BBB: Permeable Conjugate of Exogenic GABA

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ABSTRACT: Neurotransmitters are the key factors in ameliorating the symptoms of nervous system diseases. Stroke/cerebral ischemia has been proven to be caused by the excess release of excitatory amino acid glutamate in the brain, and the inhibitory neurotransmitter γ-aminobutyric acid (GABA) is considered to be the best choice to counteract the action of glutamate. Here, we show that GABA conjugated to a cytoplasmic transduction peptide (YGRRARRRRRR) by means of custom chemical synthesis could penetrate through the blood–brain barrier, increasing the GABA level in the plasma of rats and mice, which, as a result, display a state of calmness and somnolence.

1. INTRODUCTION

Stroke/cerebral ischemia remains the second leading cause of death and of lasting adult disability.1 The occurrence of stroke-induced cell death in the brain is ascribed to the excess release of excitatory amino acid glutamate, and as a compensatory effect, the inhibitory neurotransmitter γ-aminobutyric acid (GABA) is released. However, the release of GABA occurs rather slowly in vivo, that is, 3 days after the stroke, and thus only affords a mild improvement in functional recovery.2 Thus, administration of exogenic GABA is supposed to be the best choice in clinical use.

Quite a few of attempts have been made to find pharmacological therapies of GABA to treat stroke, but several of these experiments have failed because, as expected, neurotransmitters, including GABA, cannot pass freely across the blood–brain barrier (BBB) in vivo. Cell-penetrating peptides (CPPs) have been receiving much attention in recent decades as a tool for delivering various materials with low membrane permeability into the cell.3 They have been used in many research fields, including drug-delivery systems and regenerative medicine. We have also studied up on our works of TAT (YGRKKRRQRRR, one of the CPPs) carrying proteins through BBB in treatment of various kinds of nervous diseases, such as TAT-ChAT,4 TAT-TH,5 TAT-BDNF,6 TAT-CNTF,7 TAT-GH,8 etc. CPP conjugates with these enzymes or bioactive factors could efficiently cross the BBB and penetrate into the brain, consequently demonstrating their therapeutic effects. It clearly showed that the CPP conjugates had already carried the exogenic neuroactive agents across BBB.

Cytoplasmic transduction peptide (CTP) is an interesting CPP. It can permeate across the cell membrane, entering into the cytoplasm of the cell but not into the nucleus.9 Thus, if the conjugate used is not expected to enter the nucleus of the cell, the CTP moiety will be more desirable than the other CPPs to avoid side effects, if any. The permeation efficiency of CPP across the cell membrane is not influenced by whether it is conjugated to the N- or C-terminal.10–13 Here, we show that a membrane-permeable conjugate with the CPP connected to the C-terminus of GABA is also able to answer the purpose.

2. RESULTS AND DISCUSSION

2.1. Pharmacokinetic Study of GABA in Rats. First, we used rats in the experiments to make intravenous gutta easy. Six male rats (Sprague-Dawley species) were stratified into control and GABA groups by weight and assigned randomly. GABA was administered by intravenous gutta (50 mg/kg h) via the tail vein. The GABA levels in the plasma and brain samples were detected by liquid chromatography/mass spectroscopy (LC–MS) analysis.

The results showed that the plasma concentrations of GABA in the rats in which GABA was administered by intravenous gutta (50 mg/kg h) was obviously elevated within 1 h and approached a plateau of 4461 ng/mL in a dynamic equilibrium state. However, no free GABA could be detected in the plasma of the control rats, in which saline was administered intra muscularly over a period of 1 h (Figure 1a and Table 1).

The GABA concentration in the brain supernatants of rats used GABA by intravenous gutta (271.8 ± 15.2 ng/mL) seems slightly higher than that in saline control rats (247.3 ± 69.5 ng/mL), but there is no statistical significance (Table 1). However, the result might not necessarily mean that GABA cannot penetrate into the brain because during slow intravenous gutta, the speed of GABA penetration into the brain...
injected GABA rose up to 7985 ng/mL within 2 min and then sharply decreased to 141 ng/mL within 1 h (Figure 1b). However, the GABA concentration in the plasma of mice injected with GABA−CPP remained at a very low level (10−24 ng/mL) over a period of 1 h after intravenous injection (Figure 1b).

The GABA concentration in the brain supernatants of the mice was then examined. This showed that GABA at dose of 50 mg/kg essentially penetrated, as expected, into the brain tissue (474 ± 33 μg/g) and so did GABA−CPP (446 ± 44 μg/g) at a dose of 10 mg/kg (Table 2).

Table 2. GABA Concentrations in the Brain of Mice 1 h after One Bolus of Intravenous Injection of GABA or GABA−CPP

| dosage          | molar concentration ratio | GABA concentration in brain (μg/g) |
|-----------------|--------------------------|-----------------------------------|
| (mg/kg) (mmol/kg) |                          |                                   |
| saline control  |                          | 276 ± 53                          |
| GABA 50         | 0.485                    | 474 ± 33*                         |
| GABA−CPP 10     | 0.006                    | 446 ± 44*                         |

*Mean ± standard deviation (SD). */p < 0.05 compared with the saline control group (n = 9).

3. CONCLUSIONS

Comprehensive analysis of the above-mentioned data clearly shows that GABA and GABA−CPP are able to cross the BBB and penetrate into the brain cells; however, the GABA−CPP conjugate showed a much higher permeation speed than that of GABA per se. The mice injected with GABA−CPP at a dose of 10 mg/kg showed a GABA concentration in the brain tissues approximately equal to that in the mice injected with only GABA at a dose of 50 mg/kg. That is, GABA−CPP injected at one-fifth of the weight dosage (corresponding to 1/80 the molar dosage) of GABA resulted at approximately the same concentration of GABA in the brain tissue. The penetration efficacy of GABA−CPP into the brain in vivo is much higher than that of GABA alone.

The behavior of the mice after injection of GABA or GABA−CPP seemed more or less alike, but in varying degrees. All of the mice injected with GABA (50 mg/kg) or GABA−CPP (10 mg/kg) showed quiet and sleepy behavior, but the symptom of the mice injected GABA−CPP appeared a bit worse.

In summary, the brain bioavailability of the GABA−CPP conjugate is much higher than that of GABA per se. The undecapeptide CPP is a potential carrier of GABA across the BBB.

4. EXPERIMENTAL SECTION

4.1. Reagents. The synthetic peptides CTP (YGR-RARRRRRR) and GABA−CPP were synthesized by SBS Genetec Co., Ltd. in a purity of 98%. The molecular weights of the peptides were confirmed by mass spectrometry. The lyophilized peptides were prepared and diluted in 0.9% NaCl before use.

GABA was purchased from Sigma Aldrich (St. Louis, MO), with purity ≥99%.

LC−MS hypergrade acetonitrile was purchased from Merck (Darmstadt, Germany). High-performance liquid chromatography (HPLC)-grade formic acid was purchased from Dikma Technologies Inc. (Lake Forest, CA). HPLC-grade ammonium

Figure 1. LC−MS analysis of free GABA in the plasma supernatants of rats and mice. (a) GABA (50 mg/kg h) and saline were separately administered to anesthetized rats by intravenous gutta; blood from the rats was withdrawn at various time intervals, and the plasma samples were subjected to LC−MS analysis. The GABA concentration in the plasma of rats administered GABA by intravenous gutta elevated within 1 h and approached 4461 ng/mL in a dynamic equilibrium state; however, no free GABA could be detected in the plasma of the control rats. (b) The GABA concentration in the plasma of mice after one bolus intravenous injection of GABA−CPP rose up to 7985 ng/mL within 2 min and then sharply decreased to 141 ng/mL within 1 h, whereas the GABA levels in the plasma of mice injected with GABA remained at a very low level (10−24 ng/mL) over a period of 1 h after one bolus intravenous injection in mice.

Table 1. GABA Concentrations in Plasma and Brain Tissue of Intravenous Dripping Rats in the Dynamic Equilibrium State (n = 3)

|                               | plasma (ng/mL) | brain (ng/mL) |
|-------------------------------|----------------|---------------|
| saline dripping rats          | 0              | 247.3 ± 69.5  |
| GABA dripping rats            | 4461 ± 3589    | 271.8 ± 15.2  |

tissue is in dynamic equilibrium with the clearance rate of GABA from the brain.

2.2. GABA−CTP BBB Penetration Experiment. Second, nine male mice were divided into three groups (control group, GABA group, and GABA−CTP group) by weight and assigned randomly. GABA (50 mg/kg) and the GABA−CPP conjugate (GABA−YGR-RARRRRRRR, 10 mg/kg) were each administrated via the tail vein in one bolus, and in control mice, saline was used instead. The plasma and brain samples were collected for detection of the GABA content by the LC−MS assay.

The GABA concentrations in the blood stream of the saline control mice were negligible (data not shown), whereas the GABA concentrations in the plasma of the mice intravenously injected GABA rose up to 7985 ng/mL within 2 min and then...
formate was purchased from Sinopharm Chemical reagent Co., Ltd (Shanghai, China). All other chemicals and reagents of reagent grade were purchased from Sigma Aldrich (St. Louis, MO). Ultrapure water was prepared using a Milli-Q purification system (Millipore, Bedford, MA).

4.2. Instrumentation and Conditions. An Agilent 1290 Infinity HPLC system coupled with an Agilent 6410 B triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA) was employed for the analysis.

Chromatographic separation was achieved on an Thermo Scientific Synchros HILIC HPLC column (2.1 mm × 100 mm i.d., 5 μm particle size) at 30 °C. The flow rate was set at 0.4 mL/min. A gradient elution program was run for chromatographic separation, with mobile phase A (0.1% formic acid, 5 mM ammonium formate in water) and mobile phase B (0.1% formic acid in acetonitrile), as follows: the gradient elution method was used with 75% solvent B, 75–60% over 0–1.2 min, 60–50% over 1.2–2.5 min, 50–75% over 2.5–2.8 min, followed by a re-equilibration time of 2.4 min. The total run time was 5.2 min for each sample.

The optimized ion source conditions were as follows: transfer capillary temperature, 320 °C; capillary voltage, 4000 V; nebulizer, 20 psi; and gas flow, 10 L/min. The mass spectrometer was operated in the electrospray ionization (ESI)-positive ion selected reaction monitoring mode using a dwell time of 50 ms per transition to detect ion pairs at m/z 104.01–87 for GABA. The fragmentor was 62 V and collision energy was 8 eV.

Data were acquired and analyzed using the software Gastropus V9.0 (Simulations Plus).

4.3. Calibration Standards and Quality-Control (QC) Samples. The stock solution of GABA was prepared by dissolving the compound in 0.9% NaCl at a concentration of 1.0 mg/mL. Working solutions for calibration and control were prepared from the stock solution by dilution with normal saline.

All of the solutions were stored at 4 °C and brought to room temperature before use.

GABA calibration standards were prepared by spiking blank rat plasma or the brain homogenate with appropriate amounts of the working solutions. Calibration plots were constructed in the range of 20 to 5000 ng/mL for GABA in rat plasma or the brain homogenate (20, 100, 500, 1000, and 5000 ng/mL). QC samples (100, 500, 1000, and 5000 ng/mL) were prepared in a similar manner to that of the calibration standards. The calibration standards and QC samples were stored at −20 °C.

4.4. Sample Preparation. An aliquot of 20 μL of plasma was diluted to 50 μL with normal saline and precipitated by adding 100 μL of acetonitrile. After centrifugation at 13,000 rpm for 15 min, a 5 μL aliquot of the supernatant was injected into the liquid chromatography–ESI-mass spectrometry (LC–ESI-MS) system. The sample was prepared (1:9, v/v) and diluted 1:100 with normal saline. An aliquot of 50 μL of the brain homogenate was precipitated by adding 100 μL of acetonitrile. After centrifugation at 13,000 rpm for 15 min, 5 μL aliquot of the supernatant was injected into the LC–ESI-MS for analysis.

4.5. Animals. Rats (male, Sprague-Dawley species, 180–220 g) and mice (male, 18–26 g, Kunming species) were provided by the Experimental Animal Center of Academy of Military Medical Sciences, China. The health condition of the mice was examined to confirm their suitability for use in the study. The animal rooms were monitored and maintained under a 12 h light–dark cycle, with the temperature ranging from 20 to 25 °C and a relative humidity of 40–60%. All of the mice were allowed to acclimate to the laboratory environment for 2 days, provided with a standard commercial diet and drinking water ad libitum. All of the animal procedures performed in this study were reviewed and approved by the Animal Experimental Welfare and Ethical Inspection Committee of the Chinese Center for Disease Control and Prevention (Approval number IACUC#15-026).

4.6. Pharmacokinetic Study. The rats were intraperitoneally anaesthetized with 10% chloral; then, GABA (Sigma) was administrated by intravenous gutta (50 mg/kg) via the tail vein. The blood samples were taken from the plexuses oculus venosus at 5, 10, 20, 40, and 60 min intervals. The separated plasma was diluted to 40% with saline and precipitated by acetonitrile. The supernatant was collected by high-speed centrifugation. Then, the rats were killed by blood-letting from the femoral artery. The brains were dissected, homogenized in saline (1:9 w/v), diluted 100-fold, and precipitated by acetonitrile; the supernatants were obtained by high-speed centrifugation. Finally, both supernatant samples were subjected to LC–MS analyses for detection of the free GABA content.

4.7. GABA–CTP BBB Penetration Experiment. GABA (50 mg/kg) and GABA–CPP conjugate (GABA–YGRARRRRRR, 10 mg/kg), respectively, were injected via the tail vein in one bolus into the mice. The same volume of normal saline was injected into the control mice in the same manner. Samples were taken at 2, 5, 15, 30, and 60 min intervals after injection, and the concentrations of GABA in the supernatants of blood and brain tissue were examined as mentioned above.

4.8. Statistical Analysis. Descriptive statistics were expressed as mean ± SD. The GABA concentrations in plasma or brain homogenates of rats and mice were compared by analysis of variance using SPSS software (version 20.0; SPSS Inc., Chicago, IL). All tests of hypotheses were two-sided, and p values less than 0.05 and 0.01 were considered statistically significant.

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Notes
The authors declare no competing financial interest.

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