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Original Article

Determination of Brain Distribution of Amino Acid Neurotransmitters in Pigs and Rats by HPLC-UV

Si-Yu Meng[1,2#], Mo-Huan Tang[1,2#], Xue-Jiao Zhao[1,2#] and Zhao-Ying Liu[1,2*]

[1]College of Veterinary Medicine, Hunan Agricultural University, Changsha, China;; [2]Hunan Engineering Technology Research Center of Veterinary Drugs, Hunan Agricultural University, Changsha, China

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Abstract

Background and objectives: Amino acid neurotransmitters are closely correlated to the neurological function of the brain, and the imbalance of amino acid neurotransmitters can lead to a variety of neurological diseases. Therefore, the development of a simple method to detect five neurotransmitters (aspartate, glutamate, glycine, taurine, γ-aminobutyric) in the brain is urgently needed.

Methods: The sample was initially treated and derived, and analyzed using liquid chromatography. In the range of 0.300–100.000 mol/L, the linear relationship was good, and the correlation coefficient was ≥0.999. Furthermore, the intra-day accuracy of this method was 1.48–13.85%, and the inter-day accuracy was 2.13–12.61%. Moreover, the limit of detection (LOD, signal-to-noise ratio 3 [S/N = 3]) was 0.15–0.20 mol/L, and the limit of quantitation (LOQ, S/N = 10) was 0.30–0.55 mol/L. This approach was used to compare the content of amino acids in the brain of pigs and rats.

Results: The data revealed that most of the amino acid neurotransmitters were higher in the five brain tissues obtained from pigs, when compared to those obtained from rats. Aspartate and taurine had the greatest concentrations in brain tissues obtained from pigs and rats, respectively (except the cerebellum).

Conclusions: It can be concluded that there are differences in the content of neurotransmitters in brain regions among animals. The development of this method would support the detection of neurotransmitters in the brain.

Introduction

Amino acid neurotransmitters is a common substance to transmit nerve information, and plays an extremely vital role in nerve organization, particularly in terms of neural functions of the brain.[1] There are two types of amino acid neurotransmitters: excitatory and inhibitory. Excitatory neurotransmitters include aspartate (Asp) and glutamate (Glu), while inhibitory neurotransmitters include glycine (Gly), taurine (Tau), and γ-aminobutyric (GABA). These five amino acid neurotransmitters are involved in the functions of the nervous system. For instance, the excessive activation of Glu receptors would result in central nervous system disorders.[2] Variations in the amount of Asp generated and released in neuronal terminals would have an impact on the functional functions of the brain, including cognition, memory, intellect and emotion.[3] The lack of GABA can manifest itself in different ways, such as anxiety and panic.[4] Tau has a promising therapeutic potential in the central nervous system, where it protects against toxicity and damage induced by the functions of the nervous system. Furthermore, Tau may also help to treat a number of neurological conditions, including epilepsy, stroke, and neurodegenerative illnesses.[5] Gly not only improves sleep quality and prevents neurological disorders, such as epilepsy, depression and pain,[6] but is also critical for regulating hippocampal excitation and inhibiting balance.[7] Therefore, it is critical to quantitatively detect amino acid neurotransmitters in multiple brain regions, and determine how amino acid neurotransmitters change during various physiological and pathological processes.

With the advancement of techniques for examining amino ac-

Keywords: HPLC-UV; Aspartate; Glutamate; Glycine; Taurine; γ-aminobutyrica.

Abbreviations: ASP, aspartate; GABA, γ-aminobutyric; GLU, glutamate; GLY, glycine; HPLC, high-performance liquid chromatography; LOD, limit of detection; LOQ, limit of quantitation; S/N, signal-to-noise ratio; TAU, taurine.

***Correspondence to:** Zhao-Ying Liu, College of Veterinary Medicine, Hunan Agricultural University, Furong District, Changsha 410128, Hunan, China. ORCID: https://orcid.org/0000-0002-9050-2287. Tel: 86+15111179464, E-mail: liu_zhaoying@hunau.edu.cn

[#]Contributed equally to this work.

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ids, an increasing number of detection methods have been applied to detect amino acid neurotransmitters in various sample types. At present, amino acid neurotransmitters test samples include the cerebrospinal fluid, blood and urine, and the high-performance liquid chromatography (HPLC)-Triple time-of-flight (TOF) method is used.[8] This approach is highly sensitive, but the disadvantage is its complex operation. In addition, previous studies have tested amino acids obtained from the hippocampus of the brain of rats using gas chromatography-mass spectrometry (GC-MS),[9] but other areas of the brain were not tested. Furthermore, the liquid chromatography-tandem mass spectrometry (LC-MS/MS) approach has been employed to detect brain amino acid content,[10] but the high cost of equipment and maintenance has made this method less accessible. These shortcomings highlight the need to establish a simple and cost-effective detection method. Therefore, the present study developed a liquid phase pre-column derivation-based method to address this need.

Since most amino acids are not ultraviolet (UV) absorbent, derivations must be performed to quantitatively measure the amino acid content before using the liquid phase. The common derivative reagents include phenyl isothiocyanate (PITC), FOMC-Cl, 6-aminoquinolyl-N-hydroxysccinimidyl carbamate (AQC), and ortho-phthalaldehyde (OPA). However, these reagents have various disadvantages. For example, FOMC-Cl itself and its decomposition product, FOMC-OH, induces fluorescence, which affects the separation efficiency,[11] OPA derivatives are unstable, and require immediate analysis after derivatization,[12] and the presence of PITC in a sample would shorten the life of the column. A sensitive derivatization reagent that works well with amino acids is 4-fluoro-7-nitrobenzofurazan (NBD-F),[13] and this also applies for partial proteins[14] and drug concentration in plasma.[15] Pre-column derivation using 4-Fluoro-7-nitrobenzofurazan (NBD-F) can lead to several benefits, including gentle reaction conditions, consistent byproducts, and rapid assay time.[16]

As a result, HPLC was developed for the determination of amino acid neurotransmitters in the brain using the NBD-F derived reagent. This has been successfully used to compare the amino acid neurotransmitter content in the brain of pigs and rats. This technique has been proven to be straightforward and useful for estimating the amount of amino acid neurotransmitters.

The experiment

Instruments and reagents

The liquid chromatography system comprised of the following: a rapid-resolution binary pump, an SPD-20A UV detector, a SIL-20A auto-sampler (with a 600 μL injection loop), and a CBM-20A system controller; 1/100,000 electronic balance (ATX224 Shimadzu production Institute); acidity meter (Shanghai Mettler-Toledo Instrument Co., Ltd.); high-speed frozen centrifuge (Eppendorf, Germany); ultrapure water system (Millipore, Bedford, MA, USA).

The Asp, Glu, Gly, Tau and GABA were purchased from Beijing Solarbio Science & Technology Co., Ltd. The NBD-F was purchased from Shanghai McLean Biochemical Technology Co., Ltd. The HPLC grade acetonitrile and methanol were purchased from Merck (USA). The HPLC grade phosphoric acid was purchased from Comeo Chemical Reagent Co., Ltd. The analytical grade potassium tetraborate was purchased from Shanghai Jingchun Biochemical Technology Co., Ltd. All of the substances were obtained from Chinese Pharmaceutical Chemical Reagent Co., Ltd., including the sodium dihydrogen phosphate and disodium hydrogen phosphate of analytical quality. The Milli-Q ultrapure water system was used to create ultrapure water.

Experimental animals

Laboratory animals: The Hunan Agricultural University Ethics Committee authorized the use of animals for the research (No. 2020-43). Twelve male Sprague-Dawley rats (weighing 180-240 g) were obtained from SJA Laboratory Animal Co., Ltd. The blank pig brain tissue samples were obtained from Hunan New Wufeng Co., Ltd. (Liuyang, China). The Chinese Guidelines for the Care and Use of Laboratory Animals were followed in the present study. The investigators will continue to follow these guidelines in future experiments.

Standard solution

Standard reserve solution: accurate weighing of dissolved Glu, Asp, Gly, Tau and GABA in water. The first two formulations were 10 mmol/L, and the last three formulations were 100 mmol/L. Then, these were stored at −20°C. Five kinds of standard amino acids were prepared into 1 mmol/L of mixed standard working solution, with 100 mmol/L and 10 mmol/L, respectively, and stored at 4°C.

Derivative reagent: the NBD-F was accurately weighed and prepared into a reserve solution at a concentration of 0.1 mol/L, and this was kept away from light at −20°C until analysis.

Potassium tetraborate solution: the potassium tetraborate was accurately weighed and dissolved in water, and the amount was fixed to 100 mL. Using a calibrated pH meter, the pH of the solution was adjusted to 9.5 ± 0.1. Then, 40 Hz of ultrasound was applied for 10 minutes, and this was stored at 4°C through a membrane.

Sample collection and pretreatment

Sample collection and preservation

The hippocampus, cortex, striatum, brainstem and cerebellum were quickly removed from the skull of the pigs and rats, and separated from the ice plate. Before use, the separated brain tissues were kept at −80°C. After accurate weighing, normal saline was added to homogenize at a mass-volume ratio of 1:1. Then, the homogenate was centrifuged at 12,000 rpm for 10 minutes at 4°C to separate the supernatant, and this was transferred and purified using an organic membrane filter with a pore size of 0.22 μm.

Sample collection and pretreatment

The mixed amino acid solution or sample supernatant (100 μL), potassium tetraborate solution (350 μL), and NBD-F working solution (50 μL) were mixed in 1.5 mL light-proof centrifuge tubes, and allowed to react in a heated constant temperature mixer at 60°C for 10 minutes.

Chromatographic conditions

The analytes were separated in the ChromCore C18 column (150×4.6 mm, 5 μm) using the FLC automatic 2-D liquid chromatography coupling instrument. Mobile phase A was methanol, and mobile phase C was the phosphate buffer (0.02 mol/L, including 0.2 mmol/L of sodium dihydrogen phosphate and 0.2 mmol/L of sodium disodium phosphate, pH 6.0). Then, a 0.22 μm water system filter membrane was used to filter the mobile phase. The injection volume was 20 L, the column temperature was 45°C, the wavelength was 472 nm, and the mobile phase flow rate was 1.0 mL/min.

Method of validation

Five standard amino acids were selected, and these were detected from the hippocampus samples obtained from pigs. Then, the limit of detection (LOD), limit of quantitation (LOQ), linearity, precision, and accuracy were determined to confirm the dependability of the method. Next, after the five standard amino acids were selected, these were detected from the hippocampus samples obtained from pigs. Then, the standard curve was drawn using the concentration and peak area of Asp, Glu, Gly, Tau, and GABA in milli-q water. Three concentrations (QCH, QCM and QCL) of quality control samples were prepared ($n = 6$). The precision and accuracy were measured within six days, within days, and between days using the QC samples of the same concentration. The detection limit was set at a signal-to-noise ratio (S/N) of 3, and the quantitative limit was set at a S/N of 10.

Statistical analysis

GraphPad Prism 8.0.1 was used to conduct the multivariate ANOVA analysis on the experimental results, and the findings of the experiment were presented in mean ± standard deviation. The difference was considered to be significant when $p < 0.05$, while this was considered to be highly significant when $p < 0.01$.

Results and discussion

Enhancing separation conditions

Liquid chromatographic conditions

Gradient-free elution was used for the present study. Following the previous experiments, the isometric elution of 25% methanol buffer salt, 15% methanol buffer salt, and 5% methanol buffer salt for 25 minutes was determined. Due to the strong retention of GABA on this column, and in order to enable GABA to be eluted smoothly without affecting the normal peak emission of Asp and Glu, 12% methanol buffer salt was selected as the flow stage. In addition, the impact of pH (6.0, 6.5 and 6.8) in the buffer salt mobile phase on the target peak was determined, and the pH was detected to be 6.0. Therefore, the ChromCore C18 column was selected, and this was eluted for 18 minutes in 12% methanol buffer salt. Thus, the five neurotransmitter amino acids were completely separated in the ChromCore C18 column, and the peak shape was good. However, after the addition of the biological matrix, there were other unknown peaks that were not completely eluted. In order to prevent the normal injection of the next needle to be affected, the investigators decided to extend the isometric elution to 25 minutes. The results indicated that the five amino acid neurotransmitters totally separated after 25 minutes at pH 6.0, and the isometric elution with 12% methanol buffer salt after 18 minutes was determined.

Based on the previous experiments, three derivative systems were compared, and the best concentration and derivative solution of NBD-F under the best derivative environment were obtained. The following were investigated: the mixed derivative system that consisted of the sample + potassium tetraborate + NBD-F solution, the mixed derivative system that consisted of the sample + potassium tetraborate + NBD-F and methanol, and the mixed derivative system that consisted of the sample + potassium tetraborate + NBD-F + acetonitrile. In addition, the NBD-F solution of 10, 50 and 100 mmol/L under the conditions of the three mixed derivative systems were investigated. It was revealed that the 10 mmol/L NBD-F solution had the best peak shape and separation, without acetonitrile and methanol.

pH, temperature and time

In the present experiment, after studying and analyzing the derivation conditions of amino acids, it was revealed that the pH of the derivative reagents, temperature, and reaction heating time affects the sensitivity of the detection method. Therefore, the pH of the reaction medium was investigated. The effect of various pH levels (8.5, 9.0, 9.5 and 10.0) on the peak area of the derivative product of amino acid was investigated. It was revealed that the derivative yield of potassium tetraborate with pH 9.5 was the highest.

Based on this information, the effects of several derivatization temperatures (30, 45, 60 and 75°C) and time ranges (1–20 minutes) on the peak area of the derivatives were determined. It was found that the reaction time of heating for 10 minutes at 60°C can make the derivatization reaction complete, and allowed for more ideal peak areas and separations to be obtained.

Chromatography and detection

The chromatographic conditions in the experimental study were used to determine the selectivity of the five amino acid neurotransmitters, and draw the chromatogram for the five brain areas in pigs and rats (Fig. 1). Within the chosen experimental parameters, the retention time of the symmetrical resolution of the analyte in its vicinity was not disturbed by the additional substances. Furthermore, the five kinds of amino acid neurotransmitters were entirely separated in 18 minutes. As shown in Figure 1b, the peak emergence time of Asp, Glu, Gly, Tau and GABA in the chromatogram was 2.44, 3.29, 8.09, 10.01 and 16.20 minutes, respectively. As shown in Figure 1c, the peak emergence time for Asp, Glu, Gly, Tau and GABA in pigs in the chromatogram was 2.42, 3.38, 7.96, 9.88 and 17.29 minutes, respectively.

Linearity, detection and quantitative limits, precision and accuracy

The linear relationships of the five standard amino acid neurotransmitters were determined within the range of seven different concentrations (0.300–100.0 μmol/L). The regression equations for the correction curves, peak area relative standard deviations (RSDs), detection limits, and quantitation limits are presented in Table 1. The results revealed that the strong linear relationship among the five amino acids is good, with correlation coefficients totaling higher than 0.999.

Precision and accuracy were assessed by measuring the RSD of the five amino acid neurotransmitters in the hippocampus of pigs within and between six days (Table 1). The concentration of the amino acid neurotransmitters was 0.15–0.20 μmol/L and 0.30–0.55 μmol/L, respectively.

According to the experimental results, the LOD value of Asp, Glu, Gly, Tau and GABA was 0.15, 0.15, 0.20, 0.20 and 0.20 μmol/L, respectively. Although the sensitivity of this method was not high, this satisfies the basic quantification, and provides a new method for the detection of amino acids in the brain.

Results of the method application

The method has been successfully applied in two animal substrates (rats and pigs). The outcomes are presented in Figure 2. The blank contents of the five amino acid neurotransmitters in the five brain regions (hippocampus, cortex, striatum, cerebellum and brainstem) were respectively measured in rats and pigs.

The findings revealed that the method of determining the con-

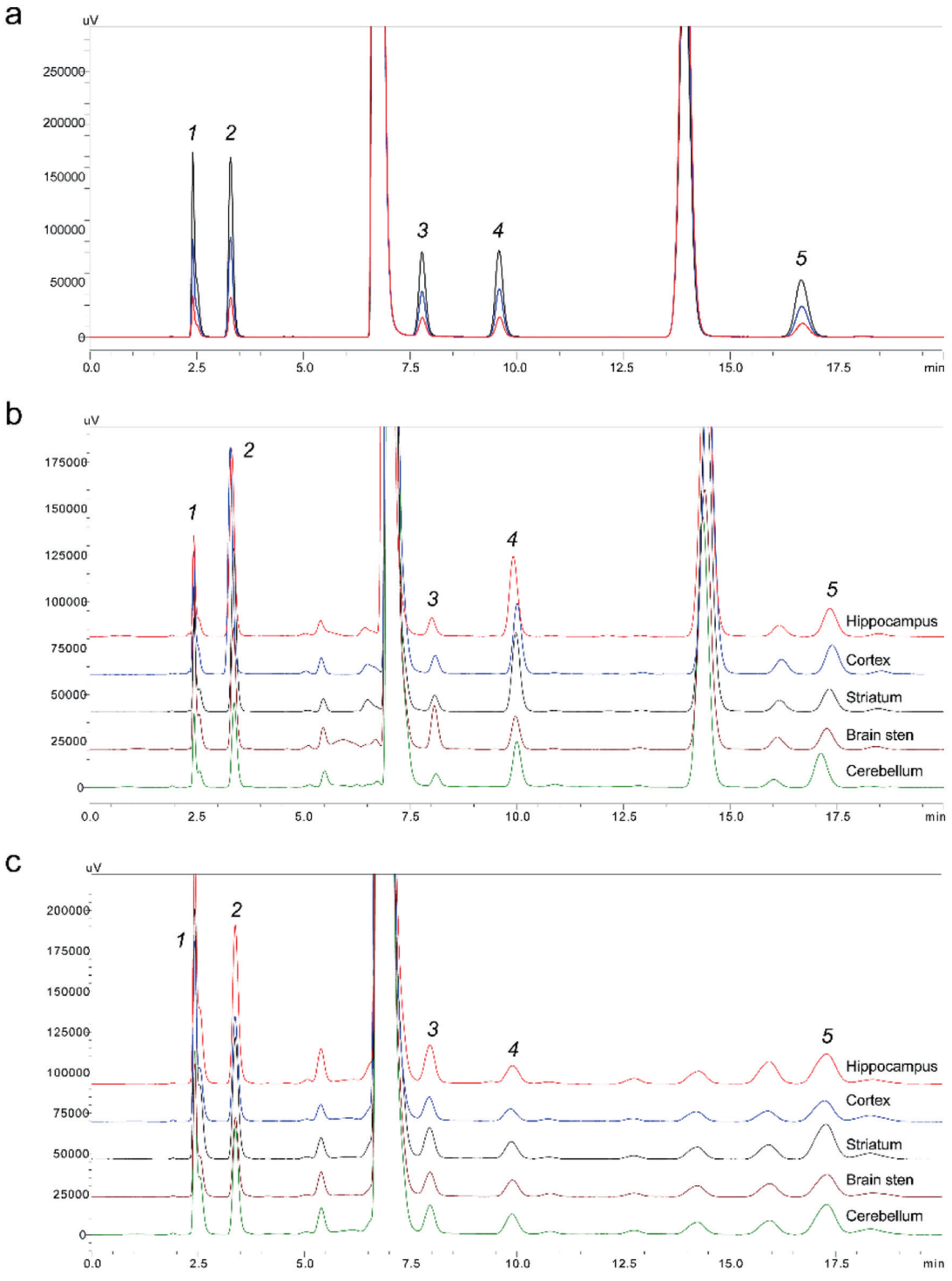

Fig. 1. High-performance liquid chromatography: (1) Asp, (2) Glu, (3) Gly, (4) Tau, and (5) GABA. (a) Standard solution for the five amino acids; (b) Specimens obtained from the rat brain regions; (c) Specimens obtained from the pig brain regions.

centration of amino acid neurotransmitters in rats and pigs has strong selectivity, because the peak area and resolution of the amino acid targets in the five separate brain areas of rats and pigs were good. In the pig samples, the content of Asp was the highest in the different brain regions. Furthermore, the results revealed that the amino acid neurotransmitters in the brain of rats were generally lower, when compared to those in the brain of pigs. However, the amount of Tau in the brain of rats was higher, when compared to that in the brain of pigs. These results show that for the amino acid neurotransmitters in both pigs and rats, Asp, Glu, Gly and GABA

Table 1. Linearity, detection limit, quantitative limit, precision and accuracy of the pig hippocampal samples

Compound	Regression equation	r^2	QC, *n* = 6	RSD (%)		Accuracy (%)		LOD (μmol/L)	LOQ (μmol/L)
				Intra-day	Inter-day	Intra-day	Inter-day		
Asp	y = 24.15x + 4,953.5	0.9999	QCH	1.48	2.13	88.98	88.14	0.15	0.3
			QCM	2.40	5.47	87.66	94.46		
			QCL	7.72	5.94	91.91	85.91		
Glu	y = 27.708x + 6869.8	0.9993	QCH	1.76	2.81	89.01	87.71	0.15	0.3
			QCM	7.74	8.66	110.80	111.98		
			QCL	13.85	12.61	101.73	108.68		
Gly	y = 17.686x + 4,103.2	0.9997	QCH	3.14	3.49	88.72	87.26	0.2	0.5
			QCM	6.14	6.68	87.66	96.94		
			QCL	4.64	3.53	85.99	85.70		
Tau	y = 19.415x + 7,971.5	0.9991	QCH	1.84	2.15	87.44	88.27	0.2	0.5
			QCM	2.94	5.86	89.47	98.89		
			QCL	3.63	4.16	93.19	88.49		
GABA	y = 20.378x + 6,738.8	0.9999	QCH	3.59	2.59	87.95	89.13	0.2	0.55
			QCM	7.13	7.35	88.43	90.14		
			QCL	4.50	3.53	91.97	86.31		

Asp, aspartate; Glu, glutamate; Gly, glycine; Tau, taurine; GABA, γ-aminobutyric; RSD, relative standard deviations; LOD, limit of detection; LOQ, limit of quantitation.

were significantly different in the hippocampus, while Tau was not significantly different. In the cortex, Asp, Glu, Gly and GABA presented with significant differences, while Tau did not present with significant differences. In the striatum, Asp, Gly, Tau and GABA presented with significant differences, but there was no significant difference in Glu. In the cerebellum, there were significant differences between Asp and Gly, and between Glu and GABA, while there was no significant difference in Tau. In the brain stem, Asp, Glu, Gly and GABA markedly varied, while Tau did not markedly vary. Interestingly, it was observed that the excitatory amino acid neurotransmitters and inhibitory amino acid neurotransmitters balanced with each other in each region of the animal brain, and this was not dominated by any one kind of amino acid neurotransmitter. Animal amino acid neurotransmitters are regulated by two different types of amino acid neurotransmitters, and these maintains the dynamic balance of the body under normal physiology.

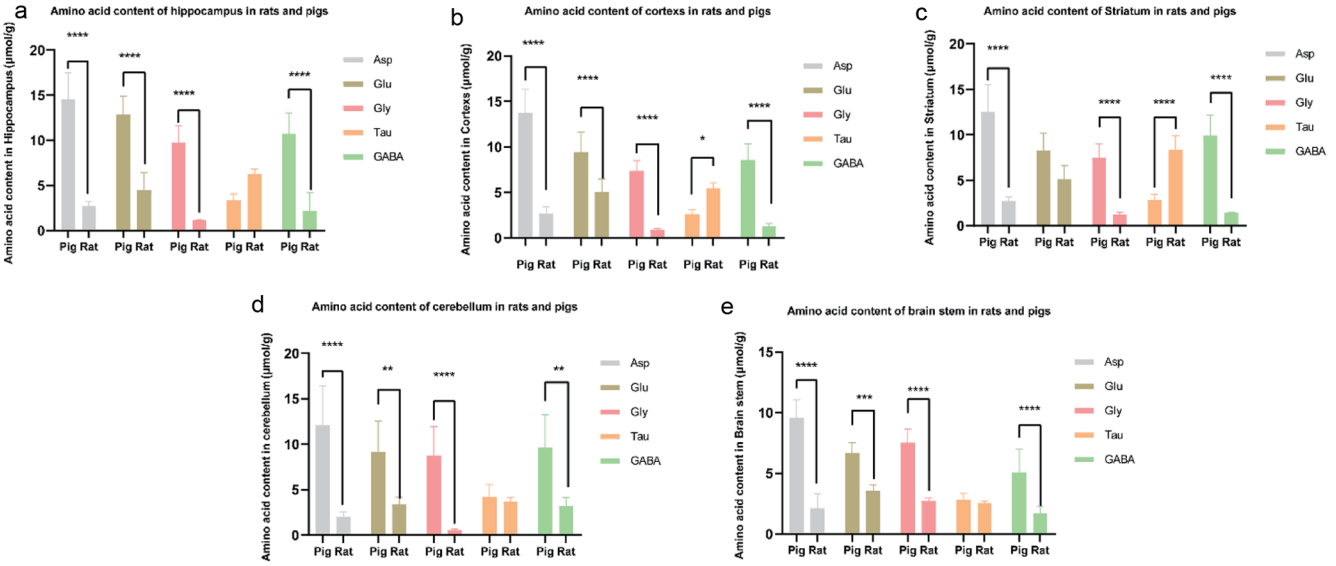

Fig. 2. Comparison of amino acid neurotransmitters in the five different brain regions in pigs and rats: (a) hippocampus, (b) cortex, (c) striatum, (d) cerebellum, and (e) brain stem. Levels of amino acids in the five different brain regions in pigs and rats. The data were expressed as mean ± standard error of the mean (SEM, *n* = 6). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$, pig vs. rat.

Comparison of the contents of amino acid neurotransmitters between pigs and rats

Earlier studies on the hippocampus of the brain of rats have revealed that this has the highest concentration of Glu, followed by Asp.[16] However, this result was slightly different from what was revealed in the present study. It was found that the Tau content was the highest in the hippocampus, cortex, striatum and cerebellum, and that the glutamic acid content was the highest in brain stem. The reason for this difference may be due to difference in conditions during processing, such as sample derivation. In previous studies, the highest concentrations of Glu were detected in the hippocampus and cortex of pigs, followed by Asp.[17] This finding differs from the present results, which revealed that the highest concentration was Asp, followed by Glu. It was analyzed that the reason for this difference may be because the sensitivity to different amino acids varied due to different methods. However, the results remained consistent. For example, in the cortex of pigs, except for Glu and Asp, which had the highest content, the ranking of the contents of the other three amino acids in descending order was GABA, Gly and Tau.

Future directions

A number of studies have revealed that the imbalance of amino acids in the brain is associated with a variety of neurological diseases, such as Alzheimer's disease, anxiety and depression. Based on this, a simple brain amino acid detection method was established, in order to provide technical support for the monitoring of these diseases, hoping to provide help in the prevention of neurodegenerative diseases. Future research would be conducted, and focus will be given on the exploration of the methodology, providing technical support for scientific research in the future.

Conclusions

The present study offers a quick and low-cost method for identifying amino acid neurotransmitters in brain tissues. This technique has been used to identify amino acid neurotransmitters in five different regions of the brain in pigs and rats. These findings demonstrate the suitability of this approach for identifying amino acid neurotransmitters in animal brain tissues.

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Conflict of interest

One of the authors, Dr. Zhao-Ying Liu, has been an editorial board member of *Journal of Exploratory Research in Pharmacology* since February 2023. The authors have no other conflict of interests to declare.

Author contributions

ZYL and SYM: contributed to study concept and design; MHT and XJZ: acquisition of the data; SYM and MHT: assay performance and data analysis; MHT: drafting of the manuscript; SYM: critical revision of the manuscript; ZYL: supervision.

Ethical statement

The Hunan Agricultural University Ethics Committee authorized the use of animals in research (No. 2020-43). The 12 male Sprague-Dawley rats, which weighed 180–240 g, were obtained from SJA Laboratory Animal Co., Ltd. The samples of blank pig brain tissues were obtained from Hunan New Wufeng Co., Ltd. (Liuyang, China). The Chinese Guidelines for the Care and Use of Laboratory Animals were followed in the study.

Data sharing statement

No additional data are available.

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