Regulatory effects of Ningdong granule on microglia-mediated neuroinflammation in a rat model of Tourette's syndrome

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SUMMARY  Tourette's syndrome (TS) is an inherited neurologic disorder characterized by involuntary stereotyped motor and vocal tics. Its pathogenesis is still unclear and its treatment remains limited. Recent research has suggested the involvement of immune mechanisms in the pathophysiology of TS. Microglia are the brain's resident innate immune cells. They can mediate neuroinflammation and regulate brain development and homeostasis. A traditional Chinese medicine (TCM), Ningdong granule (NDG), has been found to be efficacious in the treatment of TS while causing few adverse reactions. In the current study, a rat model of 3,3’-iminodipropionitrile (IDPN)-induced TS was used to explore the regulating effects and mechanisms of NDG on microglia-mediated neuroinflammation. IDNP led to robust pathological changes and neurobehavioral complications, with activation of microglia in the striatum of rats with TS. After activation by IDNP, microglia strongly responded to this specific injury, and TNF-α, IL-6, and MCP-1 were released in the striatum and/or serum of rats with TS. Interestingly, NDG inhibited the activation of microglia and decreased the abnormal expression of TNF-α, IL-6, and MCP-1 in the striatum and/or serum of rats with TS, thus controlling tics. However, there were no significant changes in the striatum and/or serum of rats with TS after treatment with haloperidol. The anti-TS action of haloperidol might occur not through microglial activation and neuroinflammation but through the DAT system, thus controlling tics. In conclusion, microglia might play key roles in mediating neuroinflammatory responses in TS, triggering the release of TNF-α, IL-6, and MCP-1. NDG inhibited tics in rats with TS, and this mechanism may be associated with a reduction in the increased number of activated microglia and a decrease in the expression of pro-inflammatory cytokines and chemokines in the striatum and/or serum.

Keywords  Tourette's syndrome (TS), Ningdong granule (NDG), microglia, immunoregulation, neuroinflammation

1. Introduction

Gilles de la Tourette syndrome, or Tourette's syndrome (TS), is an inherited neurologic disorder characterized by involuntary stereotyped motor and vocal tics, with a variety of behavioral comorbidities in most cases, such as attention deficit hyperactivity disorder, obsessive compulsive disorder, and other impulse control disorders (1). TS usually starts in childhood, with a peak age between 7 to 15 years. Its prevalence is estimated to be four to six per 1,000 children and adolescents, with an incidence in males 3-4 times higher than that in females (2). In terms of its clinical course, TS can cause lifelong impairment in 5 to 10% of patients and even life-threatening symptoms in some, including mild self-injurious behaviors and borderline personality disorders (3).

Currently, the detailed etiological and pathophysiological mechanism of TS is still unclear. The etiology is complex, with polygenic, immunological, and hormonal contributions and potential involvement of environmental factors (4). The pathophysiology involves the dysfunction of both motor and non-motor basal ganglia-thalamo-cortical circuitries, with a variety of neurotransmitters implicated including dopamine (DA), serotonin (5-TH), and gamma-aminobutyric acid (GABA) (5). Mounting evidence has shown that immune dysregulation contributes to the pathophysiology of TS. Neuroimmune interactions are increasingly appreciated as an important
regulator of normal brain development and function and a potential contributor to the pathophysiology of a range of neuropsychiatric illnesses, including TS (6).

Microglia are the principal resident immune cells of the brain involved in homeostasis and host defense against pathogens and central nervous system (CNS) disorders (7). Microglia survival and maintenance depend on cytokines and transcription factors. Activated microglia will produce pro-inflammatory tumor necrosis factor (TNF)-α, the cytokines interleukin (IL)-1 and IL-6, and other substances (8). Recent studies have suggested that there is abnormal activation of microglia in patients with TS. Lennington et al. performed the first unbiased and comprehensive characterization of changes in gene expression based on RNA sequencing of specimens from the basal ganglia of patients with TS (9). They found that the top-scoring up-regulated module was enriched in immune-related genes including TNF-α, IL-6, and IL-12, consistent with activation of microglia in patients’ striatum. The activation of microglia was mainly evident as an increased number of CD45+ cells in the caudate of patients with TS. Another study also observed bilateral inflammatory microglial activation in the caudate nuclei of children with TS (10). Therefore, the potential involvement of microglia dysregulation in TS may be an intriguing area for future study.

At present, there is still no ideal pharmacological treatment for TS. Haloperidol (Hal) is approved by the US Food and Drug Administration for treatment of TS. It can effectively inhibit the excitability of the cortical motor area by suppressing the activity of DA receptors (11). However, an extremely high proportion of patients eventually refuse further therapy with Hal because of adverse reactions, including sedation, dizziness, dyskinesia tarda, and extrapyramidal symptoms (e.g., acute dystonia and akathisia) (12). Therefore, novel drugs for treatment of TS need to be developed soon.

Traditional Chinese medicine (TCM) has been widely used in the treatment of various diseases, including nervous system diseases, in China, Japan, South Korea, and other Asian countries for thousands of years (13). Ningdong granule (NDG), a TCM used to treat TS in accordance with the therapeutic principles of TCM, has been used as an anti-tic agent in Chinese clinics for several years. A previous study by the current authors indicated that NDG had a total efficacy of 79.3% in patients with TS while causing few apparent adverse reactions or toxicities (14). Moreover, the NDG group displayed a 41.39% reduction in tic severity and frequency compared to the placebo group (10.79%) (15).

Previous studies by the current authors also indicated that NDG regulates the disturbance of DA, DA transporter (DAT), 5-TH, and GABA in animals and patients with TS (11,12,16). In addition, NDG modulates abnormal serum levels of IL-12 and TNF-α in patients with TS, and NDG might be an immune mechanism for treating TS (14). However, the possible immune mechanisms by which NDG treats TS are still unclear. The aim of the current study was to explore the possible mechanism by which NDG immunoregulates microglia in rats with TS.

2. Materials and Methods

2.1. Preparation of NDG

NDG was provided by 999 Modern Chinese Medicine Co. Ltd. (999 Co. Ltd., Shenzhen, China). As shown in Table 1, NDG contains 8 ingredients. After the ingredients were mixed in proportion, they were macerated with distilled water for 1 h at room temperature, and the whole mixture was decocted twice for 30 min each time. The filtrates were mixed and condensed and then dried with a vacuum-drier at 40°C. The resulting granules were stored at 4°C.

2.2. Laboratory animals and behavior recordings

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

After one week, the rats were randomly divided into a control group (n = 10) and TS model group (n = 30). Rats in the TS model group were intraperitoneally injected (i.p.) with 3,3′-imino dipropionitrile (IDPN) (150mg/kg, i.p.), while the control group received normal saline (NS) (5 mL/kg, i.p.). After IDPN was administered once a day for 7 consecutive days, rats in the model group with IDPN-induced TS were further divided into 3

| Components                      | Part used         | Amount used (g) |
|---------------------------------|-------------------|-----------------|
| Gastrodia elata Blume          | rhizome           | 6               |
| Codonopsis pilosula (Franch) Nannf. | root             | 9               |
| Ophiopogon japonicus (L.f) Ker-Gawl | root tuber        | 6               |
| Paeonia lactiflora Pall.       | root              | 12              |
| Ostraea gigas Thunb.           | shell             | 15              |
| Fossil fragments               | skeletal fossils  | 15              |
| Pheretima aspergillum          | whole polypide    | 6               |
| Glycyrrhiza uralensis Fisch.   | root              | 6               |
sections were mounted on slides and covered. The number of Iba1-positive cells, activated cells with large cell bodies and thick processes, was counted in five 400× non-overlapping microscopic fields in each section.

2.4. Levels of TNF-α, IL-1, IL-6, and monocyte chemoattractant protein 1 in the striatum and serum

The levels of TNF-α, IL-1, IL-6, and monocyte chemoattractant protein 1 (MCP-1) in the striatum and serum were measured using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (TNF-α: JYM0635Ra, IL-1: JYM0418Ra, IL-6: JYM0646Ra, MCP-1: JYM0495Ra, Wuhan ColorfulGene Biological Technology Co., Ltd, Wuhan, China). Briefly, dispensed antigen standards and samples were added to each well of 96-well plates pre-coated with primary antibody. After a biotin conjugate reagent and an enzyme conjugate reagent were added to each well, the plates were incubated at 37℃ for 60 min. The plates were then rinsed 5 times with distilled water. After a chromogenic reaction, absorbance was measured within 30 min at 450 nm with a microtiter plate reader.

2.5. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance ANOVA. A repeated measures ANOVA was used to analyze the stereotypic behaviors of the rats. All analyses were performed using the statistical software package SPSS (Version 21.0, SPSS Inc., Chicago, IL, USA), and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Behavioral study

Repeated measures ANOVA indicated that the IDPN-induced TS model had significant group effects. Administration of IDPN produced multiple stereotypical behaviors in rats compared to control rats throughout the study ($p < 0.01$). After treatment with NDG or Hal, scores for stereotypical behaviors in both groups: a model group ($n = 10$), an NDG group ($n = 10$), and a haloperidol (Hal) group ($n = 10$). The rats were administered normal saline by gastric perfusion (0.9%) at 10 mL/kg (control group and model group), NDG at 370 mg/kg (NDG group), or haloperidol at 1.0 mg/kg (Hal group) once a day for 8 weeks.

Stereotyped behaviors were counted according to evaluation standards described previously (Table 2). Counts were conducted once every 2 weeks by trained observers who were blinded to the group’s treatment. Each animal was observed for one min of every 10 min for a total of 6 observation periods.

At the end of the experiment, all rats were sacrificed under anesthesia and the striatal tissues were extracted from the brain by the method described by Hida et al. (18). Right striatal tissues were removed and fixed overnight at 4℃ by immersion in a 4% formalin solution for immunohistochemistry, and the left striatal tissues were stored at -80℃ until analysis.

2.3. Immunohistochemistry

Ionized calcium-binding adaptor molecule-1 (Iba-1) is a marker of microglial activation. To analyze the microglial activation in the striatal tissues of rats with TS, the expression of Iba-1 was detected immunohistochemically as follows. Three samples were randomly selected from each group. Sections of striatal tissues were routinely processed, embedded in paraffin, and sectioned in 5-μm serial sections. Two sections were randomly selected from each sample. For Iba1-immunohistochemistry, sections were washed three times in a 0.1 M phosphate buffer solution (PBS) for 10 minutes each. Afterwards, sections were treated with 3% H2O2 in PBS for 20 minutes at room temperature. Sections were incubated in a blocking solution containing PBS/10% filtered goat serum (v/v) for 1 hour at room temperature followed by incubation with goat polyclonal anti-Iba1 (dilution 1:1,000, ab5076, Abcam, Cambridge, UK) overnight at 4℃. Next, the paraffin sections were washed thrice in PBS for 8 minutes each and were then incubated with secondary antibody (dilution 1:200; KIT-9901, Maixin Biotechnologies, Fuzhou, China) for 30 min at room temperature followed by washes and colorimetric development (DAB: DAB-2031, Maixin Biotechnologies, Fuzhou, China). Immuno-stained sections were mounted on slides and covered. The number of Iba1-positive cells, activated cells with large cell bodies and thick processes, was counted in five 400× non-overlapping microscopic fields in each section.

Table 2. Standards for evaluation of stereotypical behaviors

| Score | Stereotypical behaviors                                                                 |
|-------|----------------------------------------------------------------------------------------|
| 0     | Asleep, resting in place, or normal activity in place.                                  |
| 1     | Occasional sniffing and head raising                                                    |
| 2     | Frequent sniffing and body raising                                                     |
| 3     | Frequent sniffing, self-grooming with head and body raising primarily in one place, and an occasional rapid burst of loco motor 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 rising/wall climbing whereby the forepaws do not touch the cage floor. |
the NDG group and Hal group decreased significantly compared to scores in the model group ($p < 0.01$), and there were no marked differences in scores between the two treatments ($p > 0.05$) (Figure 1).

### 3.2. Microglial activation in the striatum

The number of activated microglia (Iba-1+) in the striatal tissues of rats with TS was detected immunohistochemically. As shown in Figure 2, the number of activated microglia in the striatum increased significantly in the model group compared to that in the control group ($p < 0.01$). After treatment with NDG or Hal, NDG down-regulated the increased number of activated microglia in the striatum of rats with TS ($p < 0.05$); while there were no significant changes in the number of activated microglia in the Hal group compared to the number in the model group ($p > 0.05$) (Figure 2).

### 3.3. Levels of TNF-α, IL-6, and IL-1 in the striatum and serum

Activated microglia produce pro-inflammatory TNF-α, IL-1, IL-6, and other substances. Here, the levels of TNF-α, IL-6, and IL-1 were detected in the striatum and serum of rats with TS using ELISA. As shown in Figure 3, IDPN regulated the levels of TNF-α and IL-6 in the striatum and serum of rats. There was a significant increase in the TNF-α (Figures 3A and 3B) and IL-6 (Figures 3C and 3D) levels in the striatum and serum of the model group (TNF-α: $p < 0.01$, IL-6: $p < 0.01$) compared to levels in the control group. After treatment with NDG or Hal, the levels of TNF-α and IL-6 in the striatum and serum of the NDG group decreased significantly compared to levels in the model group (TNF-α: $p < 0.05$, IL-6: $p < 0.05$); there were no significant changes in the levels of TNF-α and IL-6 in the striatum and serum of the Hal group compared to levels in the model group ($p > 0.05$). Interestingly, there were no significant differences in the levels of IL-1 in...
the striatum and serum of the four groups ($p > 0.05$) (Figures 4A and 4B).

3.4. Levels of MCP-1 in the striatum and serum

MCP-1 is a chemokine regulating monocyte chemotaxis and T-lymphocyte differentiation, and it plays a crucial role in the pathogenesis of inflammatory diseases, atherosclerosis, and cancer (19). The current study detected the levels of MCP-1 in the striatum and serum of rats with TS using ELISA. As shown in Figure 5A, the levels of MCP-1 in the striatum and serum of the model group increased significantly compared to levels in the control group ($p < 0.01$). After treatment with NDG or Hal, the levels of MCP-1 in the striatum of the NDG group decreased significantly compared to levels in the model group ($p < 0.05$); there were no significant changes in the levels of MCP-1 in the striatum of the Hal group compared to levels in the model group ($p > 0.05$). Interestingly, there were no significant differences in the levels of MCP-1 in the serum of the four groups ($p > 0.05$) (Figure 5B).

4. Discussion

Recent research has suggested that immune mechanisms might be involved in the pathophysiology of TS. According to previous animal and post-mortem studies, microglia play a crucial role in neural-immune crosstalk in TS and other related disorders (20,21). Microglia, as the primary resident immune cells of the CNS, are the first line of defense of the brain’s innate immune response against infection, injury, and diseases (22).

They play an important role in maintaining normal brain function. When the body is healthy, they are known as surveying microglia because they examine the tissue to maintain homeostasis; when disease develops, they are activated and, along with other functions, become phagocytic to clear cellular debris. The current study found that NDG inhibits tics in rats with TS by regulating the activation of microglia.

In the CNS, microglia serve as resident phagocytes that dynamically survey the environment, playing crucial roles in CNS tissue maintenance, injury response, and pathogen defense (23). Microglia can respond quickly to various CNS injuries including trauma, ischemia, and infection, and the maintain the homeostasis of the CNS. However, this response is not always beneficial, and sometimes it worsens damage. Studies have indicated that microglia might act as a double-edged sword in various neurological diseases. In general, microglial activation and the increased expression of cytokines are intended to protect the CNS and benefit the host organism. Nonetheless, amplified, exaggerated, or chronic microglial activation can lead to robust pathological changes and neurobehavioral complications such as depression and cognitive deficits (24).

Microglial abnormalities are implicated in a range of neuropsychiatric pathologies, including TS and autism. A recent postmortem analysis of brains from patients with TS indicated an increased number of CD45$^+$ microglial cells in the striatum and revealed that these cells had morphological changes consistent with neuro-toxic activation (9). A recent positron emission tomography study similarly suggested increased microglial activation in patients with TS.
(10). These findings are consistent with the results of a study by the current authors which found that the neurotoxic drug IDNP can lead to robust pathological changes and neurobehavioral complications with microglial activation in the striatum of rats with TS. After intervention with NDG, the increase in activated microglia decreased and tics were alleviated (Figures 1 and 2).

Neuroinflammation is defined as an inflammatory response within the brain or spinal cord. Microglia play key roles in mediating these neuroinflammatory responses. For example, in infection or disease, microglia become 'activated' and function as inflammatory cellular mediators. Upon activation, resident microglial cells transform from a ramified form to an amoeboid form and acquire the ability to phagocytose and release pro-inflammatory cytokines, chemokines, and growth factors, including ILs (e.g., IL-1 and IL-6), TNF-α, and MCP-1 (25).

TS is largely genetic. Recent research has identified a hypomorphic mutation in L-histidine decarboxylase (Hdc) as a rare but high-penetrance genetic cause of TS. The Hdc-KO (knockout of the Hdc gene) model thus serves as a unique platform to probe the pathophysiology of TS and related conditions (26). After administration of lipopolysaccharide (LPS) as an inflammatory challenge, microglial activation in the striatum of Hdc-KO mice was enhanced, with greater expression of Iba1 than that in wild-type controls (27). This was accompanied by increased production of IL-1β and TNF-α, confirming an increased inflammatory response. Morer et al. evaluated the expression of genes encoding selected inflammatory factors including interferon-γ, IL-2, IL-1β, MCP-1, and CD45 in post-mortem specimens from adults with TS (20). They noted significantly increased expression of MCP-1 and IL-1 in patients with TS (a 6.5-fold and a 2.3-fold increase, respectively), supporting the notion of inflammatory processes in the basal ganglia of patients with TS.

In the current study, microglia in rats were activated by the neurotoxic drug IDNP and then strongly responded to this specific injury, releasing TNF-α and IL-6 in the striatum and serum (Figure 3). After intervention with NDG, tics were alleviated, and the levels of TNF-α and IL-6 in the striatum and serum of rats with TS decreased. TNF-α plays an integral role in immunological responses to infection as a potent regulator of the immune system and inflammatory processes, recruiting macrophages, activating T-cells, and inducing the expression of downstream cytokines and other immune mediators during infection (28). IL-6 is an important mediator of neuroinflammation and is involved in microglial priming under neuroinflammatory conditions. IL-6-mediated cell-cell interactions may be an attractive therapeutic target for brain inflammation (29). The increase in TNF-α and IL-6 may increase the permeability of the blood-brain barrier. These changes might lead to an enhanced autoimmune response and even abnormal release of neurotransmitters in the basal ganglia, which in turn contributes to the clinical symptoms of TS and related disorders.

MCP-1, also called chemokine (CC motif) ligand 2 (CCL2), is a key chemokine involved in neuroinflammation, and a MCP-1 deficiency protects against inflammation in the brain. Mounting evidence suggests that MCP-1 is significantly involved in the activation of microglia (30). The current study found that microglia were activated by the neurotoxic drug IDNP, and they strongly responded to this specific injury by releasing MCP-1 in the striatum of rats with TS (Figure 5A). After intervention with NDG, tics were alleviated, and the levels of MCP-1 in the striatum of rats with TS decreased. However, there were no significant differences in the levels of MCP-1 in the serum of the four groups (the control group, the IDNP-induced TS group, the NDG group, and the Hal group) (Figure 5B). The speculation is that MCP-1 might be mainly expressed in brain tissue, a finding that is similar to the results of a previous study which found that MCP-1 and its receptor CCR2 are primarily expressed by microglia in the mouse and human brain (31).

IL-1 is one of the most well-known pro-inflammatory cytokines that acts within the brain during insults and neurodegenerative diseases. The IL-1 system involves two essential agonists, IL-1α and IL-1β, as well as IL-1’s endogenous antagonist, IL-1 receptor antagonist (IL-1RN) (32). In the brain, IL-1 is mainly synthesized and released by activated microglia and involved in neuroinflammation during various neurological diseases. He et al. investigated the relationship between single-nucleotide polymorphisms (SNPs) of IL-1α and IL-1RN and the susceptibility to TS in the Chinese Han population (33), and they found that IL-1α rs17561 and IL-1RN rs315952 polymorphisms might not be associated with susceptibility to TS in that population. In addition, Morer et al. found that the levels of IL-1β expression were below detection limits in both patients with TS and controls (20). Interestingly, the current study found no significant differences in the levels of IL-1 both in the striatum and serum of the four groups (the control group, the IDNP-induced TS group, the NDG group, and the Hal group) (Figures 4A and 4B). The speculation is that IL-1 might be not involved in neuroinflammation and microglial activation in TS.

NDG, a TCM to treat TS in accordance with the therapeutic principles of TCM, has been used as an anti-tic agent in Chinese clinics for several years. Pharmacological studies have found that NDG contains a number of active substances such as saponins (e.g., gastrodin and paeoniflorin), steroid saponins, carbohydrates and their glycosides, alkaloids, organic acids, and flavonoids, which have proven to have antioxidant action, to protect brain neurons, to reduce...
and allay excitement (34). The current study found that NDG inhibited the activation of microglia and decreased the abnormal expression of TNF-α, IL-6, and MCP-1 in the striatum and/or serum of rats with TS, thus controlling tics. However, there were no significant changes in the striatum and/or serum of rats with TS after treatment with Hal. The anti-TS action of Hal might occur not through microglial activation and neuroinflammation but through the DAT system, thus controlling tics (16).

In conclusion, microglia might play key roles in mediating neuroinflammatory responses in TS, triggering the release of TNF-α, IL-6, and MCP-1. NDG inhibited tics in rats with TS, and this mechanism may be associated with a reduction in the increased number of activated microglia and a decrease in the expression of pro-inflammatory cytokines and chemokines in the striatum and/or serum.

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