

Gastrodin Attenuates Tourette Syndrome by Regulating EAATs and NMDA Receptors in the Striatum of Rats

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Purpose: This study explored whether gastrodin (Gas) could attenuate the symptoms of Tourette syndrome (TS) via the regulation of glutamate (Glu), its transporters (EAAT1 and EAAT2) and its receptors (NMDAR1, NMDAR2A and NMDAR2B) in rats.

Materials and Methods: Seventy-five Wistar male rats were randomly divided into five groups (n=15 each): the control, TS, Tia (tiapride, 25mg/kg), Gas60 (gastrodin, 60mg/kg) and Gas120 groups (gastrodin, 120mg/kg). Rats in all groups except the control group received intraperitoneal injection of 3,3'-iminodipropionitrile (IDPN) for 7 consecutive days to establish the TS model. Thereafter, rats in the Tia, Gas60, and Gas120 groups were gavaged with 25mg/kg Tia, 60mg/kg Gas and 120mg/kg Gas for 28 days. Rats in the control and TS groups were gavaged with 0.9% normal saline. Behavioral evaluation was performed by using stereotypy scoring, nodding experiment and autonomic activity test. The Glu level was measured by UPLC-QqQ-MS analysis. The expression of EAAT1, EAAT2, NMDAR1, NMDAR2A and NMDAR2B was measured by Western blot and quantitative real-time PCR (qRT-PCR) analyses.

Results: The results showed that rats with IDPN-induced TS exhibited an increase in stereotypy score, nodding numbers, number of times to enter the central area and autonomic total distance, which could be improved by Tia and Gas treatments. Furthermore, Tia and Gas treatments significantly decreased the IDPN-induced increase in Glu levels in rats with TS. Furthermore, the decreased expression of EAAT1 and EAAT2 and increased expression of NMDAR1, NMDAR2A, and NMDAR2B in rats with TS induced by IDPN could be substantially altered by Tia and Gas treatments.

Conclusion: Gas ameliorated the behavioral dysfunction of rats with TS by maintaining Glu at a normal level, upregulating the expression of EAAT1 and EAAT2, and downregulating the expression of NMDAR1, NMDAR2A and NMDAR2B.

Keywords: Tourette syndrome, gastrodin, Glu, EAATs, NMDARs

Introduction

Tourette syndrome (TS), a chronic neuropsychiatric disorder with onset in childhood, is characterized by rapid, involuntary, repetitive, non-rhythmic, and burst-like phonic and motor tics.^{1,2} TS affects approximately 1% of school-age children,³⁻⁵ and boys are approximately three to four times more likely to develop the disease than girls.⁶ The majority of patients with TS may have other comorbidities, such as obsessive-compulsive disorder (OCD), attention-deficit/hyperactivity disorder (ADHD), autism, anxiety, and other emotional problems.^{7,8} Both tics and

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comorbidities may hinder the social interactions and learning of patients with TS and contribute to the overall level of functional impairment.^{9–11} Although the spectrum of treatment for TS has been expanding in recent years, the current treatment strategies are often unsatisfactory and have many adverse reactions. Therefore, it is necessary to seek novel treatments and drugs.

The etiology and pathogenesis of TS are still unclear.¹ Previous studies have demonstrated that the etiology of TS is closely related to neurobiochemical factors, genetic factors, environmental factors, immunological factors, neuroinflammation, psychosocial factors, and other factors.¹² Previous studies have found that abnormalities in the neurotransmission of glutamate (Glu) and gamma-aminobutyric acid (GABA) are relevant to the pathogenesis of TS.^{13,14} Glu, the major neurotransmitter associated with TS, is the primary excitatory neurotransmitter of the central nervous system (CNS).¹⁵ It is widely accepted that there is a trend of an increase in Glu in cortico-striato-thalamo-cortical (CSTC) circuits in patients with TS.¹⁶ Under physiological conditions, the excitatory amino acid transporters (EAATs) located in neurons and glial cells can take up Glu to maintain the concentration of extracellular Glu below excitotoxic levels. EAAT1 (glutamate aspartate transporter, GLAST) and EAAT2 (glial glutamate transporter, GLT-1) have a high affinity for Glu and may take up almost 80% of extracellular Glu, playing a key role in the uptake of Glu, termination of excitability signals and protection against neuronal damage.¹⁷ However, when EAATs dysfunction occurs or the release of Glu is excessive, more Glu gathers in the synaptic cleft, thus over-activating Glu receptors, which may lead to TS.

Extracellular Glu activates postsynaptic Glu receptors to regulate brain excitability. Among Glu receptors, NMDARs have been proved to be associated with the occurrence of TS. Three groups of NMDAR subunits exist: NMDAR1, NMDAR2 (further subdivided into four distinct members NMDAR2A- NMDAR2D) and more rarely NMDAR3 (further subdivided into two distinct members NMDAR3A and NMDAR3B).^{18,19} NMDAR1 plays a key role in the ion channel function of NMDAR, which is a main modulator of Glu.²⁰ NMDAR2A and NMDAR2B are the main subunits expressed in forebrain regions (striatum, cortex, hippocampus).²¹ Previous studies have proved that GRIN2B, which encodes NMDAR2B, might be a susceptibility gene for neuropsychiatric disorders such as TS, OCD and ADHD.²² Therefore, we suggested that

NMDAR1, NMDAR2A and NMDAR2B might be closely related to the occurrence of TS.

Tian ma (also called *Rhizoma Gastrodiae*), the dried rhizome of *Gastrodia elata* Blume, is a notable traditional Chinese herb that has been used for the treatment of various conditions, including dizziness, headache, epilepsy, stroke, dementia, cardiovascular diseases, spasm, and other diseases for centuries.²³ Gastrodin (Gas) is one of the major bioactive components isolated from the *Rhizoma Gastrodiae*. Numerous studies have shown that Gas possesses a broad range of pharmacological properties, including hypnotic, sedative, anti-tic, anti-vertigo, anxiolytic, anti-epileptic, antidepressant, and memory-improving.^{24–27} It has been reported that Gas has beneficial effects on many CNS diseases, including TS, Parkinson's disease, Alzheimer's disease, epilepsy, affective disorders, cognitive impairment, and cerebral ischemia/reperfusion.^{23–25} The mechanisms of Gas include modulating neurotransmitters, anti-inflammatory, antioxidative, suppressing microglial activation, upregulating neurotrophins, and regulating mitochondrial cascades.^{23,28,29} Although there have been some animal studies on the effect of Gas treating TS,^{24,25} few studies have demonstrated an association between the anti-tic effect of Gas and the functions of EAATs and NMDARs. This study was designed to explore the underlying mechanism of Gas in treating TS based on the EAATs and NMDARs functions. Collectively, we hypothesized that Gas might decrease the Glu level and regulate the expression of EAATs and NMDARs in rats with TS, thus playing a role in the treatment of TS.

In the present study, we evaluated the effects of Gas in a rat model of TS induced by 3,3'-iminodipropionitrile (IDPN) by behavioral tests, such as stereotypy scoring, nodding experiment, and autonomic activity test. The levels of Glu, GABA and dopamine (DA) were measured by UPLC-QqQ-MS. The protein and mRNA levels of EAAT1, EAAT2, NMDAR1, NMDAR2A, and NMDAR2B in the striatum of rats with TS were measured by Western blot and qRT-PCR.

Materials and Methods

Drugs and Reagents

Gastrodin (Gas; Sigma-Aldrich, St. Louis, MO, USA), 3,3'-Iminodipropionitrile (IDPN; Sigma-Aldrich, Shanghai, China), anti-EAAT1 (Abcam, Cambridge, MA, USA), anti-EAAT2 (Abcam, Cambridge, MA, USA), anti-NMDAR1

(Abcam, Cambridge, MA, USA), anti-NMDAR2B (Abcam, Cambridge, MA, USA), anti-NMDAR2A (ABclonal Technology, Wuhan, China), anti-GAPDH (ABclonal Technology, Wuhan, China), RIPA lysate (Beyotime, Nantong, China), and Bicinchoninic acid protein assay kit (BCA; Beyotime, Nantong, China) were used.

Animals

Seventy-five specific pathogen-free (SPF) Wistar male rats aged 3 weeks and weighing 50–60 g were purchased from Shanghai Sciple-Bikai Laboratory Animal Co., Ltd. (Shanghai, China, No: SCXK(Hu) 2017–0005). All rats were raised in an SPF animal room with a stable temperature of $22\pm 2^{\circ}\text{C}$, a relative humidity of $35\pm 5\%$, and a 12-hour light/night cycle environment. All rats had free access to food and water and were acclimatized for 7 days before experiments. All animal experiments were approved by the Laboratory Animal Ethical and Welfare Committee of Xinhua Hospital Affiliated to Shanghai Jiao Tong University of Medicine (XHEC-F-2021-04), which conforms to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Animal pain was minimized during the experiment.

Grouping and Administration of Animals

All rats were randomly separated into 5 groups ($n=15$ in each group): control group, TS group, Tia group (tiapride, 25 mg/kg), Gas60 group (gastrodin, 60 mg/kg), Gas120 group (gastrodin, 120mg/kg). In the TS group, Gas60 group and Gas120 group, rats were injected intraperitoneally with 300 mg/kg IDPN once daily for 7 consecutive days to induce TS. Rats in control group were given an intraperitoneal injection with 0.9% normal saline. From the eighth day, rats in the Tia group, Gas60 group and Gas120 group were gavaged with 25 mg/kg Tia, 60 mg/kg Gas and 120 mg/kg Gas, respectively, once per day for subsequent 28 days. Rats in the control group and TS group were gavaged with an equal volume of 0.9% normal

saline. An overview of the experimental procedures is shown in Figure 1.

Behavioral Tests

Stereotypy Scoring

Stereotype scoring was performed by two trained independent examiners who were familiar with the experiments but blind to the allocation of groups. Under quiet and dark circumstances, the rats were reared in a separate observation cage. After 5 minutes of acclimation, the rats were scored by two observers for 5 minutes. The average score of each rat was calculated for statistical analysis. Stereotypy score was graded as follows: 0 points: no stereotyped movement; 1 point: rotation of body; 2 points: excessive up-and-down movement of the head and neck; 3 points: excessive up-and-down movement and rotation of the head and neck; 4 points: head swings sideways and excessive up-and-down movement of the head and neck.

Nodding Experiment

Nodding experiment was performed by two trained independent examiners who were unaware of the allocation of groups. Under quiet and dark conditions, the rats were reared in a separate observation cage. After 5 minutes of acclimation, the nodding numbers of rats were counted and recorded by two observers for 5 minutes. The average nodding numbers of each rat were calculated for statistical analysis.

Autonomic Activity Test

The experiment was conducted in a quiet and shaded environment using an open field test box with dimensions of $100\text{ cm}\times 100\text{ cm}\times 50\text{ cm}$. The inner bottom and floor of the apparatus were black, and the bottom surface was equally divided into 25 squares. A camera connected with a computer was placed directly above the box, and the behavior of each rat was recorded for 5 minutes. The computer recorded the movement track and analyzed the number of times to enter the central area, central region residence time and total movement distance of each rat.



Figure 1 A schematic representation of the experimental procedures (TS model and treatments procedure in rats).

The bottom and inner surfaces of the apparatus were cleaned with 75% ethanol after each test.

Ultra-High Performance Liquid Chromatography Tandem Triple Quadrupole Bar Mass Spectrometry (UPLC-QqQ-MS) Analysis

UPLC-QqQ-MS was used to detect the levels of Glu, GABA and DA in the striatum of rats. In brief, the rat was deeply anaesthetized, and the whole striatum was dissected. The samples were stored in a freezer at -80°C . Before the measurement, the samples were taken from the freezer and thawed at room temperature. Then, 375 μL of ice-cold sample extraction buffer (40% methanol+40% acetonitrile+20% pure water) was added to each sample. After standing, the samples were lysed for 120 s to completely homogenize the tissue. The samples were centrifuged at 12,000 rpm for 15 min at 4°C , and the supernatants were transferred to new EP tubes. A total of 375 μL of ice-cold sample extraction buffer was added to each sample residue. After shaking, the samples were centrifuged again. The supernatants were transferred to the previous supernatants. The supernatant was added with 300 μL of chloroform, shaken, and placed in a freezer at -20°C . After 30 min, the samples were centrifuged at 12,000 rpm for 15 min at 4°C . All supernatants were transferred to new EP tubes and diluted with sample diluent (50% methanol+50% pure water) to a proper concentration. Then, 50 μL of the diluted sample solution was transferred to the injection vial for testing.

The analyses were performed by using ultra-performance liquid chromatography (H-Class UPLC) coupled with Xevo TQ-XS triple quadrupole MS (Waters, Milford, MA, USA). The separation was performed on an Acquity UPLC BEH HILIC column (2.1 mm \times 100 mm, 1.7 μm) and the temperature of the column was set at 40°C . The mobile phase consisted of 0.25% formic acid aqueous containing 20 mmol ammonium formate (solvent A) and 0.25% formic acid acetonitrile (93:7) containing 5 mmol ammonium formate (solvent B). The injection volume was 1 μL . Under the selected electrospray ionization (ESI) conditions, analytes were detected by MRM in negative ionization mode. The capillary voltage was set at 3.5 kV. Nitrogen was used as the desolvation gas at a flow rate of 1000 L/h at a temperature of 500°C . The flow rate of the cone gas was set at 150 L/h.

Western Blot Analysis

Western blot was used to measure the protein levels of EAAT1, EAAT2, NMDAR1, NMDAR2A and NMDAR2B. Total proteins were extracted from the striatum of rats by using RIPA lysate, and the concentrations of the proteins were detected by using a BCA kit according to the manufacturer's instructions. The proteins were separated by 7.5% to 12.5% SDS-PAGE and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The PVDF membranes were blocked with 5% skim milk for 2 hours at room temperature. Then, the membranes were incubated with the following appropriate concentrations of primary antibodies: anti-EAAT1 (1:1000), anti-EAAT2 (1:1000), anti-NMDAR1 (1:1000), anti-NMDAR2A (1:1000), anti-NMDAR2B (1:1000) and anti-GAPDH (1:2000) at 4°C overnight. The second day, the primary antibodies were discarded, the membranes were washed with TBST four times for 10 min each time. Then, the membranes were incubated with anti-mouse or anti-rabbit secondary antibodies at room temperature for 2 hours. After incubation with the secondary antibodies, the membranes were washed with TBST as described above. With enhanced chemiluminescence (ECL), the membranes were scanned by an imaging system (Bio-Rad, Hercules, CA, USA) to obtain protein bands. Then, the optical density of the protein band was quantified by using ImageJ software.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

qRT-PCR was used to measure the mRNA levels of EAAT1, EAAT2, NMDAR1, NMDAR2A and NMDAR2B. Total RNA was extracted from the striatum of each rat by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed to synthesize cDNA by using a PrimeScript TM RT Master Mix kit (TaKaRa, Otsu, Japan) according to the manufacturers' instructions. Then, qRT-PCR was conducted by using a TB Green[®] Premix Ex Taq TM II kit (TaKaRa, Otsu, Japan). Thermal cycling conditions were as follow: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The gene expression of GAPDH was used as an internal reference, and all gene expression values were normalized against the same samples. The results were quantitatively analyzed according to the $2^{-\Delta\Delta\text{Ct}}$ method. All the sequences of primers are shown in Table 1.

Table I Primer Sequences Used for the qRT-PCR Analysis

Gene	Sequences (5'-3')	
EAAT1	Forward	GCCATCATGAGATTGGTAGCGGT
	Reverse	GGAAGTAGAGGAGAGGCAGGACGA
EAAT2	Forward	GAACCTCGGTCAATGTAGTGGGCG
	Reverse	TGGACTGCGTCTTGGTCATTTTCG
NMDAR1	Forward	CGGTATCAGGAATGCGACTC
	Reverse	GGAAAATCCCAGCTACGATG
NMDAR2A	Forward	GCTTGTGGTGATCGTGCTGAA
	Reverse	AATGCTGAGGTGGTTGTCATCTG
NMDAR2B	Forward	TGGCTATCCTGCAGCTGTTTG
	Reverse	TGGCTGCTCATCACCTCATTC
GAPDH	Forward	TTCCTACCCCCAATGTATCCG
	Reverse	CCACCCTGTTGCTGTAGCCATA

Statistical Analysis

All statistical analyses were performed using SPSS 20.0 software (IBM, Armonk, NY, USA). All the experimental data are normally presented as the mean \pm SD. One-way analysis of variance (ANOVA) was used to statistically analyze the experimental results, and the differences among groups were compared using multiple least significant difference (LSD) tests. $p < 0.05$ was considered statistically significant. All figures were obtained by GraphPad Prism 8.0 software (GraphPad Software Inc., San Diego, CA, USA).

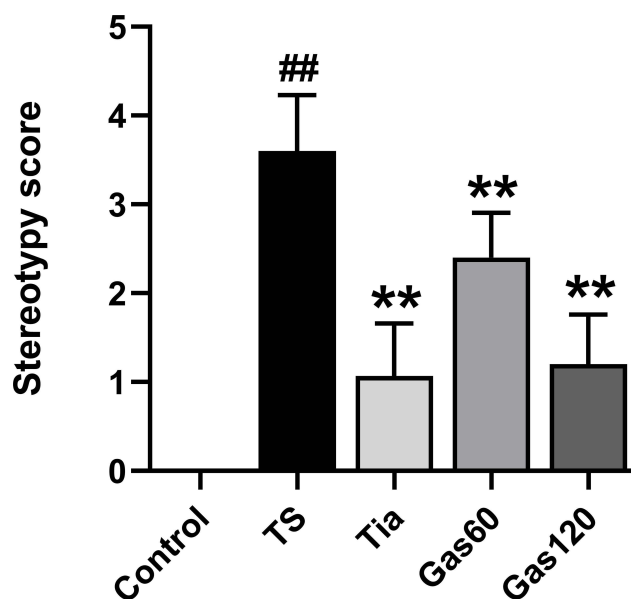
Results

Effects of Gas on the Behaviors of Rats with TS

For the stereotypy scoring, as shown in Figure 2, there was a significant increase in the stereotypy score of the rats in the TS group (3.60 ± 0.63 , $p = 0.000$) compared with the control group. However, treatment with Tia (1.07 ± 0.59 , $p = 0.000$), Gas60 (2.40 ± 0.51 , $p = 0.000$) and Gas120 (1.20 ± 0.56 , $p = 0.000$) substantially decreased the stereotypy score of the rats compared with that of the TS group.

For the nodding experiment, as shown in Figure 3, the nodding numbers of the rats in the TS group (84.80 ± 5.39 , $p = 0.000$) showed a significant increase compared with those of the control group (20.60 ± 3.72). However, treatment with Tia (44.40 ± 4.32 , $p = 0.000$), Gas60 (69.60 ± 5.07 , $p = 0.000$) and Gas 120 (50.20 ± 5.70 , $p = 0.000$) strongly decreased the nodding numbers of the rats compared with those of the TS group.

For the autonomic activity test, as shown in Figure 4A–D, the number of times the rats entered the central area (26.07 ± 11.20 , $p = 0.000$) and the autonomic total distance travelled (43041.51 ± 7831.83 mm, $p = 0.000$) were significantly greater in the TS group than in the control group (10.53 ± 6.29 ; 19570.55 ± 2376.58 mm). However, the number of times the rats entered the central area and the autonomic total distance travelled were markedly decreased after treatment with Tia (12.80 ± 9.02 , $p = 0.000$;

**Figure 2** Effects of Gas treatment on stereotypy score of rats with TS induced by IDPN.

Notes: Stereotypy score was measured as described in materials and methods. Each bar represented the mean \pm SD ($n = 15$). ## $p < 0.01$ vs control group; ** $p < 0.01$ vs TS group.

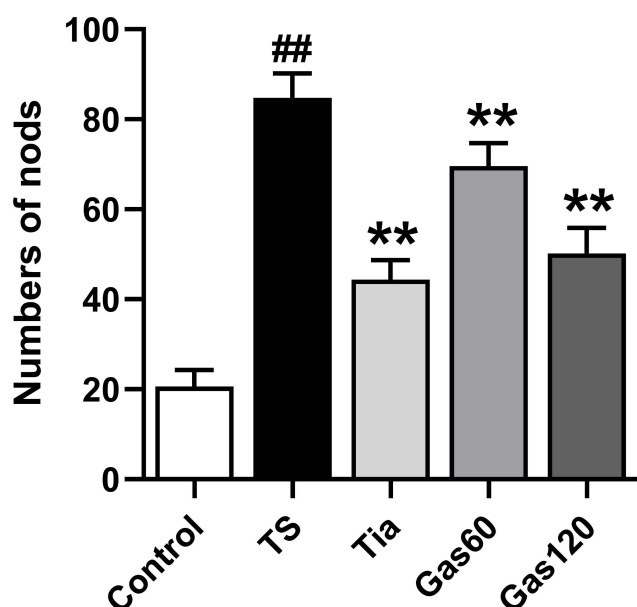


Figure 3 Effects of Gas treatment on nodding numbers of rats with TS induced by IDPN.

Notes: Numbers of nods were measured as described in materials and methods. Each bar represented the mean \pm SD ($n=15$). ## $p<0.01$ vs control group; ** $p<0.01$ vs TS group.

25567.68 \pm 2675.04 mm, $p=0.000$), Gas60 (18.73 \pm 12.35, $p=0.035$; 34630.45 \pm 1802.88 mm, $p=0.009$) and Gas 120 (15.33 \pm 6.17, $p=0.002$; 27815.35 \pm 2389.80 mm, $p=0.000$) compared with those of the TS group. The differences in the central region residence time of the rats in the control, TS, Tia, Gas60 and Gas120 groups were not significant ($p>0.05$).

Effects of Gas on the Glu, GABA and DA Levels in the Striatum of the Rats with TS

As shown in Figure 5A and B, the Glu levels in the TS group (224.46 \pm 12.43 μ g/mL, $p=0.000$) were significantly higher than those in the control group (118.79 \pm 7.39 μ g/mL). However, the Glu levels in the Tia group (154.09 \pm 11.11 μ g/mL, $p=0.000$), Gas60 group (197.01 \pm 9.80 μ g/mL, $p=0.002$) and Gas120 group (172.17 \pm 11.30 μ g/mL, $p=0.000$) were substantially decreased compared with those in the TS group.

As shown in Figure S1A and B, we found increased GABA and DA levels in the TS group (GABA: 45.50 \pm 2.80 μ g/mL, $p=0.000$; DA: 2423.25 \pm 131.86 ng/mL, $p=0.000$) compared with the control group (GABA: 34.13 \pm 2.06 μ g/mL; DA: 1778.75 \pm 90.64 ng/mL). However, the differences in the levels of GABA and DA of the rats in the TS, Gas60 and Gas120 groups were not significant ($p>0.05$).

Effects of Gas on the Protein Expression of EAAT1, EAAT2, NMDAR1, NMDAR2A, and NMDAR2B in the Striatum of the Rats with TS

As shown in Figure 6A–C, the relative protein expression levels of EAAT1 and EAAT2 in the striatum of the rats in the TS group (EAAT1: 0.38 \pm 0.05, $p=0.000$; EAAT2: 0.32 \pm 0.03, $p=0.000$) were obviously lower than those in the striatum of the rats in the control group (EAAT1: 1.00 \pm 0.04; EAAT2: 1.00 \pm 0.01). However, the protein levels of EAAT1 and EAAT2 in the Tia (EAAT1: 0.65 \pm 0.03, $p=0.000$; EAAT2: 0.83 \pm 0.02, $p=0.000$), Gas60 (EAAT1: 0.53 \pm 0.01, $p=0.000$; EAAT2: 0.45 \pm 0.03, $p=0.000$) and Gas120 (EAAT1: 0.86 \pm 0.02, $p=0.000$; EAAT2: 0.67 \pm 0.00, $p=0.000$) groups were significantly increased relative to those in the TS group.

As shown in Figure 6A, D–F, the relative protein expression levels of NMDAR1, NMDAR2A and NMDAR2B in the striatum of the rats in the TS group (NMDAR1: 2.24 \pm 0.12, $p=0.000$; NMDAR2A: 2.33 \pm 0.03, $p=0.000$; NMDAR2B: 4.08 \pm 0.10, $p=0.001$) were markedly higher than those in the control group (NMDAR1: 1.00 \pm 0.06; NMDAR2A: 1.00 \pm 0.02; NMDAR2B: 1.00 \pm 0.03). However, the upward trend was obviously weakened by treatment with Tia (NMDAR1: 1.38 \pm 0.10, $p=0.000$; NMDAR2A: 1.21 \pm 0.02, $p=0.000$; NMDAR2B: 1.84 \pm 0.02, $p=0.002$), Gas60 (NMDAR1: 1.89 \pm 0.06, $p=0.000$; NMDAR2A: 1.75 \pm 0.10, $p=0.000$; NMDAR2B: 2.67 \pm 0.16, $p=0.003$) and Gas120 (NMDAR1: 1.65 \pm 0.04, $p=0.000$; NMDAR2A: 1.34 \pm 0.07, $p=0.000$; NMDAR2B: 1.24 \pm 0.08, $p=0.000$) compared with that of the TS group.

Effects of Gas on the mRNA Expression of EAAT1, EAAT2, NMDAR1, NMDAR2A, and NMDAR2B in the Striatum of the Rats with TS

As shown in Figure 7A and B, there was a downward trend in the relative mRNA expression levels of EAAT1 and EAAT2 in the striatum of the rats in the TS group (EAAT1: 0.33 \pm 0.12, $p=0.000$; EAAT2: 0.37 \pm 0.07, $p=0.000$) compared with the control group (EAAT1: 1.00 \pm 0.14; EAAT2: 1.00 \pm 0.15). However, the decreased mRNA levels of EAAT1 and EAAT2 were significantly altered in the Tia (EAAT1: 0.67 \pm 0.05, $p=0.004$; EAAT2: 0.67 \pm 0.10, $p=0.002$), Gas60 (EAAT1: 0.63 \pm 0.11, $p=0.009$; EAAT2: 0.61 \pm 0.07, $p=0.01$)

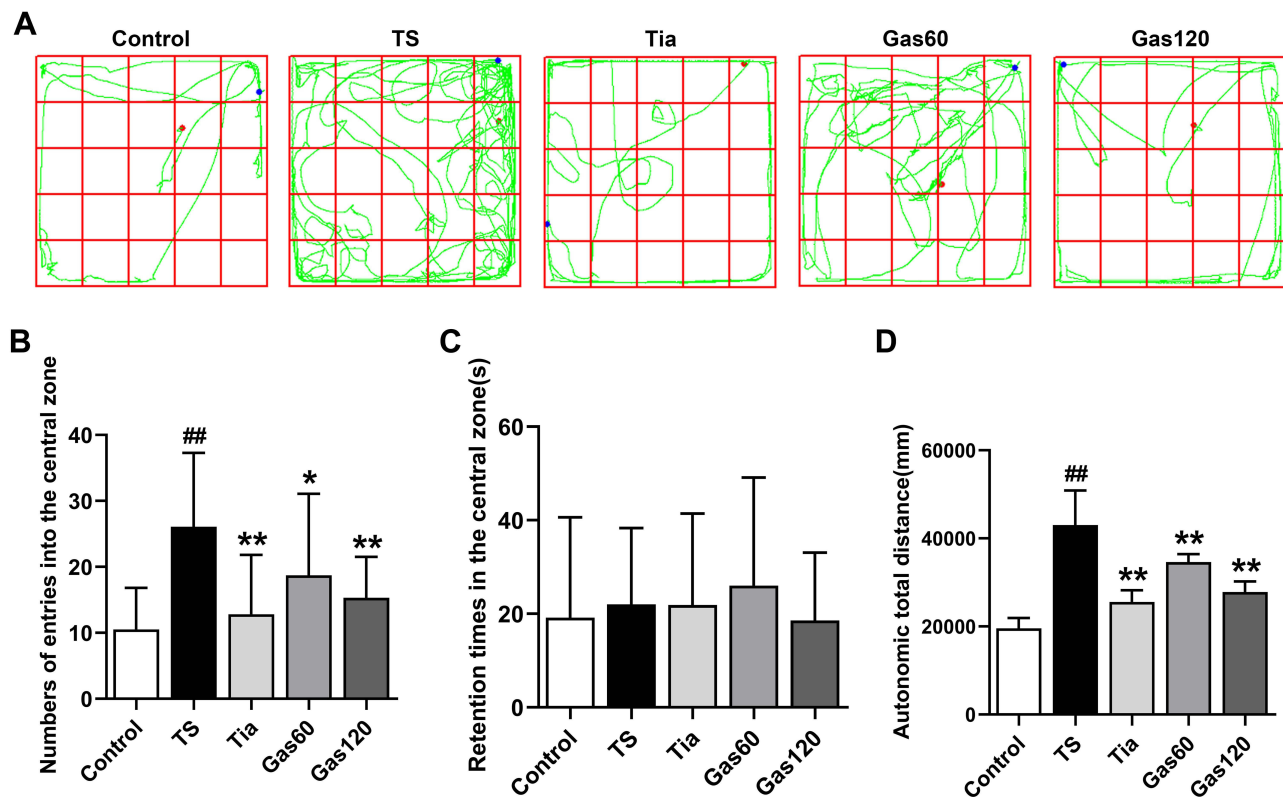


Figure 4 Effects of Gas treatment on autonomic activity of rats with TS induced by IDPN.

Notes: (A) Representative movement trajectory of each group of rats. (B) The number of times to enter the central area of each group of rats. (C) The central region residence time of each group of rats. (D) The autonomic total distance of each group of rats. The movement trajectory, number of times to enter the central area, central region residence time and autonomic total distance were detected as described in materials and methods. Each bar represented the mean \pm SD ($n=15$). ^{##} $p<0.01$ vs control group; ^{*} $p<0.05$, ^{**} $p<0.01$ vs TS group.

and Gas120 (EAAT1: 0.69 ± 0.13 , $p=0.003$; EAAT2: 0.76 ± 0.03 , $p=0.000$) groups relative to the TS group.

As shown in Figure 7C–E, the relative mRNA expression levels of NMDAR1, NMDAR2A and NMDAR2B in the striatum of the rats in the TS group (NMDAR1: 1.62 ± 0.13 , $p=0.000$; NMDAR2A: 1.74 ± 0.09 , $p=0.000$; NMDAR2B: 1.89 ± 0.11 , $p=0.000$) presented an upward trend compared with those of the control group (NMDAR1: 1.00 ± 0.15 ; NMDAR2A: 1.00 ± 0.07 ; NMDAR2B: 1.00 ± 0.21). Treatment with Tia (NMDAR1: 1.09 ± 0.05 , $p=0.000$; NMDAR2A: 1.01 ± 0.08 , $p=0.000$; NMDAR2B: 1.15 ± 0.19 , $p=0.001$) and Gas120 (NMDAR1: 1.29 ± 0.14 , $p=0.009$; NMDAR2A: 1.18 ± 0.18 , $p=0.000$; NMDAR2B: 1.32 ± 0.28 , $p=0.007$) substantially reversed the TS-induced alternation in the mRNA expression of NMDAR1, NMDAR2A and NMDAR2B compared with that of the TS group. There was a significant decrease in NMDAR2A relative mRNA expression in the Gas60 group (1.44 ± 0.12 , $p=0.01$) relative to the TS group. However, the differences between the TS group and the Gas60 group in NMDAR1 and NMDAR2B mRNA relative expression were not significant ($p>0.05$).

Discussion

The purpose of this study was to investigate the anti-tic effect of Gas on rats with TS induced by IDPN and explore the potential mechanisms underlying these anti-tic effects. We found that Gas could alleviate the behavioral symptoms of rats with TS induced by IDPN including stereotypy score, nodding numbers, and autonomic activity. More importantly, the Gas mediated amelioration of the behavioral dysfunction of rats with TS might be associated with the maintenance of Glu at a normal level, upregulation of the expression of EAAT1 and EAAT2, and downregulation of the expression of NMDAR1, NMDAR2A and NMDAR2B.

The choice of animal model plays a crucial role in the study of new therapeutic strategies and the pathogenesis of diseases. IDPN is a neurotoxin that is commonly used to establish TS animal models.³⁰ In this study, stereotypy scoring, nodding experiment, and autonomic activity test were conducted to access the severity of behavioral symptoms of rats with TS induced by IDPN. The stereotypy

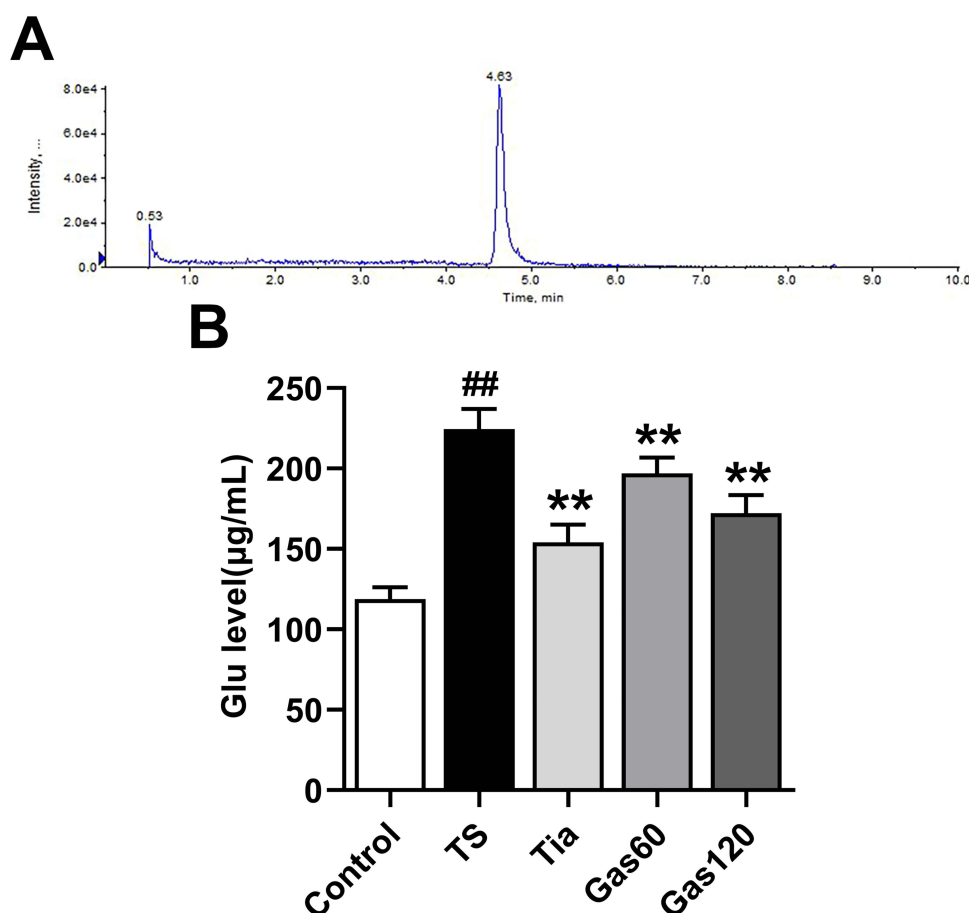


Figure 5 Effects of Gas treatment on Glu level in the striatum of the rats with TS induced by IDPN.

Notes: (A) Representative extracted chromatograms of Glu. (B) The Glu level of each group of rats. Glu level was measured as described in materials and methods. Each bar represented the mean \pm SD ($n=4$). ### $p<0.01$ vs control group; ** $p<0.01$ vs TS group.

score and nodding numbers can objectively reflect the severity of tics. The autonomic activity test can reflect the exploration characteristics of each rat and can be used to quantitatively evaluate the excitability, exploration behavior, and spontaneous behavior of rats.³¹ Here, we found that injection with IDPN remarkably increased the stereotypy score, nodding numbers, number of times to enter the central area, and total movement distance of the rats compared with those of the control group and produced persistent behavioral syndrome imitating TS. Tiapride (Tia) is a selective dopamine receptor (D2R) antagonist that has been proved to antagonize the symptoms of dyskinesia caused by injection of dopamine in the striatum.³⁰ Tia has been used to treat TS for many years and possesses anti-tic effects, as reported in clinical and experimental studies.^{32,33} Therefore, Tia was used as a positive control drug for a comparison with the effect of Gas on TS. Here, we found that Gas and Tia showed a significant improvement in stereotypy score, nodding

numbers, number of times to enter the central area, and the total movement distance of the rats with TS, which indicated that both Gas and Tia could ameliorate the severity of behavioral symptoms in the rats with TS. Our findings are consistent with the results of previous studies showing that Gas and Tia have similar therapeutic effects on the behaviors of rats with TS.^{24,25}

Currently, an increasing number of studies have demonstrated that malfunction of CSTS circuits, which are involved in cognitive, sensorimotor, and emotional functions, may be related to the development of a variety of neuropsychiatric diseases, including TS.^{34,35} Abnormal amino acid neurotransmitters, such as Glu and GABA, involved in the CSTS circuits, are closely associated with the occurrence of TS.^{13,14} GABA is the primary inhibitory neurotransmitter in the CNS of mammals. Puts et al found reduced GABA concentrations in patients with TS and, importantly, that there was a negative correlation between GABA concentration and

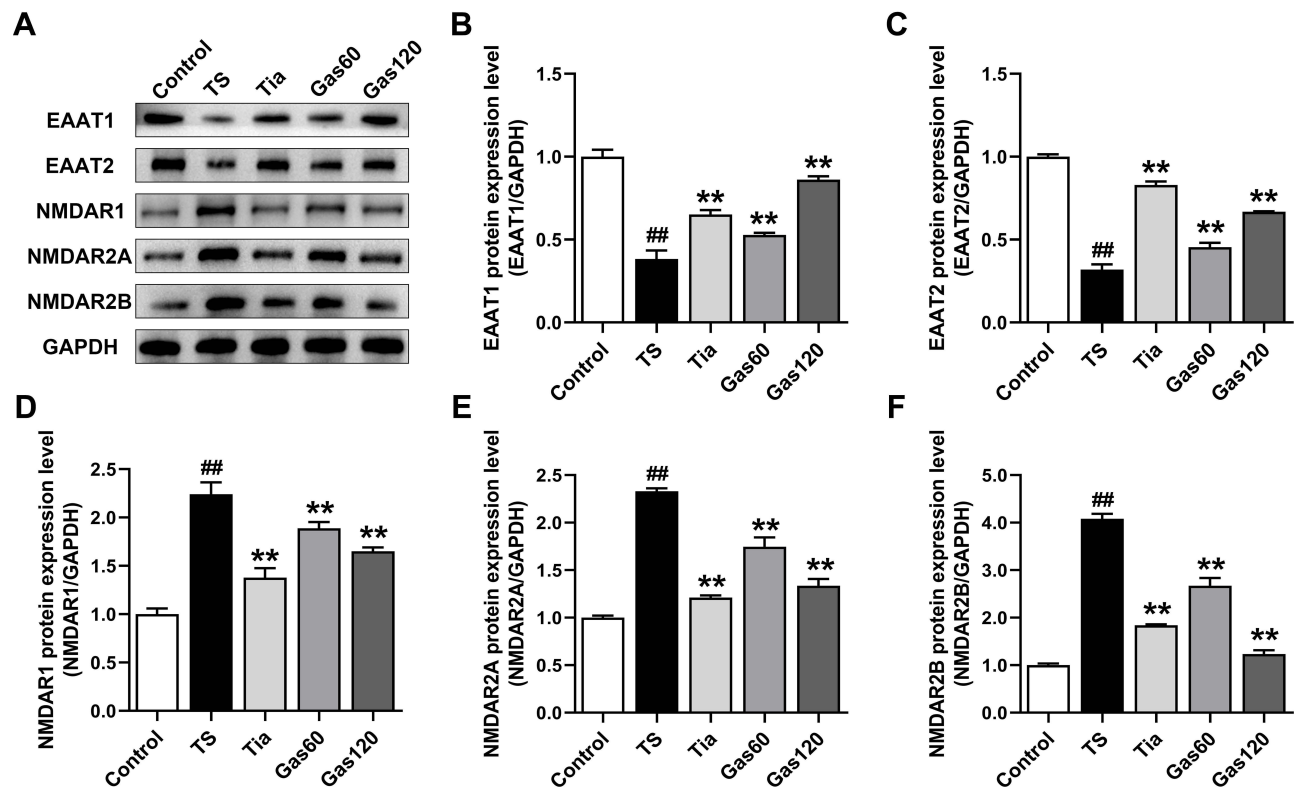


Figure 6 Effects of Gas treatment on the protein expression of EAAT1, EAAT2, NMDAR1, NMDAR2A and NMDAR2B in the striatum of the rats with TS induced by IDPN. **Notes:** (A) Representative protein bands of EAAT1, EAAT2, NMDAR1, NMDAR2A and NMDAR2B of each group of rats in the striatum. (B) The protein expression of EAAT1 in the striatum detected by Western blot. (C) The protein expression of EAAT2 in the striatum detected by Western blot. (D) The protein expression of NMDAR1 in the striatum detected by Western blot. (E) The protein expression of NMDAR2A in the striatum detected by Western blot. (F) The protein expression of NMDAR2B in the striatum detected by Western blot. The levels of these proteins were measured as described in materials and methods. Each bar represented the mean \pm SD. ### p < 0.01 vs control group; ** p < 0.01 vs TS group.

tic severity.³⁶ However, our study demonstrated that administration of IDPN caused significantly increased GABA concentrations in the striatum of the rats compared with those of the controls. Although our results were contradictory to the findings of Puts et al, they were consistent with the findings of Chen et al, in which increased GABA concentrations was observed in IDPN-treated rats.³⁰ The increased GABA concentration in the striatum, as observed in the present and previous studies, was possibly due to an adaptive response to inhibit the hyperactivity that caused repetitive and involuntary motor in rats with TS. In addition, our results demonstrated that the increased GABA levels could not be decreased by Gas treatment. Therefore, GABA might not participate in the mechanism of drug action of Gas. Glu is the primary excitatory neurotransmitter in the mammalian brain, and it is associated with essential brain functions.³⁷ Numerous neurological disorders, such as neurodegenerative diseases, epilepsy, cerebrovascular diseases, and TS, are closely related to abnormal

concentrations of Glu.³⁷ It is widely accepted that Glu shows an increasing trend in patients with TS, resulting in an overexcited state in the CNS. As reported in previous studies, the levels of Glu in patients were significantly increased, as evaluated by magnetic resonance measurements.¹⁶ When the excess release of Glu is inhibited, it can effectively ameliorate the development of TS and its complications. Animal studies showed that there was an increase in the levels of Glu in the striatum of TS model rats.³⁸ Interestingly, consistent with previous studies, our study demonstrated that the Glu levels in the striatum showed a significant increase after injection with IDPN compared with that of the control group. Furthermore, our results showed that treatment with Gas and Tia could efficiently decrease the Glu levels in the striatum. Moreover, the alleviation of the severity of behavioral symptoms in the rats with TS treated with Gas was paralleled by a prominent decrease in Glu levels in the striatum. This study confirmed that a high level of Glu was closely related to the pathogenesis of TS.

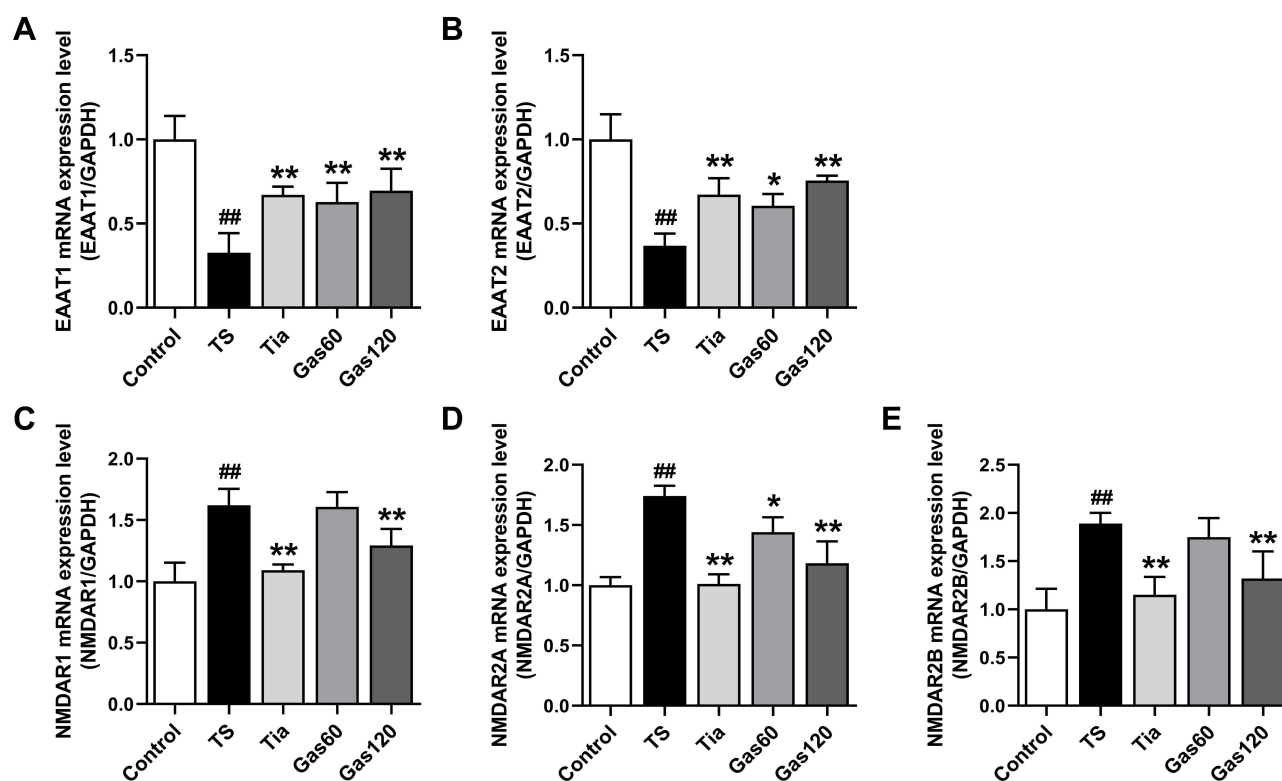


Figure 7 Effects of Gas treatment on the mRNA expression of EAAT1, EAAT2, NMDAR1, NMDAR2A and NMDAR2B in the striatum of the rats with TS induced by IDPN. **Notes:** (A) The mRNA expression of EAAT1 in the striatum detected by qRT-PCR. (B) The mRNA expression of EAAT2 in the striatum detected by qRT-PCR. (C) The mRNA expression of NMDAR1 in the striatum detected by qRT-PCR. (D) The mRNA expression of NMDAR2A in the striatum detected by qRT-PCR. (E) The mRNA expression of NMDAR2B in the striatum detected by qRT-PCR. The expressions of these mRNAs were measured as described in materials and methods. Each bar represented the mean \pm SD. ### $p < 0.01$ vs control group; * $p < 0.05$, ** $p < 0.01$ vs TS group.

The excessive accumulation of extracellular Glu indicated that the clearance of Glu might be disrupted in rats with TS induced by IDPN. EAATs, which can mediate the clearance of Glu, include a class of five transporters with different expression in neurons and glia cells.¹⁷ When Glu is released into the synaptic cleft, it is rapidly transferred out of the extracellular space by EAATs to prevent the excessive accumulation of Glu. It is a crucial functional element of glutamatergic synapses that EAATs take up Glu to maintain a suitable concentration of Glu in the synaptic cleft. Up to 80% of extracellular Glu is transferred out of the extracellular space through EAAT1 and EAAT2, which play a key role in the uptake of Glu, termination of excitability signals and neuronal damage protection.¹⁷ The dysfunction of EAAT1 and EAAT2 may lead to excessive accumulation of extracellular Glu and further activation of Glu receptors and excitatory transmission, which causes excitotoxicity of neurons and the occurrence of various neuropsychiatric disorders, including TS. Previous studies found increased Glu concentration and decreased expression of EAAT1 in the striatum of rats with TS induced by IDPN relative to those

of the control group.³⁸ Consistent with previous studies, our results showed that IDPN injection caused a significant increase in Glu concentration and a decrease in the protein and mRNA expression of EAAT1 and EAAT2 in the striatum compared with those of the control group. In the present study, the decreasing EAAT1 and EAAT2 expression suggested that the clearance of Glu was weakened in the rats with TS, leading to excessive accumulation of Glu. Thus, the inhibition of EAAT1 and EAAT2 might underlie the behavioral symptoms observed in the rats with TS induced by IDPN. In addition, we found an increase in EAAT1 and EAAT2 expression after treatment with Gas and Tia. Our results suggested that Gas could ameliorate tic-like symptoms by upregulating the expression of EAAT1 and EAAT2 to increase the clearance of Glu and maintain the concentration of extracellular Glu at a suitable level. These findings revealed that EAAT1 and EAAT2 might participate in the mechanism of Gas drug action.

Among Glu receptors, NMDARs have been proved to be associated with the occurrence of TS. NMDARs, which are widely distributed in the mammalian brain, constitute a main

class of Glu receptors.³⁹ NMDARs consist of NMDAR1, NMDAR2 and NMDAR3 subunits, and the physiology of NMDARs is the basis of brain development and function.³⁹ NMDARs can mediate glutamatergic neurotransmission and play a key role in several basic functions in the CNS, such as the regulation of neurodevelopment and synaptic plasticity, excitotoxicity, cognitive processes, and learning and memory formation.¹⁸ NMDARs are closely related to the development of several neuropsychiatric disorders, including neurodegenerative disorders, autism, traumatic brain injury, and affective disorders.^{40,41} Neurons release Glu to the synaptic cleft, and extracellular Glu activates Glu receptors (including NMDARs) located in neurons and glial cells to regulate brain excitability. Nevertheless, under pathological conditions, excessive accumulation of extracellular Glu can over-activate NMDARs and lead to excitotoxicity, resulting in various neuropsychiatric disorders. As reported in a previous study, a significant increase in the stereotypy score and total movement distance in rats with TS induced by IDPN was paralleled by prominently increased Glu levels and over-activated NMDAR1 relative to those of the control group.⁴² In this study, we found that the expression of NMDARs including NMDAR1, NMDAR2A and

NMDAR2B were increased after IDPN injection, which might be the reason for the increases in the stereotypy score, nodding numbers, number of times to enter the central area and total movement distance. The Western blot and qRT-PCR findings confirmed that excessive accumulation of extracellular Glu could over-activate NMDAR1, NMDAR2A and NMDAR2B. In addition, our study showed that Gas treatment could depress the over-activated NMDAR1, NMDAR2A and NMDAR2B, suggesting that NMDAR1, NMDAR2A and NMDAR2B might participate in the mechanism of Gas drug action.

Gas is one of the major bioactive components isolated from the *Rhizoma Gastrodiae*. It has been reported that Gas has beneficial effects on many CNS diseases including TS.^{23–25} Here, we found that Gas could ameliorate tic-like symptoms, including stereotypy score, nodding numbers, number of times to enter the central area and the total movement distance of the rats with TS induced by IDPN, which were consistent with previous research.^{24,25} In the present study, we further elaborated the anti-tics effects of Gas on the rats with TS induced by IDPN and the potential mechanism. Our results confirmed that Gas could decrease the Glu concentration in the

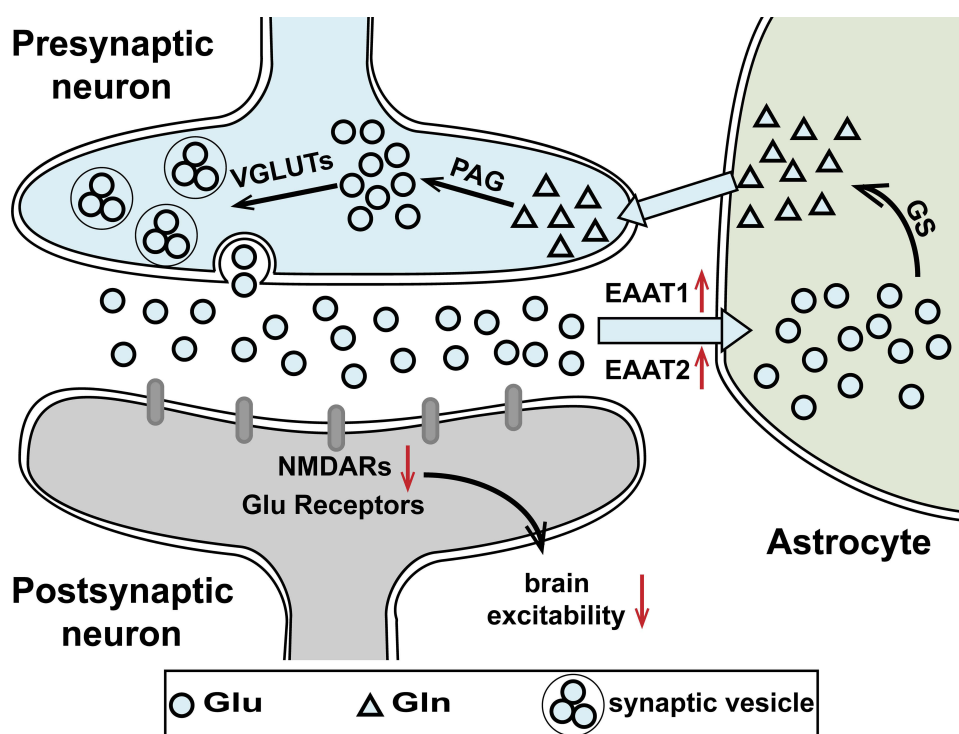


Figure 8 A schematic illustration of the proposed mechanism by which Gas alleviates Tourette syndrome in rats.

Notes: The red arrows indicate the possible mechanism of Gas. Gas might maintain the concentration of extracellular Glu at a suitable level by upregulating the expression of EAAT1 and EAAT2 to increase the clearance of Glu. The decreased Glu level might inhibit over-activation of NMDAR1, NMDAR2A and NMDAR2B and depress glutamatergic neurotransmission and brain excitability.

Abbreviations: Glu, glutamate; Gln, glutamine; EAAT1, Glutamate aspartate transporter (GLAST); EAAT2, Glial glutamate transporter (GLT-1); NMDARs, N-methyl-D-aspartate receptors; VGLUTs, vesicular glutamate transporters; GS, Glutamine Synthetase; PAG, phosphate activated glutaminase.

striatum of the rats with TS induced by IDPN, which was consistent with the results using high performance liquid chromatography shown by previous research.²⁴ We also found that the effect of Gas on decreasing extracellular Glu concentration was closely related to the downregulation of EAAT1 and EAAT2 expression. When the extracellular Glu concentration is suitable level, NMDARs might not be over-activated. Therefore, our findings showed a significant decrease in the protein and mRNA expression of NMDAR1, NMDAR2A and NMDAR2B after treatment with Gas. Together, these findings indicated that TS rats induced by IDPN could lead to tic-like symptoms, an increase in Glu concentration in the striatum, a decrease in EAAT1 and EAAT2 expression and an increase in NMDAR1, NMDAR2A and NMDAR2B expression, which could be altered by Gas treatment. EAAT1, EAAT2, NMDAR1, NMDAR2A and NMDAR2B might participate in the mechanism of Gas drug action. Our findings provide a pharmacological basis for Gas as a clinical treatment for patients with TS. A schematic illustration of the proposed mechanism for Gas to alleviate TS rats is shown in Figure 8.

However, this study also has some limitations. First, there are differences between rats and humans, so further trials conducted on patients with TS can provide more clinical information for TS treatment. Second, in addition to glutamatergic neurotransmission, other pathways may have a crucial effect on the development of TS. These questions need to be explored in further research.

Conclusion

Gas treatment might be effective in improving the behavioral dysfunction of rats with TS induced by IDPN. This beneficial effect of Gas was closely related to the decreased Glu concentration, upregulated expression of EAAT1 and EAAT2, and downregulated expression of NMDAR1, NMDAR2A and NMDAR2B in the striatum of rats with TS after Gas treatment, which could depress glutamatergic neurotransmission and brain excitability. These effects might be the reason that Gas treatment could improve the severity of behavioral symptoms of rats with TS induced by IDPN. Taken together, our findings provide a pharmacological basis for the advantageous effects of Gas treatment for patients with TS.

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Disclosure

The authors report no conflicts of interest in this work.

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