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·Original Article·

Thrombin-induced microglial activation contributes to the degeneration of nigral dopaminergic neurons *in vivo*

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Abstract: Objective To evaluate the role of thrombin-activated microglia in the neurodegeneration of nigral dopaminergic neurons in the rat substantia nigra (SN) *in vivo*. **Methods** After stereotaxic thrombin injection into unilateral SN of rats, immunostaining, reverse transcription polymerase chain reaction (RT-PCR) and biochemical methods were used to observe tyrosine hydroxylase (TH) immunoreactive positive cells, microglia activation, nitric oxide (NO) amount and inducible nitric-oxide synthase (iNOS) expression. **Results** (1) Selective damage to dopaminergic neurons was produced after thrombin injection, which was evidenced by loss of TH immunostaining in time-dependent manner; (2) Strong microglial activation was observed in the SN; (3) RT-PCR demonstrated the early and transient expression of neurotoxic factors iNOS mRNA in the SN. Immunofluorescence results found that thrombin induced expression of iNOS in microglia. The NO production in the thrombin-injected rats was significantly higher than that of controls ($P < 0.05$). **Conclusion** Thrombin intranigral injection can injure the dopaminergic neurons in the SN. Thrombin-induced microglia activation precedes dopaminergic neuron degeneration, which suggest that activation of microglia and release of NO may play important roles in dopaminergic neuronal death in the SN.

Keywords: thrombin; dopaminergic neuron; microglia; Parkinson's disease; NO

1 Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by slow and progressive degeneration of dopaminergic neurons in the substantia nigra (SN). To date, the cause of this chronic nigral cell death in PD and its underlying mechanisms remains elusive. There is an urgent need to acquire a deeper understanding of both etiological and pathogenic factors implicated in PD. Increasing evidence strongly suggested a role for inflammation in the pathogenesis of PD and activated microglia played a critical role in the degeneration of nigral dopaminergic neurons[1-4]. Thrombin is generated from the precursor prothrombin endogenously expressed in human, mouse and rat brain, including dopaminergic neurons in the SN. Thrombin induces various biological responses in the central nervous system (CNS). Its effect on neurons and astrocytes is either protective or toxic, depending on the thrombin concentration. Increased thrombin in brain had been shown to lead to the degeneration of the hippocampal neurons, spinal motoneurons and astrocytes. Previously, Lee and Debeir reported that thrombin was directly toxic to dopaminergic neurons in mesencephalic cultures containing few of microglia. Choi's results suggested that thrombin could activate microglia *in vivo* and this microglial activation could mediate degeneration of dopaminergic neurons in the SN by increased expression of inducible nitric-oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and proinflammatory cytokines from activated microglia[5-10]. However, there is no substantial evidence ob-

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serving the effect of thrombin on the degeneration of dopaminergic neurons in the longer survival time of rats. So we stereotaxically injected thrombin into the SN and observed the effects of thrombin on the numbers and morphology of the dopaminergic neurons positive for tyrosine hydroxylase (TH) and the activated microglia in order to explore the relationship between inflammation and PD.

2 Materials and methods

2.1 Animal care and treatments All work was performed in accordance with the Instructions for the Institutional Animal Care and Use of Huazhong University of Science and Technology. One hundred and ten adult female Sprague-Dawley (SD) rats (Center of Animal Experiment, Tongji Medical College of Huazhong University of Science and Technology, China) weighing 200-260 g were used. The rats were randomly divided into groups for the different time (3 h, 4 h, 6 h, 12 h, 1 d, 2 d, 3 d, 7 d, 14 d, 30 d). PBS injection and normal rats were used as controls. Five rats were used for each time point.

2.2 Stereotaxic thrombin injection Female SD rats were anesthetized through chloral hydrate injection (360 mg/kg, ip), positioned in a stereotaxic apparatus (Kopf Instrument, Tujunga, CA) and received a unilateral administration of thrombin into the right SN pars compacta (SNpc). A hole in the skull was drilled using the following coordinates: 5.0 mm posterior to the bregma, 2.0 mm lateral to the midline and 8.0 mm dorsoventral to the surface of skull, according to the atlas of Paxinos and Watson (1998). Thrombin (Sigma, St. Louis, MO, USA) was dissolved in 1% Monastral Blue in saline solution; Four microlitre of 20 U thrombin was injected at a rate of 0.4 μL/min by a 26 gauge Hamilton syringe attached to an automated microinjector. After injection, the needle was left in place for an additional 5 min before slow retraction. After surgery, the rats were injected with penicillin (100 000 IU). Untreated (normal) or PBS-treated animals were used as controls. Animals were killed by an overdose of chloral hydrate.

2.3 Immunohistochemistry and confocal double-label immunofluorescence Animals were perfused with a saline solution containing 0.5% sodium nitrate and heparin (10 U/mL) and fixed with 4% paraformaldehyde dissolved in 0.1 mol/L phosphate buffer (PB). Brains were removed from the skull, post-fixed overnight in buffered 4% paraformaldehyde at 4 °C and stored in a 30% sucrose solution at 4 °C until they sank, then frozen and sectioned on a sliding microtome. Coronal sections (30 μm thick) were cut through the nigral complex and collected in six separate series and processed for immunohistochemical staining as previously described[5,10]. After washing with PBS, sections were incubated in 0.2% Triton X-100 for 30 min and rinsed three times with 0.5% PBS. The brain sections were immunostained with the following cell-type specific antibodies: rabbit monoclonal anti-TH antibody (1:2 500 dilution, Sigma, USA) for dopaminergic neurons, mouse monoclonal OX-42 antibody (1:100) for microglia (BD Pharmingen, San Diego, CA, USA; 1:100), immunostaining was performed by the SP method (Zhongshan, Beijing, China), followed by color development with diaminobenzidine (DAB, Zhongshan, Beijing, China). Labeled tissue sections were then mounted on gelatin-coated slides and observed under HPIAS-1000 light microscope. For confocal double-label immunofluorescence, the sections were incubated in a combination of a rabbit polyclonal antibody to iNOS (Cell Signaling; 1:100) and a mouse monoclonal antibody against OX-42 overnight at 4 °C. After washing with PBS, the sections were incubated simultaneously with a mixture of FITC-conjugated goat anti-mouse IgG (Zhongshan Goldenbridge, Beijing, China; 1:100) and Rhodamine (TRITC) -conjugated goat anti-rabbit IgG (Zhongshan Goldenbridge, Beijing, China; 1:100) for 1 h at room temperature. Slides were then rinsed with PBS, coverslipped with Vectashield medium (Linfei, Wuhan, China) and viewed using an IX71 confocal laser scanning microscope (Olympus Optical, Tokyo, Japan). To analyze the localization of different antigens in double-stained samples, images were obtained from the same area and merged using interactive software. All washing were three times for 5 min. All antibodies were diluted with PBS.

2.4 Measurement of nitric oxide (NO) Brains were removed from the cranium, the ipsilateral SN was rapidly dissected out and frozen on dry ice after intranigral injection of 0 U(control), 5 U, 10 U, 20 U, 40 U thrombin or PBS for 48 h (control). Tissues were homogenized in buffer of Tris-EDTA (50 mmol/L, pH 7.4) and centrifuged at 10 000 r/min at 4 °C for 20 min. The supernatant was collected and frozen at –20 °C. The amount of nitrite formed from NO was measured by mixing the supernatant (50 μL) with an equal volume of Griess reagent (0.1% naphthyle-thylene diamine, 1% sulfanylamide and 2.5% H_3PO_4) (Jiancheng, Nanjing, China) for 10 min at room

temperature, and absorbance of diazonium compound was measured at 540 nm with ultraviolet (UV) spectrophotometer (Pharmacia Biotech, American). Absolute levels of nitrite were determined with reference to a standard curve obtained from defined concentrations of sodium nitrite.

2.5 Reverse transcription polymerase chain reaction (RT-PCR) Rats were killed at 0, 3, 6, 12 and 24 h after thrombin injections, and the SN region was rapidly removed from rat brains and frozen at –70 °C. Total RNA was extracted from dissected tissue using RNAzol B (Huashun, Shanghai, China) according to the manufacturer's instructions and quantified with UV spectrophotometer. Reverse transcription (RT) was performed using Superscript II reverse transcriptase (Invitrogen, Rockville, MD) according to the manufacturer's instructions. The sequences of PCR primers were as follows: (Sense) 5'-TCACTGGGACAGCACAGAAT-3', (Anti-sense) 5'-TGTGTCTGCAGATGTGCTGA-3' for iNOS (250 bp); (Sense) 5'-TGTGATGGTGGGAATGGGTCAG-3', (Anti-sense) 5'-TTTGATGTCACGCACGATTTCC-3' for β-actin (514 bp). The PCR condition consisted of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 90 s for 30 cycles, and a final extension at 72 °C for 10 min. PCR products were separated by electrophoresis in 1.5% agarose gels, stained with ethidium bromide, and then detected under UV light (Bio-Rad, Hercules, CA).

2.6 Image analysis and statistical analysis The sections were taken photos with Canon 350 D digital camera. The loss of dopaminergic neurons was determined by counting the TH-marked soma in SN. All cells were counted under bright-field illumination at 400 × magnification, and with blind method by two independent investigators. Cells were counted using five fields per section and five sections per animal. At least five animals were counted for each group.

2.7 Statistical analysis Data are expressed as mean±SD. Results were analyzed by one-way ANOVA. $P < 0.05$ was considered to be statistically significant.

3 Results

3.1 Thrombin induced degeneration in rat SN in a time-dependent manner Thrombin (20 U) was injected into rat SNpc to study the time course effect of thrombin on the dopaminergic neurons. The average number of TH immunoreactive cells in each SN section of thrombin-treated rats was significantly different from the control groups (Fig. 1A-E). TH immunoreactive positive (TH^+) neurons began to decrease 3 d after injection. Seven days later, thrombin-treated SN displayed a significant loss of TH^+ cells in the SN (Fig. 1B, E) compared with PBS-treated SN (Fig. 1A, D), and the decrease was 53% ($P < 0.01$). In high magnification photographs, thrombin-induced degenerating neurons appeared as shrunken and rounded cell bodies with fewer or shorter processes (Fig. 1E), in contrast to healthy and large dopaminergic neurons with long and branched neural processes in PBS-treated controls (Fig. 1D). And the TH^+ cells number decreased to 21% on the 14 d and to 12% about on the 30 d (Fig. 1C; $P < 0.01$, compared with controls).

3.2 Thrombin induced microglial activation in the SN *in vivo* When thrombin (20 U) was injected into rat SNpc, thrombin triggered profound activation of microglia at different times in the injection site of SN, with enhanced staining intensity and activated morphology in OX-42-ip cells. At 4 h, microglia began to change their morphology from ramified shape into activated amoeboid shape (Fig. 1G). At 12 h, most microglia were activated with characteristic amoeboid morphology (Fig. 1H). At 24 h, nearly all of the positive microglia became fully activated with the characteristic amoeboid morphology. At 3 d, the thrombin-injected SN was occupied by a markedly increased number of activated microglia (Fig. 1I; $P < 0.05$), whereas microglia retained their population and ramified appearance in the contralateral SN (Fig. 1F) and fewer microglia showing amoeboid morphology could be observed at 3 d with PBS-injected SN, which probably resulted from mechanical damage caused by needle insertion. These results that thrombin induced microglial proliferation and activation in the SN indicate that microglial activation occurred before the lesion of nigral dopaminergic neurons (Fig. 2).

3.3 Thrombin induced expression of the iNOS and NO release Given the neurotoxic effects of thrombin-activated microglia, we next sought to determine whether thrombin-activated microglia produced the microglia-derived NO and iNOS. RT-PCR analysis showed that the iNOS mRNA was induced as early as 3 h after thrombin treatment and was sustained up to 24 h after thrombin treatment (Fig. 3A, B; $P < 0.05$, compared with the untreated controls). NO, released by microglia as a result of thrombin-induced iNOS expression, is a candidate for mediating neurotoxicity. Thus, we also examined the levels of NO in the SN after 5-40 U/mL thrombin treated rats (Fig. 3C). Compared with the untreated and PBS

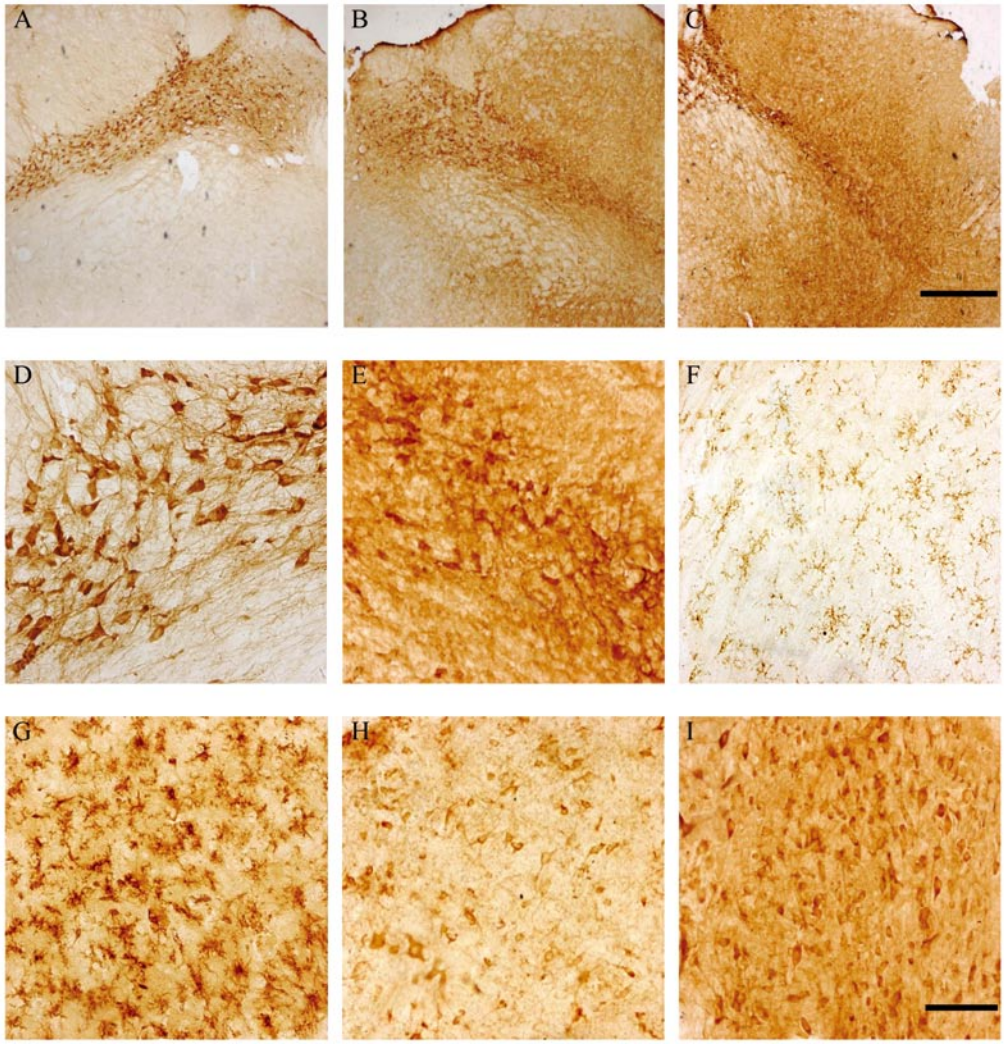

Fig. 1 Effects of thrombin on dopaminergic neurons and microglial activation in substantia nigra . Strong TH^+ neuron loss was observed in the SN of thrombin-treated animals. A, D: PBS-treated SN 7 d; B, E: Thrombin-treated SN 7 d, displayed a significant loss of TH^+ neurons; C: Thrombin-treated SN 14 d; F: PBS-treated SN 12 h, ramified shape microglia; G: Thrombin-treated SN 4 h, microglia began to change their morphology from ramified shape into activated amoeboid shape; H: Most microglial cells were activated with the characteristic amoeboid morphology at 12 h; I: Thrombin-injected SN was occupied by a markedly increased number of activated microglia at 3 d. Scale bar, 500 μm in A-C; 100 μm in D-I.

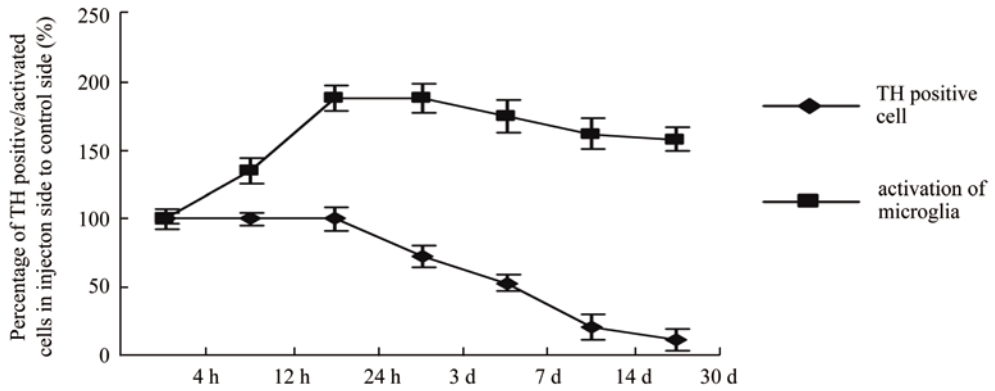

Fig. 2 The dynamical change of TH positive cell and activation of microglia after thrombin injection. All cells were counted under bright-field illumination at 400 × magnification. Cells were counted using five fields per section and five sections per animal. At least five animals were counted.

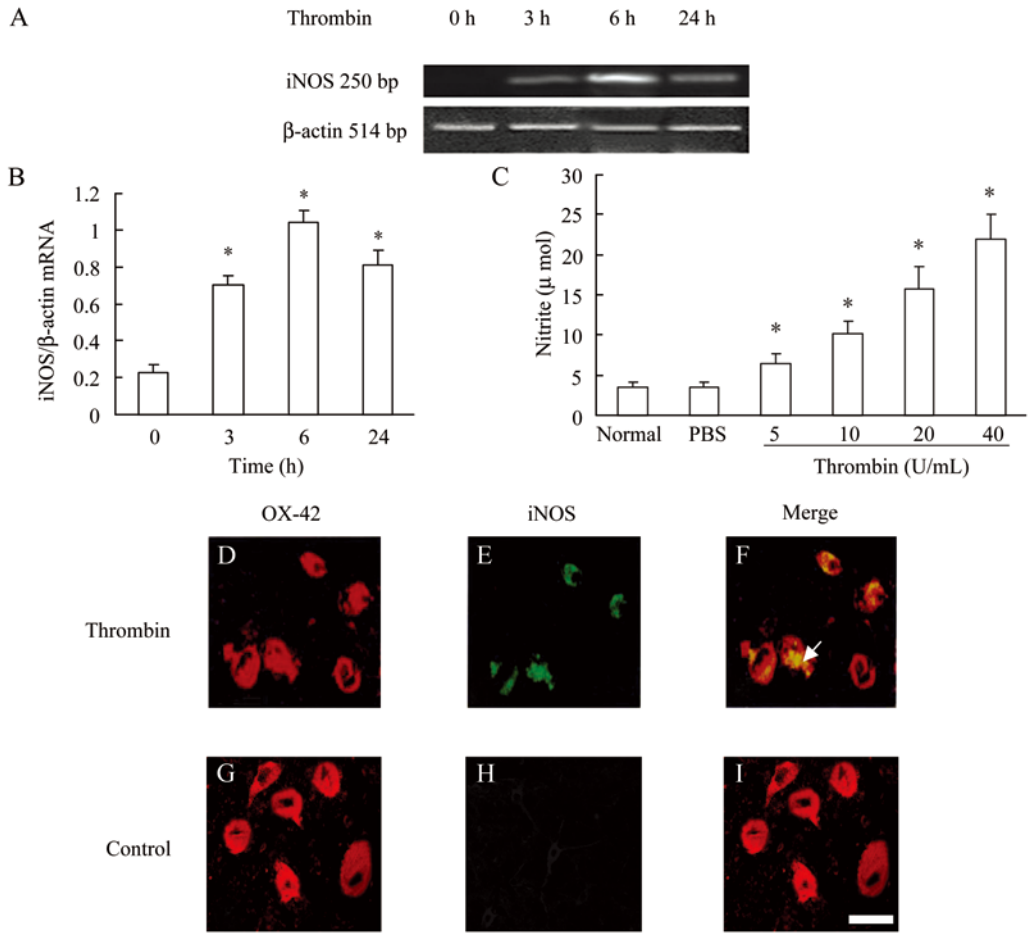

Fig. 3 Thrombin induces the iNOS expression and NO release. A, B: RT-PCR analysis showing the mRNA expression of iNOS with 20 U/mL thrombin for the indicated times in rats SN *in vivo*; * $P < 0.05$, compared with control (0 h); C: Levels of nitrite were measured at 48 h after treatment with thrombin (5-40 U/mL), which showed the levels of NO increased significantly in dose-dependent manner; * $P < 0.05$, compared with control (normal or PBS-treated); D-F: Confocal immunofluorescence images of iNOS (green) and OX-42 (red) at the ipsilateral SN after 24 h of thrombin injection; G-I: PBS injection was used as control. Arrowheads indicate iNOS-positive microglia. Scale bar = 25 μm.

controls, the levels of NO significantly increased in a dose-dependent manner after treatment with thrombin, respectively ($P < 0.05$). To further evaluate the cellular location of iNOS, sections were examined by double-immunofluorescence staining using antibodies of iNOS and OX-42 as a marker of microglia. The data showed that iNOS (Fig. 3D-F) were localized in OX-42-positive microglia 24 h after thrombin injection. Injection with PBS, in contrast, had no effect on the expression of these proteins (Fig. 3G-I).

4 Discussion

The progressive degeneration of dopaminergic neurons in the SN is a hallmark of PD. The exact mechanism through which dopaminergic neurons die in PD remains unknown. However, *in vivo* data from PD patients and animal models of PD induced by 6-hyroxydopamine (6-OHDA), lipopolysaccharide (LPS) or 1-methy-4-pheny-1, 2, 3, 6-tetrahydropyridine(MPTP) revealed that activated microglia and their cytotoxic agents played a crucial pathological role in the degeneration of nigral dopaminergic neurons[2].

Thrombin is a member of the serine protease family that plays multifunctional roles in wound healing and blood coagulation. Recently, thrombin-induced neurotoxicity had been reported *in vivo* and *in vitro* in nigral, cortical and hippocampal neurons[5-6,11]. To further investigate changes in microglial activity associated with a dramatic loss of dopaminergic neurons induced by thrombin in the SN *in vivo*, we investigated the effect of microinjection of thrombin into the

SN of rats. These neurotoxic actions of thrombin were further confirmed by our results, where thrombin induced the degeneration of nigral neurons in time-dependent manner, in addition to local inflammatory reactions involving microglial activation. Contrary to the loss of TH immunoreactivity, thrombin triggered profound activation of microglia in the injection site of SN, with enhanced staining intensity and activated morphology in OX-42-ip cells. We also found that microglial activation occurred before the degeneration of nigral dopaminergic neurons. Our results are consistent with Choi's results in which the majority of microglia displayed activated morphology (larger cell bodies with short, thick, or no process, like "amoeboid morphology") as early as 4 h after thrombin injection[5]. It suggested that microglia-mediated inflammation plays a deleterious role in the pathogenesis of PD.

Microglia are well-known intrinsic immune effector cells in the CNS and activation of microglia is a common phenomenon in response to neural tissue injury[3,12-14]. Activated microglia can produce inflammatory mediators such as TNF-α, as well as potentially neurotoxic factors including NO and prostaglandins, which are synthesized by iNOS and COX-2, respectively. NO produced by activated microglia is toxic to neighboring cells[11,13,14], although the presence of iNOS in human microglia is disputed. In the current study, RT-PCR analyses showed that the mRNA for iNOS was detected within 3 h after 20 U/mL thrombin treatment and sustained up to 24 h after thrombin treatment *in vivo*. Also the levels of NO significantly increased in a dose-dependent manner after treatment with thrombin, respectively. Additional immunoflurescene staining further revelaled colocalization of iNOS immunoreactivity within microglia after thrombin treatment. The results strongly supported the hypothesis that production of NO and expression of iNOS from thrombin-activated microglia may participate in the death of dopaminergic neurons. These results are consistent with previous studies showing that in iNOS-deficient mice, nigral dopaminergic neurons were protected from MPTP toxicity[13] and that LPS-induced iNOS expression and NO production in microglia led to the death of dopaminergic neurons in the SN[15]. However, it cannot be ruled out that other microlgia-derived proinflammatory cytokines may contribute to this degeneration.

In conclusion, the results showed that intranigral injection thrombin can induce the degeneration of dopaminergic neurons in the SN. Thrombin-induced microglia activation precedes dopaminergic neuron degeneration. These results suggest that activation of microglia may play important roles in the degeneration of dopaminergic neurons. Further studies are needed for elucidating the molecular mechanism of microglia activation induced by thrombin. Further exploration of relationship between early stage of inflammation in the SN and later degeneration of dopaminergic neurons would provide a new theory for PD treatment.

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References:

[1] Vila M, Jackson-Lewis V, Guégan C, Wu DC, Teismann P, Choi DK, *et al*. The role of glial cells in Parkinson's disease. Curr Opin Neurol 2001, 14: 483-489.

[2] Li G, Sun SG, Ma R, Xiao WZ, Tong ET. Protective effect of S-methylisourea against the degeneration of dopaminergic neurons induced by intranigral injection of lipopolysaccharide. Chin J Neurosci 2004, 20: 270-274. (Chinese, English abstract)

[3] Wu DC, Jackson-Lewis V, Vila M, Tieu K, Teismann P, Vadseth C, *et al.* Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine mouse model of Parkinson disease. J Neurosci 2002, 22: 1763-1771.

[4] Kim WG, Mohney RP, Wilson B, Jeohn GH, Liu B, Hong JS. Regional difference in susceptibility to lipopolysaccharide-induced neurotoxicity in the rat brain: role of microglia. J Neurosci 2000, 20: 6309-6316.

[5] Choi SH, Joe EH, Kim SU, Jin BK. Thrombin-induced microglial activation produces degeneration of nigral dopaminergic neurons *in vivo*. J Neurosci 2003, 23: 5877-5886.

[6] Carreño-Müller E, Herrera AJ, de Pablos RM, Tomás-Camardiel M, Venero JL, Cano J, *et al.* Thrombin induces *in vivo* degeneration of nigral dopaminergic neurones along with the activation of microglia. J Neurochem 2003, 84: 1201-1214.

[7] Lee DY, Oh YJ, Jin BK. Thrombin-activated microglia contribute to death of dopaminergic neurons in rat mesencephalic cultures: dual roles of mitogen-activated protein kinase signaling pathways. Glia 2005, 51: 98-110.

[8] Fujimoto S, Katsuki H, Ohnishi M, Takagi M, Kume T, Akaike A. Thrombin induces striatal neurotoxicity depending on mitogen-activated protein kinase pathways *in vivo*. J Neurosci 2007, 144: 694-701.

[9] Lee da Y, Park KW, Jin BK. Thrombin induces neurodegeneration and microglial activation in the cortex *in vivo* and *in vitro*: proteolytic and non-proteolytic actions. Biochem Biophys Res Commun 2006, 36: 727-738.

[10] Choi SH, Lee DY, Kim SU, Jin BK. Thrombin-induced oxidative stress contributes to the death of hippocampal neurons *in vivo*: role of microglial NADPH oxidase. J Neurosci 2005, 25: 4082-4090.

[11] Choi SH, Lee DY, Ryu JK, Kim J, Joe EH, Jin BK. Thrombin induces nigral dopaminergic neurodegeneration *in vivo* by altering expression of death-related proteins. Neurobiol Dis 2003, 14: 181-193.

[12] Bani-Hani MG, Greenstein D, Mann BE, Green CJ, Motterlini R. Modulation of thrombin-induced neuroinflammation in BV-2 microglia by carbon monoxide-releasing molecule 3. J Pharmacol Exp Ther 2007, 318: 1315-1322.

[13] Liberatore GT, Jackson-Lewis V, Vukosavic S, Mandir AS, Vila M, McAuliffe WG, *et al*. Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease. Nat Med 1999, 5: 1403-1409.

[14] Kaktsuki H, Okawara M, Shibata H, Kume T, Akaike A. Nitric oxide-producing microglia mediate thrombin-induced degeneration of dopaminergic neurons in rat midbrain slice culture. J Neurochem 2006, 97: 1232-1242.

[15] Iravani MM, Kashefi K, Mander P, Rose S, Jenner P. Involvement of inducible nitric oxide synthase in inflammation-induced dopaminergic neurodegeneration. J Neurosci 2002, 110: 49-58.

凝血酶诱导的小胶质细胞激活促进多巴胺能神经元变性

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摘要：目的 探讨凝血酶 (Thrombin) 诱导小胶质细胞 (Micoglia) 激活与黑质多巴胺能神经元变性的关系。**方法** 采用立体定向术注射凝血酶至大鼠黑质，在不同时间点观察酪氨酸羟化酶 (tyrosine hydroxylase, TH) 神经元的表达及小胶质细胞的激活情况；同时检测黑质NO量及iNOS mRNA表达。**结果** (1) 凝血酶注入大鼠黑质导致明显的黑质多巴胺能神经元变性，呈时间依赖性，TH阳性细胞数在第3 d开始下降，第7 d有大量的TH阳性细胞丢失，与对照侧相比下降达约53% ($P < 0.01$)；高倍镜下可见胞体皱缩、突起明显缩短或减少；14 d时细胞数下降至21%，30 d时下降至12% ($P < 0.01$)。(2) 凝血酶注射入黑质4 h后小胶质细胞开始呈现为“灌木丛样”或少量呈现“阿米巴样”；12 h后小胶质细胞数目明显增加且绝大部分呈现“阿米巴样”；24 h后细胞已完全激活，“阿米巴样”细胞达高峰；3 d维持高峰；14 d后小胶质细胞染色变淡，体积变小，“阿米巴样”细胞数目下降。(3) 与对照组相比，iNOS mRNA表达明显上调及NO合成增加 ($P < 0.05$)，并且有iNOS在小胶质细胞表达。**结论** 凝血酶对多巴胺能神经元具有一定的损毁作用，小胶质细胞的激活先于多巴胺能神经元变性，其激活后释放的NO有可能参与多巴胺能神经元变性。

关键词：凝血酶; 多巴胺能神经元; 小胶质细胞; 帕金森病；一氧化氮