anesthetized with 5% chloral hydrate (450 mg/kg, i.p.). In one group, rats received ^{125}I-BBH/HP-β-CD inclusion complex solution (IN, 0.15 mg/kg calculated as weight of BBH, bilateral nostrils). And in the other group, rats were treated with equivalent amount of ^{125}I-BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system. Rats were then put in a specimen chamber at predetermined time intervals for radioactivity imaging. Two images were overlapped to show the exact location and intensity of radioactivity. The gamma images and radio intensity were obtained by the Kodak IS in vivo FX imaging system (Carestream Health, USA).

Gamma counting analysis

For gamma counting analysis, rats were randomly divided into two groups (N=36 per group). In BBH.IN group, rats were anesthetized with 5% chloral hydrate (250 mg/kg, i.p.) and then given with ^{125}I-BBH/HP-β-CD inclusion complex thermoresponsive

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hydrogel system (10 μCi, IN administration). In BBH.IG (Intragastric administration) group, after administration of ^{125}I-BBH inclusion complex (50 μCi, IG administration), rats were anesthetized with 5% chloral hydrate correspondingly. Blood samples were collected from the retinal venous plexus at predetermined time intervals, including 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 8, 12 and 24 h. Radioactivity of ^{125}I-BBH in the blood was analyzed by 2470 automatic gamma counter (Perkin Elmer, USA).

LC-MS/MS analysis of BBH in hippocampus (Pharmacokinetic analysis)

In order to accurately investigate the pharmacokinetic behavior of BBH in hippocampus, the concentration of BBH in hippocampus was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS), in which tetrahydropalmatine (2 ng/mL) was used as an internal standard. Firstly, rats were randomly divided into two groups (n=6 per group/time point), and then given with BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system (IN administration, 0.15 mg/kg calculated as weight of BBH) or BBH/HP-β-CD inclusion complex (IG administration, 5 mg/kg calculated as weight of BBH), respectively. Then hippocampi were collected at 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 12 and 24 h post-dosing. Hippocampi were excised carefully and stored at -80 °C for use.

Hippocampal samples were ground into powder under liquid nitrogen in a pre-cooled mortar. Powdered tissue (50 mg) was weighed and 9 volumes of methanol/acetonitrile/acetone/water (30/30/30/10; v/v/v/v) pre-chilled to -20 °C was added. The samples were incubated on ice for 30 min and centrifuged for 10 min at 16,000 × g and 4 ºC. The supernatants containing BBH were transferred to labeled cryotubes and stored at -80 °C for LC-MS/MS analysis. LC-MS/MS analysis was performed using electrospray ionization (ESI) with multiple reaction monitor (MRM)

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in positive mode. The MRM transition for BBH was m/z 336.0→m/z 292.1 and tetrahydropalmatine at m/z 356.3→m/z 192.1. Pharmacokinetic parameters were processed by DAS software (version 1.0) with a non-compartmental analysis. The C_{max} and T_{max} were obtained from concentration-time curves, and ($AUC_{0\text{-}24\ h}$) was calculated by the trapezoidal rule with extrapolation. Relative bioavailability was calculated according to Equation. (1):

$$\text{Relative biovailability} = \frac{AUC_{IN}}{AUC_{IG}} \times \frac{Dose_{IG}}{Dose_{IN}} \times 100\% \quad (1)$$

AUC_{IN} and AUC_{IG} is the area under the concentration-time curve from time 0 to 24 h after IN or IG administration of BBH, respectively. $Dose_{IN}$ and $Dose_{IG}$ is the dosage of BBH/HP-β-CD hydrogel system and BBH/HP-β-CD inclusion (calculated as weight of BBH), respectively.

Analysis of monoamine neurotransmitters in reserpine-induced depression model

Rats were randomly divided into eight groups (N=6 per group): control (normal saline), reserpine-induced depression model (normal saline), FLX (IG, 10 mg/kg), BBH/HP-β-CD inclusion complex (BBH.IG, 5 mg/kg calculated as weight of BBH), and BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system (BBH.IN, 0, 0.05, 0.10 and 0.15 mg/kg calculated as weight of BBH) groups. After received normal saline, FLX, BBH/HP-β-CD inclusion complex or BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system for 3 h, rats were intraperitoneally injected with reserpine (1.5 mg/kg) except for control group. Another 1.5 h later, rats were sacrificed, then hippocampus and striatum tissues were obtained and stored at -80 °C for further analysis. Monoamine neurotransmitters such as 5-HT, 5-HIAA and NE in hippocampus as well as 5-HT, DA and NE in striatum were analyzed using HPLC coupled with electrochemical detector (ECD).

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Metabolomic analysis of hippocampus in CUMS-induced rats

The CUMS procedures were performed as reported [15]. In brief, rats were randomly assigned to control or CUMS group (Eight rats in each group). Control rats without stress were given saline (10 μl/g). The chronic stress was given sequentially and randomly per day for 35 d including a 10-min strong light exposure, inversion of day/night light cycle, 5-min swim in ice-cold water (10 ºC), 2-min shaking on a rotator or 2-min tail clipping, 24-h food or water deprivation. Following four weeks of stress exposure, rats with stress-induced depression-like behaviors were treated daily with saline, fluoxetine, BBH/HP-β-CD inclusion complex via intragastric gavage or BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system via intranasal administration for 3 days: CUMS (CUMS rats with saline, 10 μl/g); Flx (CUMS rats with fluoxetine, 10 mg/kg); BBH.IG (CUMS rats with BBH/HP-β-CD inclusion complex, 5 mg/kg calculated as weight of BBH) and BBH.IN (CUMS rats with BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system, 0.05, 0.10 and 0.15 mg/kg calculated as weight of BBH). To exclude the interference of anesthesia, the anesthetics of chloral hydrate (dose) was set alone as control. The CUMS procedures continued during the day of drug treatment. The body weight and sucrose preference test (SPT) were performed every 7 days (0, 7, 14, 21, 28 and 35 d) during the experiments. The open field test (OFT) was conducted before and after drug treatment. The number of horizontal crossings (defined as at least three paws in a square) and vertical (rearings) activities during a 4-min period was counted. After drug treatment, heparinized plasma and fecal samples were collected, and the animals were sacrificed to collect the hippocampi. All the samples were snap-frozen in liquid nitrogen and stored at -80 ºC for metabolomic analysis.

Metabolites extraction was performed as described with minor changes [16]. Briefly,

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Hippocampal samples were ground into powder under liquid nitrogen in a pre-cooled mortar. Powdered tissue (50 mg) was weighed and 9 volumes of methanol/acetonitrile/acetone/water (30/30/30/10; v/v/v/v) pre-chilled to -20 °C was added. The samples were incubated on ice for 30 min and centrifuged for 10 min at 16,000 × g and 4 ºC. The supernatants containing the extracted metabolites were transferred to labeled cryotubes and stored at -80 °C for metabolites analysis.

Metabolomic analysis was performed using a LC-MS/MS system equipped with Shimazu LC20A (Shimazu, Japan) coupled with Qtrap 5500 (ABSCIEX, USA) as reported previously [17, 18]. LC-MS/MS analysis was performed by multiple reaction monitoring (MRM) under Analyst v1.6.1 (AB SCIEX, Framingham, MA, USA) software. Two injections were conducted, one on positive mode and the other one on negative mode. Ten microliters of the respective extracts were injected by PAL CTC autosampler into a 250 × 2 mm, 5 μm Luna NH2 aminopropyl HPLC column (Phenomenex, Torrance, CA, USA) held at 25 °C for chromatographic separation at a flow rate of 0.6 mL/min. A total of 279 metabolites were targeted.

Metabolomic data were log2-transformed. The OPLS-DA and volcano plots were constructed in metaboanalyst (www.metaboanalyst.ca). Metabolites with VIP scores greater than 1.5 were considered as significant. Metabolic pathways were analyzed in metaboanalyst.

Statistical analysis

One-way ANOVA was used for determining the statistical significant differences between the values of various experimental groups. *In vitro* experiments, data were expressed as means ± SD from three independent experiments. Data was expressed as means ± SE for *in vivo* experiments. Graph generation and statistical analyses were performed using Origin 8.0 software (OriginLab, USA). *P* values less than or equal to

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0.05 were considered significant.

Results

Synthesis and optimization of thermoresponsive hydrogel system for drug delivery of BBH.

To increase the solubility of BBH, the hydrophobic compound of BBH was improved by the inclusion of HP-β-CD with hydrogen binding and hydrophobic interaction. The BBH/HP-β-CD inclusion complex was characterized. XRD patterns clearly confirmed the crystalline nature of BBH and the amorphous state of HP-β-CD. After formation of BBH/HP-β-CD inclusion complex, the characteristic diffraction peaks of BBH disappeared (Figure 2A). The possible inclusion mode of inclusion complex was further explored with ^{1}H NMR spectra and 2D ROESY. The chemical shifts of protons in BBH was observed (Figure 2B) as following: ^{1}H-NMR (D_2O, 500MHz) δ: 9.52 (1H, s, H-h), 7.89 (1H, d, J=9.5Hz, H-k), 7.77(1H, d, J=9.0Hz, H-l), 8.26 (1H, s, H-m), 4.75 (2H, t, J=6.0Hz, H-f), 3.12 (2H, t, J=6.0Hz, H-e), 7.27(1H, s, H-n), 6.83 (1H, s, H-d), 6.00 (2H, s, H-b), 4.02 (3H, s, C_j-OCH_3), 3.97 (3H, s, C_i-OCH_3), which suggested that BBH could enter into the cavity of the HP-β-CD rings. 2D ROESY indicated that cross peaks were observed between the H-b protons of BBH and the H-5 protons of HP-β-CD, and the same condition was also observed between the H-d, H-n, H-m protons of BBH and the H-c proton of HP-β-CD (Figure 2C). These results revealed that BBH was deeply included in the hydrophobic cavity of HP-β-CD.

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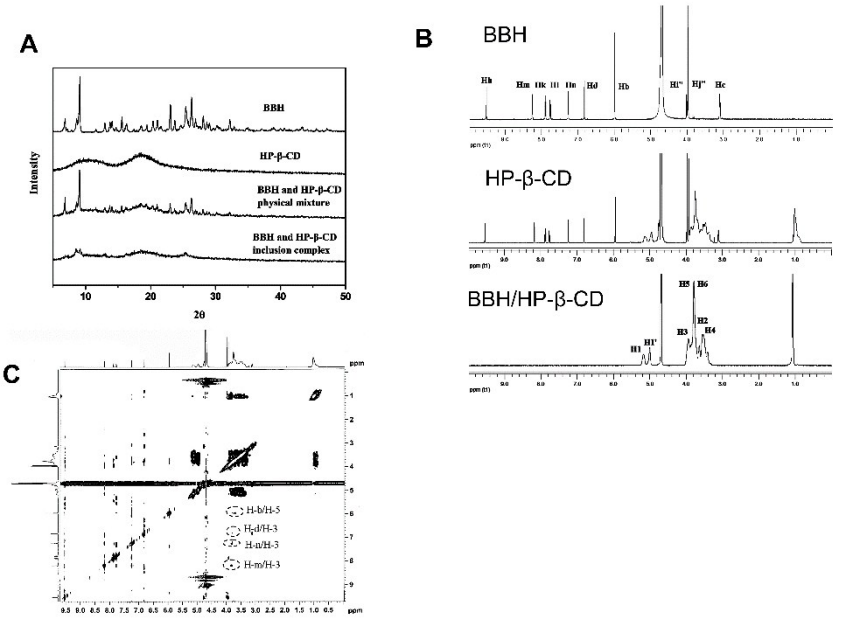

Figure 2. Characterization of BBH/HP-β-CD inclusion complex. (A) XRD spectra. (B) ^{1}H NMR spectra of BBH, HP-β-CD and BBH/HP-β-CD inclusion complex. (C) 2D ROESY of BBH/HP-β-CD inclusion complex.

The above result indicated that the inclusion of BBH could happen when 1, 3-benzodioxole groups of BBH were embedded into the cavity of the HP-β-CD rings (Figure 3A). Moreover, temperature plays a key role in the micelle formation of the polymer through the hydrophobic interaction of PPO. When the temperature rose (rising to body temperature), the PPO block of poloxamers aggregated to form micelle with a hydrophobic core and hydrophilic shell (Figure 3B). To obtain the ideal thermoresponsive hydrogel for intranasal delivery of BBH, the gelation temperature of different hydrogel formulae (Consisting of HP-β-CD, P407 and P188) were investigated. We found that the gelation temperature decreased with the increase of P407 concentration, while positively correlated with P188 concentration (concentration <10% in this system) (Table S1), which might be the higher ratio of PEO/PPO in P188 than P407. The introduction of HP-β-CD has been demonstrated that there was no significant change on gelation temperature of the system. The

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hydrogel formulae (P407/P188, 16:2), phase-transition temperature at about 30 °C, slightly lower than rat nasal cavity temperature, was optimized for further studies. The release profile of BBH in hydrogel formulae was shown in Figure 3D. A sustaining release of BBH was observed after 6 h to the environment and the cumulative release rate was about 83.29 ± 3.98%. Several mathematical models, including the Higuchi release model, zero-order release model and first-order release model, were applied to describe the release behavior of BBH from the hydrogel system. The drug-release profile followed the Higuchi release model (R^2 = 0.9981) in the formula (Table S2). The loading efficiency was 22.86% analyzed by HPLC method.

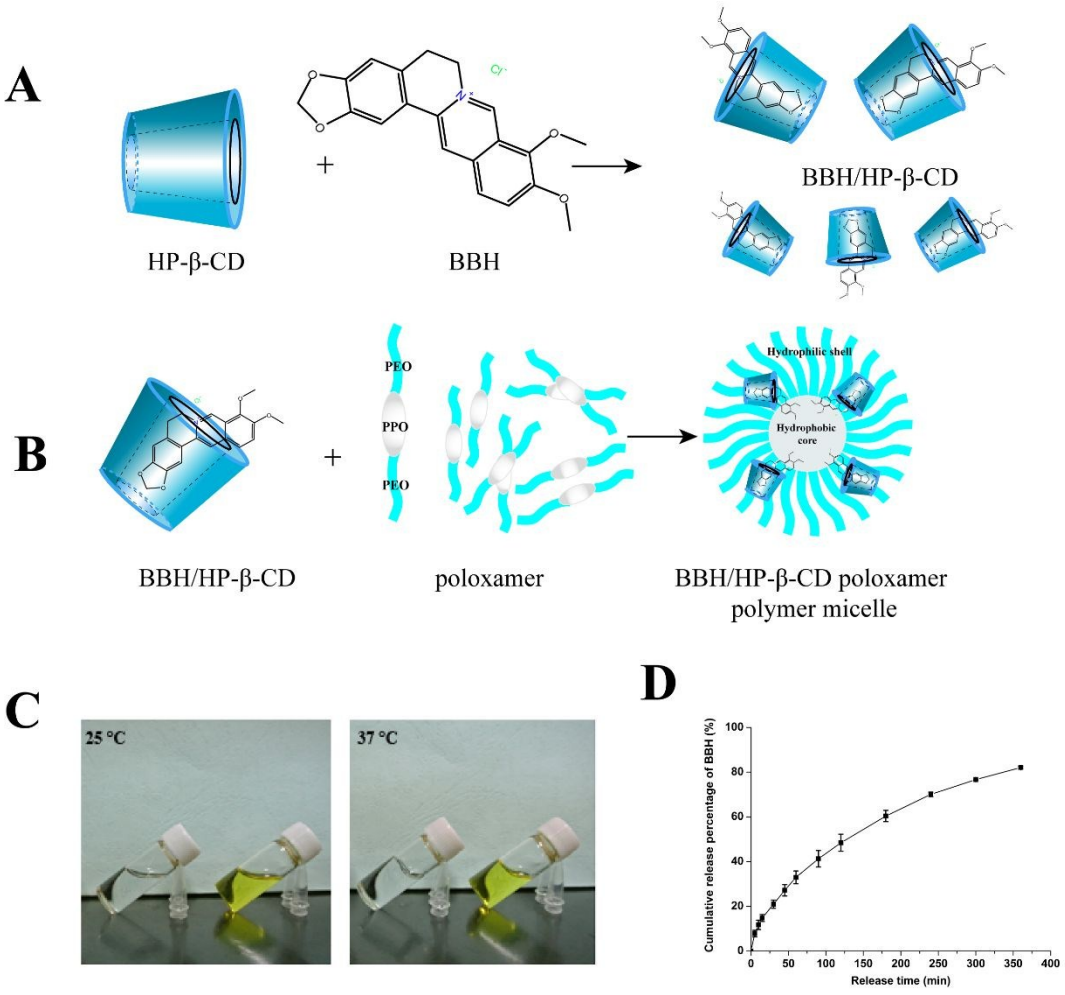

Figure 3. The schematic of the BBH/HP-β-CD hydrogel system. (A) Synthetic route of BBH/HP-β-CD inclusion complex; (B) Preparation of

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poloxamer/BBH/HP-β-CD polypseudorotaxanes micelle. (C) Poloxamer based polymers before and after gelation (left: without BBH/HP-β-CD; right: with BBH/HP-β-CD). (D) The *in vitro* release of BBH in hydrogel system (P407/P188, 16:2, w/w) was determined by a dialysis bag method in PBS at 34 ± 0.2 °C.

Brain delivery of BBH via thermoresponsive hydrogel system following intranasal (IN) administration.

The non-invasive intranasal delivery of therapeutic agents bypassing the blood brain barrier (BBB) via drug carriers has been paid more attention in recent years [19]. The direct nose-to-brain drug delivery following intranasal administration is a direct transport route from the nasal cavity to the brain as the particular anatomical, physiological and histological characteristics of the nasal cavity [20]. This also would offer an exciting mode of delivering neurotherapeutic agents. Before investigating the antidepressant-like effects of BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system, we prepared the ^{125}I labeled BBH to study the brain delivery of BBH/HP-β-CD *in situ* hydrogel *in vivo*. The ^{125}I-BBH/HP-β-CD inclusion complex was prepared and characterized (Figure S1). As confirmed by radioactive thin-layer chromatography scanning, the radiolabeling yield, radiochemical purity of ^{125}I-BBH was more than 73% and 99%, respectively, and the stability remained above 99% at 24 h of post purification (Figure S1A). UV-Vis spectra were given in Figure 4B. The intense absorption peaks of both BBH and ^{125}I-BBH located at 225, 261 and 342 nm. Only the absorption peak of ^{125}I-BBR at 225 nm was slightly intense than BBH. HP-β-CD had no characteristic peaks at range of 200-800 nm. The spectrum of the ^{125}I-BBH/HP-β-CD inclusion complex showed a same intense absorption peaks as ^{125}I-BBH, which indicated the formation of ^{125}I-BBH/HP-β-CD inclusion complex without affection by Na^{125}I labeling. The thermogram of ^{125}I-BBH showed it existed

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in a crystal state with a fusion endothermic peak at 240 °C. The DSC thermal curve of the physical mixture showed that the fusion endothermic peak of ^{125}I-BBH was lower than that of crystalline form and shifted to a relatively lower temperature. The variation may be due to the interaction between ^{125}I-BBH and HP-β-CD induced by the thermal energy supplied by DSC scan. No sharp peak at 240 °C was observed in the thermal curve of ^{125}I-BBH/HP-β-CD inclusion complex (Figure S1C), which indicated that ^{125}I-BBH was converted into an amorphous state instead of a crystalline form. The crystalline nature of ^{125}I-BBH and the amorphous state of HP-β-CD showed distinctive diffraction pattern (Figure S1D). However, no crystalline peak was observed in the diffractogram of the inclusion complex, indicating the formation of ^{125}I-BBH/HP-β-CD inclusion complex. Moreover, compared with HP-β-CD, the decreased intensity of ^{125}I-BBH/HP-β-CD inclusion complex strengthened the hypothesis that an amorphous complex had been formed.

Then, the rats were administrated with equivalent ^{125}I-BBH/HP-β-CD inclusion complex solution or ^{125}I-BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system following intranasal administration, and the drug absorption was mimicked in Figure 4B. After 1, 2, 4, 8 and 24 h, the radioactive signals from nose to brain were visualized, and obviously, there was stronger radioactive intensity in brain in ^{125}I-BBH/HP-β-CD hydrogel (IN administration) group than ^{125}I-BBH/HP-β-CD solution (IN administration) group until 8 h (Figure 4C). The total radioactive intensity from nose to brain in ^{125}I-BBH/HP-β-CD hydrogel group was also higher compared to ^{125}I-BBH/HP-β-CD solution group (Figure 4D). In comparison with the traditional nose drops (BBH/HP-β-CD solution), BBH/HP-β-CD thermoresponsive hydrogel system could prolong nasal residence to sustain drug release for brain-targeting BBH delivery.

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Improved bioavailability of BBH in rat hippocampus.

Due to the poor bioavailability of BBH, the pharmacological effects were restricted with the traditional administration of BBH. In order to increase the poor bioavailability of BBH, in this study, BBH/HP-β-CD inclusion complex was prepared and loaded into hydrogel system, and the variation of bioavailability was investigated.

Firstly, BBH was labeled with radioactive ^{125}I and the pharmacokinetic profile was analyzed by the gamma counting. Compared with IG administration of ^{125}I-BBH/HP-β-CD inclusion complex solution (50 μCi of ^{125}I-BBH), the IN delivery of ^{125}I-BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system even in a lower dose (10 μCi of ^{125}I-BBH) showed higher bioavailability in plasma (Figure 4E). More specifically, the peak concentration (Cmax) and the area under the curve ($AUC_{0\text{-}24\ h}$) of ^{125}I-BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system (IN, dose of 10 μCi of ^{125}I-BBH) was 2.8-fold and 5.3-fold higher than ^{125}I-BBH/HP-β-CD inclusion complex solution (IG, dose of 50 μCi of ^{125}I-BBH), respectively. Secondly, to further accurately investigate the bioavailability of BBH in hippocampus, the pharmacokinetic profile of BBH was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) after rats receiving BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system (IN, 0.15 mg/kg, calculated by weight of BBH) or BBH BBH/HP-β-CD inclusion (IG, 5 mg/kg, calculated by weight of BBH) for 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 12 and 24 h. The BBH/HP-β-CD hydrogel system via IN delivery could decrease the peak time (T_{max}) (3.5 h in the hippocampus) in comparison with BBH/HP-β-CD inclusion complex via IG administration (4 h in the hippocampus) (Figure 4F). This indicated that the BBH/HP-β-CD hydrogel system (IN delivery) exerted a relatively rapid treatment than BBH/HP-β-CD inclusion complex (IG administration). Similarly, the C_{max} of

BBH (IN administration) increased 3.1-fold, and AUC_{0-24h} increased 1.7-fold compared to BBH/HP-β-CD inclusion complex (IG administration) in hippocampus. The relative bioavailability of BBH via BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system (IN administration) was enhanced approximately 110 folds than BBH/HP-β-CD inclusion complex (IG administration) in hippocampus. The enhanced bioavailability in brain was attributed to: (1) The BBH/HP-β-CD inclusion complex enhanced the solubility of BBH, and thermoresponsive BBH/HP-β-CD hydrogel system maintained a sustaining releases of BBH; (2) The BBH/HP-β-CD hydrogel system by IN administration directly delivered BBH to brain via olfactory pathway and trigeminal neural pathway bypassing the blood-brain barrier and first-pass effect; (3) The abundant capillaries in the nasal cavity increased the absorption of BBH.

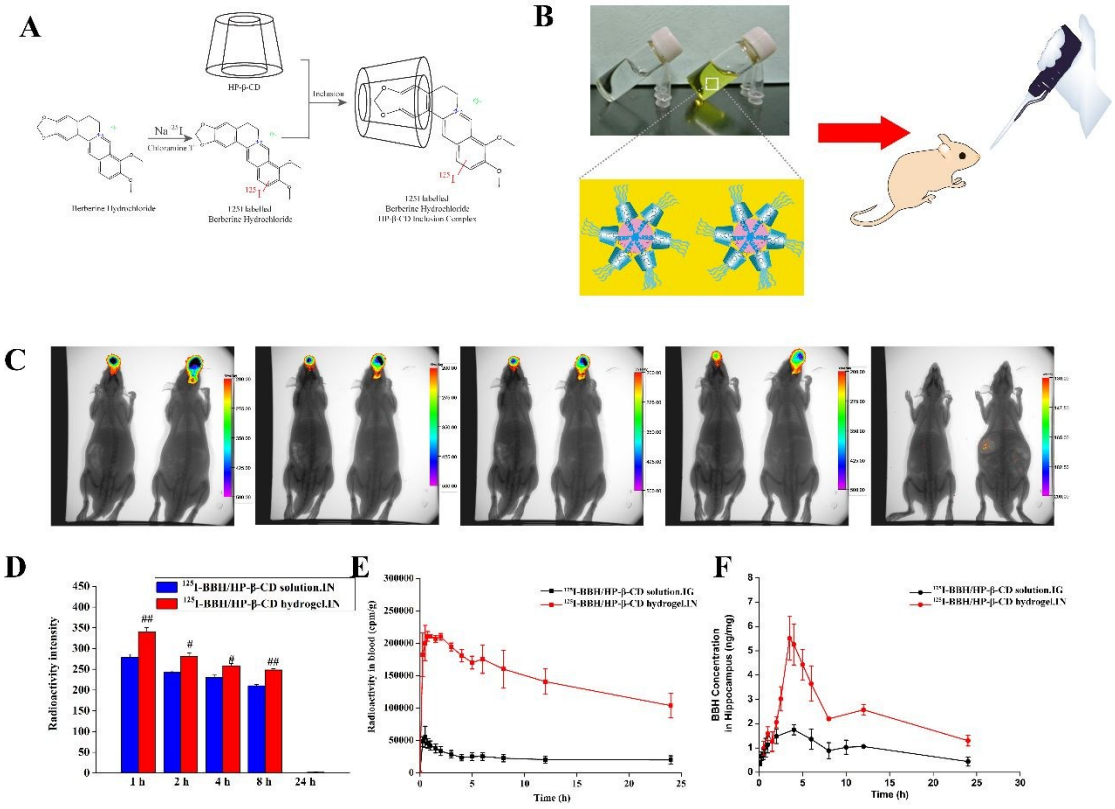

Figure 4. *In vivo* imaging of BBH absorption from nose to brain and pharmacokinetic profiles. (A) Schematic of ^{125}I-BBH/HP-β-CD inclusion complex; (B) The prepared

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BBH/HP-β-CD hydrogel system adheres to the nasal mucosal surface; (C) *In vivo* imaging of rats IN administrated with ^{125}I-BBH/HP-β-CD inclusion complex solution (left) or ^{125}I-BBH/HP-β-CD inclusion complex hydrogel system (right); (D) The relative radioactivity intense of Na^{125}I. Values were the mean ± SEM with 3 mice in each group. #$P < 0.05$ or ##$P < 0.01$ vs.^{125}I-BBH/HP-β-CD solution. IN group; (E) Mean radioactivity-time profiles of ^{125}I-BBH in rat blood after oral administration of ^{125}I- BBH/HP-β-CD inclusion complex (^{125}I-BBH, 50 μCi) or IN delivery of ^{125}I-BBH/HP-β-CD inclusion complex hydrogel system (^{125}I-BBH, 10 μCi); Mean concentration-time profiles of BBH in rat hippocampus (F) after IG administration of BBH/HP-β-CD inclusion complex (5 mg/kg) or IN administration of BBH/HP-β-CD inclusion complex hydrogel system (0.15 mg/kg).

Improvement of monoaminergic dysfunction by BBH/HP-β-CD hydrogel system. Depressive disorder is associated with disturbances of brain monoamine neurotransmitters. Reserpine is known to deplete the monoamine neurotransmitters in the synapses and induce depression-like behaviors in rats [21]. Before investigating the antidepressant-like efficiency of BBH/HP-β-CD hydrogel system, the component of hydrogel system in this study, P407, P188, and HP-β-CD were firstly verified that they had no effect on neural excitability and behavioral despair (Figure S2). Reserpine remarkably decreased the levels of monoamine neurotransmitters in rat hippocampus (5-HT and NE) and striatum (5-HT, NE and DA) after injection (1.5 h) compared to control group (Figure 5, $P < 0.01$). Not only fluoxetine hydrochloride (FLX), a reuptake inhibitor of 5-HT, but BBH/HP-β-CD inclusion complex (BBH.IG) and BBH/HP-β-CD hydrogel system (BBH.IN) restored the dysfunction of 5-HT, 5-HIAA, NE and DA induced by reserpine ($P < 0.05$ or $P < 0.01$), while there was no effect in the blank hydrogel system. Both BBH/HP-β-CD inclusion complex and

BBH/HP-β-CD hydrogel system also displayed effective regulation on tryptophan metabolism to 5-HT, and tyrosine metabolism to NE and DA in hippocampus or striatum tissue. Compared to BBH/HP-β-CD inclusion complex, BBH/HP-β-CD hydrogel could reverse 5-HT depletion significantly by retarding the convert of 5-HT to 5-HIAA in hippocampus tissue ($P < 0.01$, Figure 5B). BBH/HP-β-CD hydrogel could elevate the NE and DA levels significantly than FLX group in striatum tissue ($P < 0.05$, Figure 5E and F). Although BBH/HP-β-CD hydrogel could elevate the 5-HT, NE and DA in hippocampus or striatum tissue compared to BBH/HP-β-CD inclusion complex, there was no significance ($P > 0.05$). Therefore, BBH/HP-β-CD hydrogel system by IN administration with lower dose (0.15 mg/kg) performed the comparable or enhanced effect on the improvement of monoamine neurotransmitters than BBH/HP-β-CD inclusion (5 mg/kg) and FLX by IG administration.

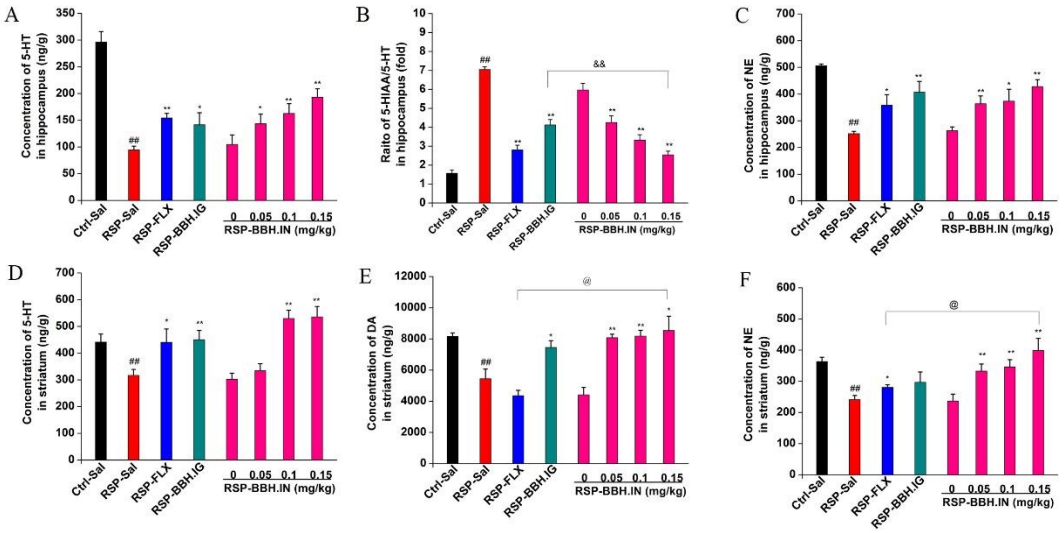

Figure 5. Evaluation of the BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system on monoamine neurotransmitters. (A) 5-HT, (B) ratio of 5-HIAA/5-HT and (C) NE in rat hippocampus. (D) 5-HT, (E) DA and (F) NE in rat striatum. $^{\#\#}$ $P < 0.01$ vs. control group, $^{*}P < 0.05$ or $^{**}P < 0.01$ vs. reserpine group, $^{\&\&}$ $P < 0.01$ vs. BBH.IG group, $^{@}$ $P < 0.05$ vs. FLX group.

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Improved therapeutic efficacy of BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system on CUMS-induced rats.

The CUMS procedure is playing an important role in the evaluation of antidepressants in animal models of depression, which is classical to mimic the stress of daily human life one of the most common risk factors for depression (Figure 6A). After a continuous CUMS stimulation for 35 days, the number of crossing and rearing was significantly decreased compared with the control group. With the intervention of FLX, BBH/HP-β-CD inclusion complex (BBH.IG) or BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system (BBH.IN) for 3 days, the locomotor activity, appetite, and emotion were remarkably increased ($P < 0.05$ or $P < 0.01$). Furthermore, BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system exerted more potent on improving anhedonia than BBH/HP-β-CD inclusion complex ($P < 0.05$ or $P < 0.01$, Figure 6B and C). BBH/HP-β-CD inclusion complex or BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system reversed the reduction of sucrose intake in rats with chronic stress (Figure 6D). It was interesting that the sucrose preference increased obviously in BBH/HP-β-CD inclusion complex treatment than FLX or BBH/HP-β-CD hydrogel system. This was due to the tasted bitter of BBH, which led to the sharply increasing of the sugar water consumption after IG administration of BBH/HP-β-CD inclusion complex. Although FLX, BBH BBH/HP-β-CD inclusion or BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system could reverse the reduction of body weight, there was no significance (Figure 6E). Moreover, no alteration was found in chloral hydrate-treated group compared with the CUMS group. These results suggested that BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system manifested stronger therapeutic efficacy than BBH/HP-β-CD inclusion complex.

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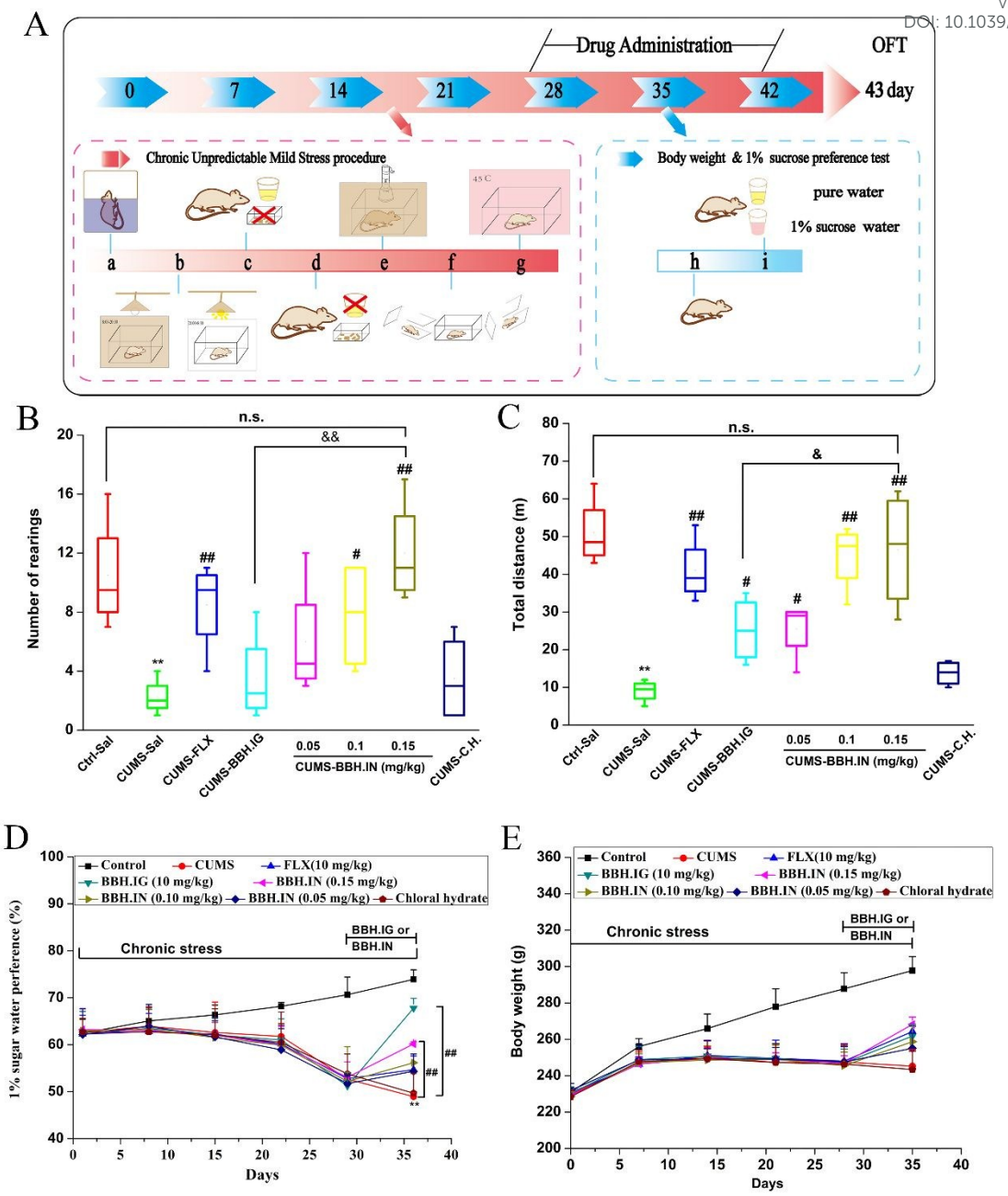

Figure 6. Antidepressant evaluation of BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system on CUMS-induced rats. (A)Time schedule of CUMS protocol and drug administration; (B) Numbers of rearing was quantified during a period of 4 min in the open field test. $^{*}P < 0.01$ vs. control group, $^{\#}P < 0.05$ or $^{\#\#}P < 0.01$ vs. CUMS group, $^{\&}P < 0.05$ or $^{\&\&}P < 0.01$ vs. BBH.IG group; n.s., not statistically significant. (C) The total distance was also quantified during a period of 4 min in the open field test. $^{*}P < 0.01$ vs. control group, $^{\#}P < 0.05$ or $^{\#\#}P < 0.01$ vs.

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CUMS group, $^{\&}P < 0.05$ or $^{\&\&}P < 0.01$ vs. BBH.IG group; n.s., not statistically significant. (D) Sucrose preference and (E) body weight at the baseline and during the consecutive CUMS period were tracked. $^{*}P < 0.01$ vs. control group, $^{\#\#}P < 0.01$ vs. CUMS group.

BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system produces more dramatic restoration of metabolic abnormalities in rat hippocampus.

We next compared the changes in the hippocampal metabolism between BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system by IN administration (BBH.IN) and BBH/HP-β-CD inclusion complex by IG administration (BBH.IG) treatment using metabolomics. Multivariate partial least squares discriminate analysis (PLS-DA) of metabolomic data revealed a distinct separation of metabolic profiles between CUMS rats treated with saline, BBH/HP-β-CD inclusion complex (IG administration) and BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system (IN administration) in hippocampus (Figure 7A). The hippocampal metabolic profile of CUMS rats with BBH/HP-β-CD hydrogel (IN administration) was closer to the normal controls than BBH/HP-β-CD (IG administration). Impaired mitochondrial function affected cellular processes, synaptic function and energy production, leading to neurodegeneration [22]. Recently, sphingomyelins (SM) and ceramides were paid more attention to the pathogenesis of depression [23, 24]. In this study, we found that compared to BBH.IG, BBH.IN uniquely restored hippocampal sphingolipids and phospholipids metabolism (Figure 7B). In addition, both BBG.IG and BBH.IN showed significant effects on tryptophan, tyrosine, glycolysis, glutathione and COA metabolism (Figure 7B). However, BBH.IN showed more dramatic influence than BBH.IG. Tryptophan–kynurenine metabolism and tyrosine-catecholamine metabolism are the common distributed pathways in

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depression. We showed that the levels of hippocampal monoamines in tyrosine metabolic pathways were significantly higher when BBH/HP-β-CD hydrogel system was delivered through the nasal canal (Figure 7C). For example, dopamine and norepinephrine were about 50% higher in BBH.IN group. Similarly, as shown in Figure 7D serotonin and melatonin were higher, while the conversion of tryptophan to kynurenine was lower in BBH.IN group. All these data demonstrated that IN delivery of BBH/HP-β-CD hydrogel system exerted stronger antidepressant-like effects than the classic oral administration.

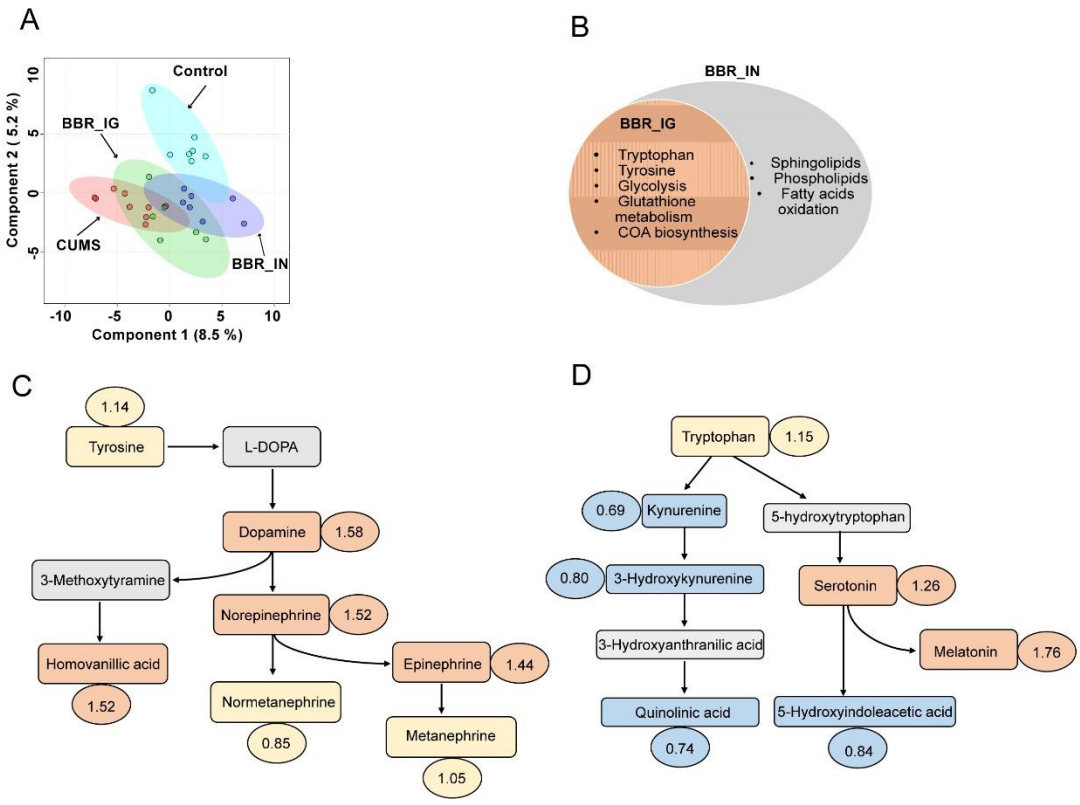

Figure 7. Novel targets of BBH/HP-β-CD hydrogel (IN administration) on CUMS-induced depressive-like rats. (A) PLS-DA analysis showed the dramatic differences in rat hippocampal metabolism among control, BBH.IN, BBH.IG and CUMS groups; (B) The shared and unique metabolic pathways altered by BBH.IN and BBH.IG treatment compared to CUMS rats treated with saline. The effect of

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different BBH delivery methods on tyrosine metabolism (C) and tryptophan metabolism (D) in rat hippocampus. The brown color indicated higher in BBH.IN group and blue indicated lower in BBH.IN group. The yellow color indicated no change. The numbers in the oval shape circle were the fold change (BBH.IN/BBH.IG).

Discussion and conclusion

Evidence from animal and clinical studies have highlighted the possibility that nasal route can directly transport drugs form nose to brain [25, 26]. Intranasal administration delivery is superior to overcome poor bioavailability, slow absorption, side-effects in the gastrointestinal tract and avoid the hepatic first-pass metabolism [27]. Among those strategies used for IN drug delivery, nasal hydrogel is a popular formulation [28]. Hydrogels are three-dimensional, hydrophilic, nontoxic, stable and swellable polymer networks [29]. The high fluid-absorption property and low surface tension of hydrogels lead to good biocompatibility, high drug loading efficiency and prolonged drug release [30]. Before preparing the thermoresponsive hydrogel system for IN delivery, how to improve the solubility of berberine is the key factor. Firstly, berberine was loaded into the hydrophobic core region of HP-β-CD, and the hydrophilic shell stabilized the hydrophobic core, which increased the solubility of berberine significantly. Then, BBH/HP-β-CD inclusion complex was loaded into poloxamers to establish the *in situ* thermoresponsive hydrogel system for IN delivery. Intermolecular interactions among HP-β-CD thread along poloxamer P407 and P188 result in the formation of supramolecular matrix which notably modifies the rheological characteristics of the system. The constructed *in situ* thermoresponsive hydrogels are given two prospects: (i) Performing a sustained release of berberine as the drug reservoir of hydrogel system; (ii) Enhancing the bioavailability of berberine with *in*

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situ thermoresponsive hydrogel system via intranasal administration.

The hydrogel formulation can be rapidly dropped or sprayed using available devices into the nasal cavity, spread along the nasal mucosa with solution state and transformed into a viscous hydrogel at body temperature [31]. Our radioactive tracing results showed that the nasal-resident ^{125}I-BBH/HP-β-CD/poloxamer hydrogel system displayed higher concentration gradient of ^{125}I-BBH to the brain for 8 h than ^{125}I-BBH/HP-β-CD inclusion complex solution. It may be attributed to: (1) The hydrogel system reduced nasal mucociliary clearance rate, prolonging ^{125}I-BBH release; (2) BBH/HP-β-CD inclusion complex is stabilized by poloxamers via the formation of polypseudorotaxanes; (3) HP-β-CD inhibits the ATPase activity at the olfactory epithelium and endothelial cells surrounding the olfactory bulb [32]. Compared with IG administration of BBH/HP-β-CD inclusion complex (5 mg/kg), IN delivery of BBH/HP-β-CD/poloxamer hydrogel system (0.15 mg/kg) increased the brain targeting bioavailability of BBH by 110 fold. This may be the reason that HP-β-CD had a P-gp inhibition by restraining the Pgp ATPase activity to increase the bioavailability[33]. Though the total amount of the hydrogel that can be incorporated was restricted by nasal cavity dimensions, the unique nasal to brain transport showed great advantages for brain potentials with a low dosage and high bioavailability. Because the strength and viscosity of hydrogel would influence the pharmacokinetics parameters such as Tmax, further preclinical testing should be performed to optimal hydrogel formulations for human [34].

Efficacy of the described BBH/HP-β-CD/poloxamer hydrogel system demonstrated excellent antidepressant-like activities even with low dose in reserpine and CUMS-induced depressant-like rats. To further understand the mechanisms of different delivery methods of BBH, we conducted the pharmacometabolomic analysis

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of the changes in rat hippocampal metabolism. Metabolomics provides powerful biochemical knowledge about therapeutic potential, mechanism of action and adverse effects, which is beneficial for drug discovery and development [35]. First, our metabolomic results showed that hippocampal monoamines levels in tyrosine and tryptophan metabolic pathways varied with different drug delivery methods. Compared with IG administration, IN delivery of BBH/HP-β-CD hydrogel system showed higher levels of dopamine, norepinephrine, serotonin and melatonin. Neuroinflammatory pathogenesis has been postulated in stress-induced depression such as activation of indoleamine 2, 3-dioxygebase (IDO), resulting in tryptophan depletion, serotonin reduction, while increase of 3-hydroxykynurenine, quinolinic acid [36, 37]. In the tryptophan-kynurenine metabolic pathway, 3-hydroxykynurenine and its metabolite quinolinic acid are neurotoxic to stimulate neurodegeneration and apoptosis [38]. Serotonin and its metabolite melatonin are positive for circadian rhythms [39]. Our results indicate that IG administration of BBH/HP-β-CD inclusion complex was prone to increase the conversion of tryptophan to kynurenine and 3-hydroxykynurenine, which might cause neurotoxicity. However, IN delivery of BBH/HP-β-CD inclusion complex induced the conversion of tryptophan to serotonin and melatonin. Second, the unique mechanism of BBH/HP-β-CD inclusion complex hydrogel (IN administration) was discovered different from BBH/HP-β-CD inclusion complex (IG administration) on restoration of mitochondrial dysfunction, correction of sphingolipids and phospholipids metabolism. BBH/HP-β-CD inclusion complex hydrogel (IN administration) could restore the abnormalities of mitochondrial function in the hippocampus by the decrease of acylcarnitines and the increase of oxaloacetic acid and cardiolipins to reduce the neurodegeneration, which were not found in BBH/HP-β-CD inclusion complex (IG administration) treatment.

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Furthermore, chronic unpredictable stress could profoundly affect sphingolipid and phospholipid metabolism [40], which were restored by BBH/HP-β-CD inclusion complex hydrogel (IN administration) only. In general, the differential metabolic responses between two different BBH deliveries have been identified in CUMS-induced depressant rats via metabolomics. The common point of two different BBH administrations (BBH/HP-β-CD inclusion complex (IG administration) and BBH/HP-β-CD inclusion complex hydrogel (IN administration)) was regulating hippocampal monoamines levels to exert antidepressant-like effects, and the later exhibited more excellent effects on change of hippocampal monoamines levels. The different point was that BBH/HP-β-CD inclusion complex hydrogel (IN administration) could restore the abnormalities of mitochondrial function, phingolipid and phospholipid metabolism, which was the unique mechanism for treatment of depression. To account for these differences, more evidence is required to explore the causes of site-specific alterations.

In summary, we demonstrated that the constructed *in situ* thermoresponsive hydrogel system by intranasal administration could deliver more amount of berberine to the brain for the treatment of depression, which would be promising for antidepressant development in comparison to oral administration. The BBH/HP-β-CD inclusion complex thermoresponsive hydrogel system by intranasal administration could exert higher bioavailability and more effective antidepressant effects even with a lower dose than oral administration, which exhibits “smaller dosage” with enhanced antidepressant-like effects. In addition, the pharmacometabolomic analysis revealed that the developed hydrogel system dramatically expanded the metabolic pathways corrected by BBH. The evaluation system applied in this study also promotes the development of nose to brain drug delivery system for brain targeting therapeutics of

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CNS disorders. It will be interesting to further investigate nose to brain delivery with high bioavailability and low adverse-effect for clinical antidepressant treatment.

Supplementary Material

The gelation temperature of different hydrogel formulae (Table S1), different mathematical models on the release behavior of BBH from the hydrogel system (Table S2), Characterization of ^{125}I-BBH and ^{125}I-BBH/HP-β-CD inclusion complex (Figure S1), Effect of HP-β-CD, P407 and P188 on mice behavioral despair model (Figure S2).

Competing Interests

The authors declare that there are no conflicts of interest.

Acknowledgments

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