Original Article

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Dual regulating effect of Ningdong granule on extracellular dopamine content of two kinds of Tourette's syndrome rat models

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Summary **Tourette's syndrome (TS) is an inherited chronic neuropsychiatric disorder characterized by involuntary stereotyped motor and phonic behaviors called tics. Its pathogenesis is still unclear and its treatment remains limited. Our previous basic and clinical studies have shown that traditional Chinese medicine (TCM) preparation Ningdong granule (NDG) is effective for the treatment of TS with little side effects. In the current study, two TS rat models (Apomorphine (Apo)- and 3,3'-iminodipropionitrile (IDPN)-induced) were used to explore the dual regulating effects and mechanisms of NDG on extracellular DA concentration. We found that NDG could regulate the extracellular DA concentration dually: it could make a gradual recovery in extracellular DA content from both an up-regulated level in Apo-induced rats and down-regulated level in IDPN-induced rats measured by high-performance liquid chromatography (HPLC). The protein expression of DA transporter (DAT) was measured by Western blot and the result showed that NDG could elevate DAT expression when DA release was up-regulated and could decrease DAT expression when extracellular DA concentration was down-regulated. The main mechanism of the dual regulating effect of NDG on extracellular DA release might be related to DAT protein expression in TS, through which the released DA is re-uptaken into nerve terminals. Taken together, compared with conventional single-target anti-tics drugs such as haloperidol (Hal), NDG with the dual regulating effect would be more significant for TS treatment.**

Keywords: Tourette's syndrome (TS), dual regulating effect, Ningdong granule (NDG), dopamine hypothesis, dopamine transporter

1. Introduction

Tourette's syndrome (TS) is an inherited chronic neuropsychiatric disorder characterized by involuntary stereotyped motor and phonic behaviors called tics. Initial symptoms of TS usually start in childhood with a peak age between 7 to 15 years old (*1*). The prevalence of this syndrome is estimated to be four to six per 1,000 children and adolescents with an incidence in males 3-9 times higher than in females (*2*). TS can cause lifelong impairment and about 5% of TS patients have life-threatening symptoms including mild self-injurious behaviors and borderline personality disorders (*3*).

Currently, the detailed etiological and pathophysiological mechanism of TS is still unclear. It is widely believed that abnormalities of dopamine (DA) neurotransmission play a primary role in the pathophysiology of TS (*4*). DA content in postmortem striatum of neurologically normal subjects increased two to three fold from birth to 9 years of age, and then gradually declined from a probable peak in late adolescence (*5*), which is concordant with the natural history of TS. DA is a

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key monoamine neurotransmitter released by nerve terminals originating from midbrain neurons. It plays an important role in the regulation of motor and limbic functions through stimulation of DA receptors, such as movement, learning, moods, neurobehavioral abilities, problem solving, and so on (*6,7*). TS is reported to be correlated with the content and activity of DA, and the density and sensitivity of DA receptors in striatum. However, the relationship between the pathophysiology of TS and DA is far from been clearly elucidated. Interestingly, in some pathological and functional imaging studies the increased DA release in TS patients has been confirmed, while other investigations of the striatal DA content have either failed to find differences between normal controls and TS patients or have yielded contradictory results (*8-10*). Furthermore, the available dopaminergic system related TS agents, including the classical neuroleptics with D2 receptors antagonistic activity, are all considered as decreasing "the effects of DA release" in striatum, while a number of DA agonists (*e.g.*, levodopa) with the effect of promoting DA release have been shown to suppress tics (*8,11,12*). These seemingly conflicting findings suggest that, DA release, either in an increased or decreased manner, would play a role in the generation of TS (*8*).

Dopamine transporter (DAT) is a high-affinity glycoprotein localized exclusively at the presynaptic membrane of DA neurons. It is responsible for modulating extracellular DA concentration by re-uptaking the released DA into nerve terminals (*4*). In recent years, some findings using molecular, pharmacological and genetic techniques have established the importance of DAT in the maintenance of DA homeostasis, which might be an essential role for the control of normal brain function (*8*). Furthermore, the activity of DAT in regulation of DA homeostasis might underlie subsequent pathological states in TS (*4,13*). All these might provide us a new way for TS management.

Haloperidol (Hal) is approved by the Food and Drug Administration (USA) for treating TS. It can effectively inhibit the excitability of the cortical motor area through restraining the activity of DA receptors (*14*). However, a very high proportion of patients eventually refused further therapy with Hal because of the side effects including sedation, weight gain, extrapyramidal symptoms, and QT prolongation (*7*). Therefore, development of novel drugs for treatment of TS is urgently needed.

Traditional Chinese medicine (TCM) has been widely used in the treatment of various diseases such as nervous system disease, infectious diseases, and cancer, in China, Japan, South Korea and other Asian countries for thousands of years (*13,15*). Ningdong granule (NDG), a TCM preparation dedicated to treating TS with the guidance of therapeutic principles of TCM, has been used as an anti-tic agent in clinics in China for several years (*16-18*). Our previous studies showed that NDG has had a total effective rate of 73.3-83.3% in TS patients with few apparent side or toxic effects (*16,18*). Our previous studies also showed that NDG could regulate the disturbance of DA, DRD2, 5-TH and so on in animals and patients with TS (*7,14*). However, the possible pharmacological mechanism of NDG for treating TS is still unclear. Thus, in the current study, two TS rat models (Apomorphine (Apo)- and 3,3'-iminodipropionitrile (IDPN)-induced) were used to explore the regulating effects and possible mechanisms of NDG on extracellular DA concentration.

2. Materials and Methods

2.1. *Reagents*

Apomorphine (Apo), 3,3-iminodipropionitrile (IDPN) and bovine serum albumin (BSA) was purchased from Sigma-Aldrich Co. Ltd. (USA), and Haloperidol (Hal) was purchased from Shanghai Pharmaceutical Group Co. Ltd. (Shanghai, China). Primary antibody against DAT was bought from Abcam (Cambridge, MA, USA). Enhanced chemiluminescence agents (ECL) were bought from Millipore Co. (Billerica, MA, USA). Peroxidase-conjugated affini-pure goat anti-rabbit antibody IgG and anti β-actin antibody were purchased from ZSGB-BIO Inc. (Peking, China).

2.2. *NDG Preparation*

NDG was provided by 999 Modern Chinese Medicine Co. Ltd. (999 Co. Ltd., Shenzhen, China). NDG formulation includes main components (*18*): Rhizoma Gastrodiae, Codonopsis pilosula, Dwarf lilyturf tuber, Radix Paeoniae Alba, Fossil fragments, Oyster shell, and Pheretima asiatica. The proportions of these eight components in NDG granule were 2:3:2:4:5:5:2:2. After being mixed in proportion, all of them were macerated with distilled water for 1 h at room temperature, and the whole mixture was decocted twice for 30min each time. The filtrates were mixed and condensed and then dried by vacuum-drier at 60°C. The yield granule was stored at 4°C.

2.3. *Experimental Animals*

Seventy male Wistar rats (4 weeks old, 100 ± 20 g) were bought from Shandong Experimental Animal Center (Jinan, China), and were housed in an air-conditioned animal room with 12 h light/dark cycle, temperature of 22 ± 2°C and humidity of 50 ± 10%. Rats were constantly provided with a laboratory diet and water *ad libitum*.

After a week, the rats were randomly divided into control group (n = 10), Apo-induced TS model group (n = 30) and IDPN-induced TS model group (n = 30).

Rats in the Apo-induced TS model group and IDPN-induced TS model group were intraperitoneally injected (*i.p.*) with Apo (2mg/kg) and IDPN (150mg/kg, *i.p.*) respectively, while the control group received normal saline (NS) (5 mL/kg, *i.p.*). Both Apo-induced and IDPN-induced TS model groups were further divided into 3 groups, that is Apo + NS group (n = 10), Apo + NDG group (n = 10), Apo + Hal group (n = 10); IDPN + NS group (n = 10), IDPN + NDG group (n = 10), IDPN + Hal group (n = 10), and the rats were administered normal saline by gastric perfusion (0.9%) at 10 mL/kg (Control group, Apo + NS group and IDPN + NS group), NDG at 370 mg/kg (Apo + NDG group and IDPN + NDG group), and Hal at 1.0 mg/kg (Apo + Hal group and IDPN + Hal group) respectively once a day for 8 weeks (*8,14*).

All animal experimental protocols were handled in accordance with the Code of Ethics of the World Medical Association, and all research procedures were approved by medical ethics committee of Provincial Hospital Affiliated to Shandong University.

2.4. *Behavior recordings*

Stereotypy actions of rats were recorded according to the evaluating standards of stereotypic actions (*19*) (Table 1). These were conducted once a week by trained observers who were blind to the group condition. Each animal was observed for one min of every 5 min for a total of 6 observation periods. One or more episodes recorded with the grades received the corresponding score. The average scores were calculated on the basis of results from observers as the objective indicator of behavioral changes.

2.5. *Intracranial surgery*

The procedure of current section was conducted according to our previous study (*8*). In brief, for surgical implantations, the rats were anesthetized with chloral hydrate (400 mg/kg, *i.p.*) and fixed in a stereotaxic instrument. A guide cannula was implanted in striatum (AP: 0.0, ML: +2.0, DV: -7.0mm from bregma) and secured with two stainless steel skull screws and dental cement. Rats were then singly housed and allowed to recover for one day before microdialysis.

On the day of microdialysis, a polyarylene-ether-sulfone MAB/6 probe (4 mm in length, 15 kDa molecular weight cut off) was slowly lowered into the position of striatum as mentioned above and perfused at 2 μL/min using 0.9% normal saline. Before experimental sampling began, the rats were perfused with saline for 2 h to maintain equilibrium of fluid exchange. Subsequently, the dialyzate samples (20 μL) were collected every 20 min. The microdialysis samples were stored at -80°C until analyzed.

2.6. *HPLC analysis*

The dialyzate samples from the striatum were analyzed by high-performance liquid chromatography with electrochemical detection (HPLC-ECD) according to the previous reported methods (*8*). The levels of extracellular DA and HVA content in striatum were detected use this method. Compound separation was achieved on a C18 reverse-phase analytical column (50 mm × 2.1 mm, 1.9 μm particle size) with a mobile phase consisting of 150 mM citric acid, 150 mM trisodium citrate dihydrate, 100 mM ethylenediamine tetraacetic acid disodium salt (EDTA • 2Na), 1 mM sodium 1-heptanesulfonate and 10% methanol (v/v). The mobile phase was passed through the system at 0.2 mL/min, while the column was maintained at 28°C and the potential of the electrochemical detector was set at 0.8 V.

2.7. *Western blot analysis*

After the intracranial microdialysis, rats were sacrificed and striatum tissues were dissected and quickly homogenized on ice in RIPA lysis buffer (50 mM Tris-Hcl (PH 7.4), 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS)) with protease inhibitors (PMSF) and centrifuged at 12,000 rpm for 15min at 4°C. After determination by BCA protocol, the Protein amounts in the supernatant were diluted in 5× loading buffer and then boiled at 100°C for 5 min. SDS polyacrylamide gel (SDS-PAGE) electrophoresis was carried out on 10% Tris-glycine gels. The separated proteins were then electrophoretically transferred to PVDF membranes (0.45 μm) that were treated previously with methanol, and blocked with 1% BSA in TBS-T (Tris-buffered saline containing 0.1% Tween 20) for 1 hour at room

Table 1. Scales for stereotypic behaviors

Score	Stereotypic behaviors
0	Asleep, resting in place or normal activity in place
1	Increased sniffing and head raising
2	Discontinuous increased sniffing with body raising
3	Discontinuous increased sniffing, licking with head and body raising primarily in one place, with occasional rapid burst of locomotor activity (2-5 steps)
4	Continuous sniffing, biting, head bobbing and repetitive body raising/wall climbing in place
5	Continuous sniffing, biting, licking, head bobbing, and continuous body raising/wall climbing wherein forepaws do not touch cage floor

temperature. After washing in TBS-T, the membranes were incubated with primary antibody against DAT (1:1,000) overnight at 4°C, and then were further incubated with peroxidase-conjugated affini-pure goat anti-rabbit antibody IgG (1:10,000) for 1 h at room temperature. Blots were developed with enhanced chemiluminescence agents before exposure to X-ray film. To confirm equivalent loading of samples, the same membranes were incubated with anti β-actin antibody (1:1,000) and visualized *via* enhanced chemiluminescence as mentioned earlier.

2.8. *Statistical analysis*

Data were expressed as mean ± S.D. and statistical differences between groups were determined by one-way analysis of variance (ANOVA). Repeated-measure analysis of variance was used to analyze the differences of the stereotypic scores of the rats. The data were analyzed using the SPSS® statistical package, version 16.0 (SPSS Inc., Chicago, IL, USA) for Windows®. A two-tailed significance level of $p < 0.05$ was used for all statistical analyses.

3. Results

3.1. *Assessment of stereotypic behaviors*

Administrations of both Apo and IDPN could produce significant stereotypic multiple behaviors in rats ($p < 0.01$). After being treated with NDG and Hal respectively, the dyskinetic-hyperkinetic syndromes in Apo-induced rats were dramatically improved, and there were no remarkable differences between the two treatments ($p > 0.05$). Moreover, in the IDPN-induced rats, which received the same therapies as Apo-induced rats, the general tendency of stereotypic behavioral improvements in the NDG groups showed no differences compared with the Apo groups. In conclusion, both NDG and Hal could make significant improvements in stereotypic abnormalities of rats with no distinctive differences between these two treatments (Figures 1A and 1B).

3.2. *Levels of extracellular DA content in striatum*

The two pharmacological manipulations used to induce stereotypic behaviors of rats caused completely opposite effects on extracellular DA content in the striatum of the rats. There was a dramatic increase in extracellular DA content in Apo + NS group ($p < 0.01$), while in IDPN + NS group the extracellular concentration of DA was found decreased significantly ($p < 0.01$), when both were compared with the control group. Furthermore, the dual regulating effect of NDG on extracellular DA content in striatum was found in the current study. It could down-regulate elevated DA content in Apo-induced rats (Apo

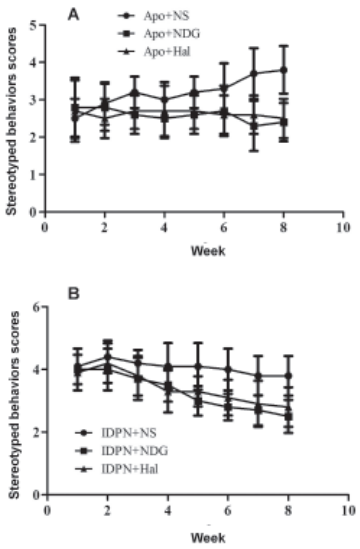

Figure 1. Stereotypic behavior of rats in the three experimental groups during an 8-week period. The data represent mean ± S.D. ($n = 10$). Administration of both Apo **(A)** and IDPN **(B)** could produce significant stereotypic multiple behaviors in rats ($p < 0.01$). The stereotypic behavior scores at baseline showed no differences among groups ($p > 0.05$). After being treated respectively with NDG and Hal, both dyskinetic-hyperkinetic syndrome scores in Apo-induced and IDPN-induced rats decreased significantly ($p < 0.01$).

+ NDG group) ($p < 0.05$ *vs*. Apo + NS group) while up-regulate decreased DA content in IDPN-induced rats (IDPN + NDG group) ($p < 0.01$ *vs*. IDPN + NS group) by moving them towards a normal extracellular DA level. However, in the Hal treated groups, no significant differences were found in the concentration of extracellular DA after the treatment (Apo + Hal group *vs*. Apo + NS group, $p > 0.05$; IDPN + Hal group *vs*. IDPN + NS group, $p > 0.05$) (Figures 2A and 2B).

3.3. *Levels of extracellular HVA content in striatum*

The effects of DNG on the extracellular HVA concentration in striatum of the rats are shown in Figure 3. Compared to the control group, neither NDG nor Hal caused any conspicuous changes in extracellular HVA content in Apo-induced rats ($p > 0.05$) (Figure 3A). Furthermore, in IDPN-induced rat groups, no significant differences were found in extracellular HVA content either, whether they were treated by NDG or by Hal ($p > 0.05$) (Figure 3B).

3.4. *DAT protein expression in striatum*

The effects of NDG on DAT protein expression in striatum of the rats were detected by *Western blot* as shown in Figure 4. Both of the pharmacological manipulations, Apo and IDPN, could significantly increase DAT protein expression in striatum compared with the control group. The treatment of NDG caused totally opposite effects on DAT protein expression in striatum: there was a dramatic decrease in DAT protein

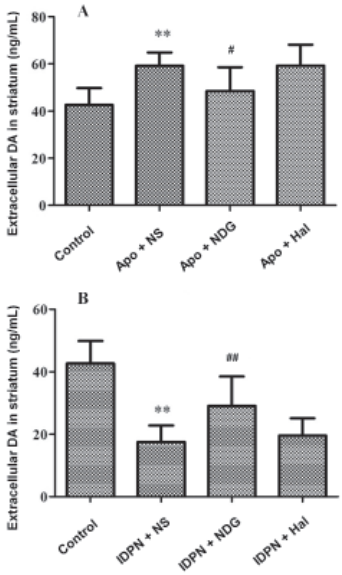

Figure 2. Levels of extracellular DA content in striatum. The data represent mean ± S.D. (n = 10). **(A)** Effects of NDG on extracellular DA content in the striatum of the Apo-induced rats. There was dramatic increase in extracellular DA content in Apo + NS group ($^{**}p < 0.01$ *vs*. control group). After being treated with NDG, the content of extracellular DA decreased ($^{\#}p < 0.05$ *vs*. Apo + NS group). **(B)** Effects of NDG on extracellular DA content in the striatum of the IDPN-induced rats. There was a dramatic decrease in extracellular DA content in IDPN + NS group ($^{**}p < 0.01$ *vs*. control group). After being treated with NDG, the content of extracellular DA increased significantly ($^{\#\#}p < 0.01$ *vs*. IDPN + NS group).

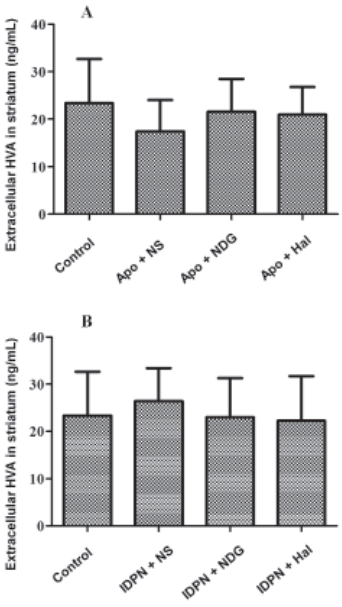

Figure 3. Levels of extracellular HVA content in striatum. The data represent mean ± S.D. (n = 10). Compared with the control group, neither NDG nor Hal made any conspicuous changes in extracellular HVA content of Apo-induced and IDPN-induced rats ($p > 0.05$).

expression in Apo + NDG group ($p < 0.01$), while in IDPN + NDG group, DAT protein expression was found elevated significantly ($p < 0.01$). However, in the Hal treated groups, no significant difference was found in the concentration of DAT after the treatment (Apo + Hal

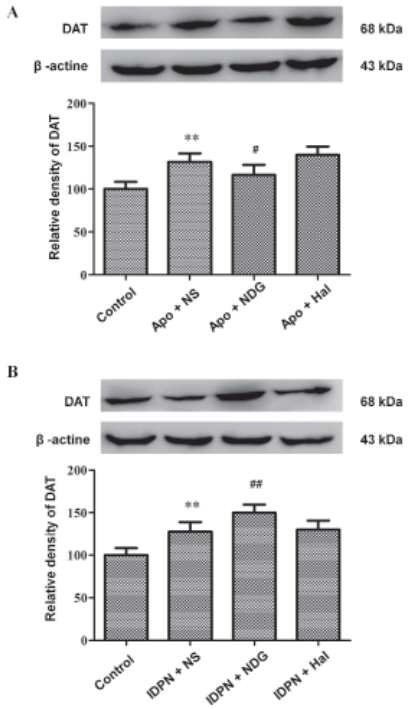

Figure 4. DAT protein expression in striatum. Both of the pharmacological manipulations, Apo and IDPN, could significantly increase DAT protein expression in striatum ($^{**}p < 0.01$ *vs*. control group). The treatment of NDG caused totally opposite effects on DAT protein expression in striatum: there was a dramatic decrease in DAT protein expression in Apo + NDG group ($^{\#}p < 0.05$ *vs*. Apo + NS group), while in IDPN + NDG group, DAT protein expression was found elevated significantly ($^{\#\#}p < 0.01$ *vs*. IDPN + NS group). Hal made no conspicuous changes in DAT protein expression in striatum of Apo-induced and IDPN-induced rats.

group *vs*. Apo + NS group, $p > 0.05$; IDPN + Hal group *vs*. IDPN + NS group, $p > 0.05$) (Figures 4A and 4B).

4. Discussion

The TCM preparation NDG has been used as an anti-tic agent for several years in clinics in China, which mainly includes 8 Chinese herbal medicines as described in the previous section. According to TCM theory, NDG is well known to have effects on nourishing heart, soothing liver, and relieving convulsion and spasm (*16,17*). Pharmacological studies have found that NDG contains a number of active substances such as saponin (*e.g.*, gastrodin and paeoniflorin), steroid saponin, carbohydrates and their glycosides, alkaloids, organic acids, and flavonoids, which have been proved to have positive antioxidant, protect brain neurons, tranquilize and allay excitement effects (*20*). Gastrodin, as one of the main active ingredients of NDG, was previously found to have the effect of regulation of extracellular DA concentration dually in TS rats: it could make a gradual recovery in extracellular DA content from both up-regulated and down-regulated levels (*8*). We wondered if NDG also possesses a dual regulating effect on extracellular DA content in TS. Thus, in the current study, the regulating effects and possible mechanisms of

NDG on extracellular DA concentration was explored.

As is well known, TS is correlated with the content and activity of DA and the density and sensitivity of postsynaptic receptors in striatum. However, the relationship between the pathophysiology of TS and DA system has far from been clearly elucidated. Apo, a dopamine receptor D1/D2 agonist, could produce similar stereotypic behaviors including biting, licking and sniffing on rodents (*21*). IDPN has been widely used as a tool for neuropathological studies and can induce a series of neurobehavioral disturbances such as dyskinesia and repetitive motor-defects similar to the characteristics of TS (*22,23*). IDPN is also known to interfere with a range of neurotransmitters including DA (*23*). Currently, Apo and IDPN are two manipulations that are commonly used to develop TS models (*14,23,24*). Accumulating evidence suggested that both Apo and IDPN manipulated dyskinesia of TS models exhibited marked alterations in DA: rats exposed to Apo showed significantly increased DA content (*14*) while rats exposed to IDPN showed significantly decreased DA content (*23*). The TS models with different alterations in DA make it possible to investigate multiple therapeutic values of drugs on the neurotransmitter. Thus, in the current study, to investigate the regulating effect of NDG on extracellular DA content in TS, we selected these two pharmacological manipulations, Apo and IDPN, to imitate both of these pathogenic forms of the DA hypothesis.

DA is a key monoamine neurotransmitter in the brain with a regulatory role for motor and limbic functions (*7*). Lots of evidence gave us a signal that dopamine is the final common neurobiological pathway for the expression of TS symptoms (*25,26*). It is transported across the pre-synaptic neuron membranes by DAT. After reuptake by DAT, DA was transformed into HVA in neurons and released into the blood. In the present study, we found that NDG similar to Hal could effectively improve stereotypic behaviors including continuous sniffing, biting, licking, head bobbing, body climbing and so on in both Apo and IDPN induced TS rat models. We also found that NDG could help make a gradual recovery of the abnormal striatal DA content from both increased and decreased levels, while Hal caused no significant alternation of extracellular DA level in either TS rat model. These findings indicated that NDG could regulate striatal DA concentration dually in these two TS rat models, which made quite distinct effects on striatal DA content compared to Hal.

Since the pathogenesis of TS is complicated, the dual regulating effect of NDG might have superiority compared to the Hal with a single-target pharmacological mechanism. Because of the highly heterogeneous nature of the human body, such as age, gender, states, and comorbidities, there could be large differences in real pathological changes among TS patients, even though the anatomical structures and physiological functions of their bodies are nearly the same. The chief pathological alterations of some TS patients, who respond well to Hal, might be the up-regulated density and/or sensitivity of D2 receptors. However, in some other TS patients, the elevated striatal DA release might be a main aspect for the occurrence of the disorder. In this case, the ideal effect of Hal on them must be hard to achieve. Thus, with the displayed properties in normalizing the dopaminergic dysfunction caused by more than one mechanism, NDG might have a positive effect in either of them.

To further uncover the probable mechanism of the dual regulating effect of NDG on extracellular DA release, extracellular HVA content and DAT protein expression were measured. HVA is generally regarded as a main metabolite of DA in the central nervous system. DAT is a high-affinity glycoprotein localized exclusively at the presynaptic membrane of DA neurons. It plays an important role for maintaining sufficient DA levels for release into the synaptic cleft in the striatum, in other words, it is responsible for modulating extracellular DA concentration by uptake of the released DA into nerve terminals (*27,28*). Furthermore, recent studies have highlighted the primary role of DAT, not only in the regulation of the extracellular concentration of DA, but also in the homeostatic maintenance of presynaptic function (*29*). Our findings indicate that NDG could elevate DAT expression when DA release was up-regulated and decrease DAT expression when extracellular DA concentration was down-regulated, while in the extracellular concentration of HVA, the NDG treatment caused no remarkable changes. It could therefore be speculated that the main mechanism of the dual regulating effect of NDG on extracellular DA release might be related to DAT protein expression in TS. The DAT-mediated re-uptake system controls the intensity as well as the duration of dopamine actions at synaptic receptors. In addition, since the NDG treatment made no remarkable changes in the extracellular HVA content, it could therefore be speculated that the dual regulating effect of NDG on extracellular DA release might not be through the metabolic pathways. In all, DAT might be critically involved in the dopaminergic dysfunction associated with TS and might be an essential factor for the management of TS.

In conclusion, to make a significant improvement in stereotypic behavioral abnormalities of TS rats Hal is given and NDG could regulate the extracellular DA concentration dually: it could make a gradual recovery in extracellular DA content from both up-regulated and down-regulated levels, while Hal showed no conspicuous effect on the same neurotransmitter. The main mechanism of the dual regulating effect of NDG on extracellular DA release might be related to DAT protein expression in TS, through which the released DA is re-uptaken into nerve terminals. Taken together, compared with conventional single-target anti-tics drugs such as Hal, NDG with the dual regulating effect would

be more significant for the treatment of TS. The unique therapeutic property of NDG might be meaningful for the treatment of TS.

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