Original Article

BoNT-A attenuated pain of endometriosis by inhibiting microglia activation

Fubo Tian1*, Hua Xu1*, Xueya Yao2, Jianying Hu3, Shen Sun2, Hui Chen1, Shaoqiang Huang3, Yuanchang Xiong2

1Department of Anesthesiology, Changhai Hospital, Second Military Medical University, Shanghai 200433, China; 2Hebei North University School of Medicine, Zhangjiakou, Hebei, China; 3Department of Anesthesiology, Shanghai Obstetrics and Gynecology Hospital, Fudan University, Shanghai 200090, China. *Equal contributors.

Received April 12, 2017; Accepted May 10, 2017; Epub June 15, 2018; Published June 30, 2018

Abstract: Pain induced by endometriosis with high incidence was common which was reported to be associated with activation of microglia. Botulinum toxin A (BoNT-A) had been used to treat clinical pathological pain including migraine, musculoskeletal pain and refractory trigeminal neuralgia effectively. We evaluated the effect of BoNT-A on microglia activation induced by lipopolysaccharide (LPS) in vitro and microglia activation of spinal cord using a endometriosis mouse model (EMS). Oxytocin-induced writhing test on EMS mice was performed to investigate the effect of BoNT-A on endometriosis pain. QPCR was used to measure mRNA expression and western blotting combined with immunofluorescence and immunochemistry were used to detect protein expression. Inflammatory factors were measured through ELISA. Our results showed that BoNT-A decreased the levels of IL-1β, IL-6, TNFα and PGE2 in the supernatant of BV-2 cells which were activated by LPS. BoNT-A suppressed Iba-1 and OX42 expression mentioned as markers of microglia activation, down-regulated P2X7, CX3R1, p-P38MAPK and p-JNK protein expression in BV-2 activated by LPS and spinal cord of mice underwent endometriosis pain. BoNT-A may have clinical potential in therapy of endometriosis pain.

Keywords: BoNT-A, endometriosis pain, microglia activation, P2X7, CX3CR1

Introduction

Endometriosis is one of gynecological diseases in which active endometrial cells implant in the endometrial accident locations. The incidence of endometriosis in women of childbearing age is about 10% [1]. Endometriosis is often accompanied with complications including infertility. The most typical symptoms of endometriosis are dysmenorrheal and secondary progressive pain, even analgesic doses of analgesics are ineffective for severe pain induced by endometriosis.

As reported, the mechanism of pain induced by endometriosis might be due to periodic bleeding which stimulated local inflammatory response in endometriosis lesions, intraperitoneal chronic inflammation, abnormal growth of the lesion caused peripheral and central nervous sensitization which induced pain sensitization [2, 3]. Moreover, endometriosis lesions induced increased release of prostaglandins, leading to much more severe uterine muscle contracture and dysmenorrheal. Recent studies reported that neuroinflammatory response was an important reason for the initiation and maintenance of chronic pain, whether it was inflammatory or neuropathic pain, and activation of glial cells in chronic pain played an important role in the integration and conduction of pain [4]. Microglia in spinal cord might be important in triggering the functional chronic visceral pain [5].

Surgical resection of endometriosis is the main method of treating endometriosis, but long-term drug treatment is still needed. It is necessary to select drugs with anti-inflammatory, anti-nociceptive and anti-neuropathic pain activity to effectively treat endometriosis pain. Botulinum toxin type A (BoNT-A) as an exotoxin
produced by botulinum, affects peripheral motor nerve, neuromuscular junction and synapses to suppress release of neurotransmitters-Acetylcholine from the presynaptic membrane. BoNT-A could cause muscle relaxation paralysis so as to be mainly used for the treatment of muscle spasms and dystonia. In recent years, BoNT-A has been reported to be used in the treatment of primary headache, postoperative pain in mastectomy and clinical pathological pain including migraine, musculoskeletal pain and refractory trigeminal neuralgia effectively, and the analgesic effect may last for several months [6-8]. BoNT-A relieves neuropathic pain in animal experiments by down-regulating the expression of the capsaicin receptor (TRPV1) [9] and it is worth noting that injecting BoNT-A into the neural stem and its surroundings did not cause neurological damage [10]. The role of BoNT-A in treating endometriosis pain remains unclear.

In present study, we investigated the effects of BoNT-A on the microglia in vitro and in vivo and explored its mechanism. It was expected that reference and experimental data could provide support for potential application of BoNT-A in the treatment of endometriosis pain, which might be a new opportunity for the therapy of endometriosis pain.

Materials and methods

Reagents and antibodies

Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich Co. (Cat#L439, Saint Louis, MO, USA). Botulinum Toxin Type A (BoNT-A) was supplied by Lanzhou Institute of Biological Products Co. (China). IL-1β, IL-6, TNF-α ELISA assay kit were supplied by Cloud-Clone Corp (TX, USA) and Mouse Prostaglandin E2 (PGE2) ELISA assay kit was obtained from (CUSABIO and CusAb, Hubei, China, Cat#CSB-E07966m). Antibodies included those against GAPDH (EaRthOx Life Sciences, Cat#ED2010-01), Iba1 (Cat#ab178680), P38MAPK (Cat#ab7952), p-P38MAPK (Cat#ab4822) (Abcam, Cambridge, UK), P2X7 (Cat#sc-514962), CX3CR1 (Cat#sc-31561), OX42 (Cat#sc-53086) (Santa Cruz, CA, USA), JNK (Cat#9252s), p-JNK (Cat#9251), AKT (Cat#4691) and p-AKT (Cat#4060) (Cell Signaling Technology, CST). Secondary antibodies including Alexa Fluor 488 rabbit anti mouse IgG (Cat#1639131) (Life Technologies CA, USA) and 4', 6-diamidino-2-phenylindole (DAPI, Molecular Probes, Life Technologies) were used for immunofluorescence confocal microscopy.

Cell lines and cultures

BV-2 microglia was kindly provided by Shanghai R&S Biotechnology Co., Ltd (Shanghai, China). Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum both from Gibco-Invitrogen (Grand Island, NY, USA) and maintained at 37°C in a humidified atmosphere consisting of 5% CO₂ and 95% air.

Microglia activation induced by LPS and treated by BoNT-A+ LPS

BV-2 cells were treated with different LPS concentrations (1 µg/ml, 5 µg/ml and 10 µg/ml). Cells with LPS were maintained at 37°C in a humidified atmosphere consisting of 5% CO₂ and 95% air for 24 h. After confirming that the cell were activated by one determinate dose of LPS, microglia was treated by BoNT-A (1 U/ml and 5 U/ml) combined with the determinate dose of LPS that induced microglia activation.

Immunofluorescence

At 24 h after activation of BV-2 cells induced by LPS as well as microglial in spinal cord of animals, Iba1 and OX42 were detected by immunofluorescence staining. Alexa Fluor 488 rabbit anti mouse IgG and Alexa Fluor 594 goat anti rabbit antibody were used for secondary antibodies. Nuclei were also counterstained with DAPI and the stained cells were examined with fluorescence microscopy (TCS SP5 Leica, Germany). For immunofluorescence analysis, the sections were respectively stained with Iba1 and OX42 antibody, and the resulting images were merged with DAPI stained images. Iba1 and OX42 were factors associated with activation of BV-2.

Measurement of IL-1β, IL-6, TNF-α and PGE2 by ELISA

To assess the inflammatory response after LPS stimulation, concentration of IL-1β, IL-6, TNF-α and PGE2 in BV-2 cell supernatant were measured by ELISA. After being activated by LPS for 24 h, cultured BV-2 cells were collected and
BoNT-A inhibited microglial activation to release pain in endometriosis

A
Control 1µg/ml 5µg/ml 10µg/ml

B
Control LPS LPS+BoNT-A 1U LPS+BoNT-A 5U

C
DAPI Iba1 Merge
Control
BoNT-A
LPS
LPS+BoNT-A

D
DAPI OX42 Merge
Control
BoNT-A
LPS
LPS+BoNT-A

E
Concentration of L-SP (ng/ml)
Control BoNT-A LPS LPS+BoNT-A

F
Concentration of TNF-α (pg/ml)
Control BoNT-A LPS LPS+BoNT-A

G
Concentration of IL-6 (pg/ml)
Control BoNT-A LPS LPS+BoNT-A

H
Concentration of PGE2 (pg/ml)
Control BoNT-A LPS LPS+BoNT-A
BoNT-A inhibited microglial activation to release pain in endometriosis

**Figure 1.** BoNT-A reversed microglia activation and inflammatory factors releasing induced by LPS. BoNT-A reversed microglia morphology changes (A, B; above: ×100; below: ×200), activation of microglia through inhibiting Iba-1/OX42 expression measured by immunofluorescence method (C, D; left: ×1000; right: ×630) and IL-1β, IL-6, TNF-α and PGE2 expression increasing induced by LPS (E-H). Mean ± SD, n=3. **P<0.01 versus control, ##P<0.01 versus LPS group.

analyzed using assay Kits according to instructions provided by manufacturer. The results were expressed as picogram per milliliter (pg·ml⁻¹).

**RNA extraction and quantitative real-time PCR (qRT-PCR)**

Using trizol reagent (invitrogen, USA), total RNA was extracted from cell lines and spinal cord according to the manufacturer's instructions. To monitor the PCR in real-time, iQ-5 (Bio-Rad) was used. The PCR cycling profile was pre-denatured at 95°C for 2 min, followed by 40 cycles of denaturing at 95°C for 15 sec, annealing at 60°C for 20 sec and extending at 72°C for 20 sec. The mean cycle threshold (Ct) from three independent experiments was used for further calculations. Relative expression levels were normalized to control. The endogenous GAPDH was chosen as the internal control. The 2-ΔΔCt (ΔΔCt = (Ctarget mRNA - CtGAPDH) - (control - CtGAPDH)) method was used to quantify the relative amount of target mRNA.

**Western blot analysis**

Activated microglial induced by LPS in vitro and cells in spinal cord were lysed in 1×SDS-PAGE loading buffer (120 μL per well of 6-well plate). The samples of lysis were heated to 95-100°C for 10 min and subsequently cooled on ice and centrifuged at 10,000× g for 1 min at 4°C, supernatant of which was run on 10% SDS-PAGE gel and then transferred electrophoretically to a polyvinylidene fluoride membrane (PVDF, Millipore, Shanghai, China). 5% skim milk in Tris-buffered saline containing Tween-20 (TBST) was used to block the blots for 1 h at 25°C followed by incubation with primary antibodies against GAPDH, P38MAPK, p-P38MAPK, P2X7, CX3CR1, JNK, p-JNK, AKT and p-AKT at 4°C overnight. After being washed with TBST, the membranes were incubated with proper secondary antibodies. Blots were then incubated and visualized with enhanced chemiluminescence (ECL, Thermo Scientific, Shanghai, China). The results were normalized to GAPDH to correct for loading.

**Establishment of animal model of endometriosis and animal behavior observation**

All care and handling of animals were performed in accordance with the guidelines of the animal ethics committee of Second Military Medical University (Shanghai, China). Mouse model of endometriosis was established in BALB/c mice. Female BALB/c mice (weighting 18-20 g) were purchased from Sino-British Sippr/BK Laboratory Animal Co., Ltd. (Shanghai, China). Six to eight week-old female mice were intraperitoneally (i.p.) administered β-estradiol with a dose of 5 mg·kg⁻¹ every 3 days 6 days before model establishment. Endometriosis dysmenorrhea was established as previously reported. In brief, donor mice were anesthetized intraperitoneally with 10% Chloral Hydrate (400 mg/kg), then both uterine horns were removed and transferred into Dulbecco's modified Eagle's medium (10% fetal calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Gibco, Life Technologies, USA) maintained at 37°C. Subsequently, the uterine horns were opened longitudinally and attached to the internal surface of the abdominal wall and sutured diagonally with 4-0 absorbable suture. Mice in sham group underwent surgeries using the same steps as the endometriosis surgeries, but no tissue was sutured to the abdominal wall. After confirming hemostasis, the incision was sutured and closed in layers, and mice were injected intraperitonealy with penicillin at a dose of 20 million units per kilogram daily for 3 days to against infection.

The endometriosis model mice were randomly divided into 3 groups according to the administration 7 days after surgery by subcutaneous injection. One mouse was given BoNT-A at a dose of 0.5 U and 1 U and an equal volume of normal saline (NS) was used as control. All animals were treated only once. The writhing test was performed according to the method in previous reports [11]. 20 IU·kg⁻¹ oxytocin was used to induce writhing response by intraperitoneal injection at 1, 3, 5 and 7 days and
BoNT-A inhibited microglial activation to release pain in endometriosis

![Graphs showing mRNA expression of various proteins under different conditions.](image-url)

F: Western blot analysis showing protein expression levels of P2X7, CX3CR1, Akt, p-Akt, p38MAPK, p-p38MAPK, JNK, and p-JNK under control, LPS, BoNT-A, and LPS+BoNT-A conditions.

G: Graph showing relative P2X7/GAPDH expression.
H: Graph showing relative CX3CR1/GAPDH expression.
BoNT-A inhibited microglial activation to release pain in endometriosis

writhes were counted for 30 minutes after oxytocin injection.

**Immunohistochemistry**

Immunohistochemical staining was performed to evaluate P2X7 and CX3CR1 expression in the spinal cord. Paraffin-embedded tissue sections (5 μm thick) were prepared. Slides were deparaffinized, rehydrated, and subjected to microwave heat antigen retrieval in 0.01 M citrate buffer (pH 6.0) for 20-25 min. After blocking endogenous peroxidase activity with 3% H2O2 for 15 min, the sections were incubated with primary antibodies against P2X7 (1:500, Santa Cruz, CA) and CX3CR1 (1:250, Santa Cruz, CA) overnight at 4°C. After washing with phosphate-buffered saline (PBS), the sections were incubated with the corresponding second antibody. Chromogen, 3, 3’diaminobenzidine (DAB) (Maixin Bio, China) was used to visualize P2X7 and CX3CR1 expression.

Figure 2. Effects of BoNT-A on mRNA and protein expression of P2X7, CX3R1, AKT, P38MAPK and JNK in microglia of BV-2. mRNA expression of P2X7, CX3R1, AKT, P38MAPK and JNK was measured by qPCR (A-E) and protein expression was detected by western blotting (F-N). Mean ± SD, n=3. *P<0.05, **P<0.01 versus control, #P<0.05, ##P<0.01 versus LPS group.
BoNT-A inhibited microglial activation to release pain in endometriosis

Statistical analysis

Results were presented as means ± standard deviations (SD). Significant differences in the mean values between two groups were evaluated by Student’s unpaired t-test. Places needing multiple comparisons were evaluated by one-way ANOVA with Bonferroni correction. SPSS software 18.0 (IBM, New York, USA) was used to perform statistical analysis. P-value of 0.05 or less was considered to be statistically significant.

Results

BoNT-A reversed microglial activation induced by LPS

To evaluate the effect of BoNT-A on microglial activation induced by LPS, we used LPS with different concentrations to stimulate the BV-2 microglia for 24 h to induce activation, to which BoNT-A was also added. LPS induced changes of microglia morphology in a dose-dependent manner (Figure 1A). Compared with control group (0 µg/ml of LPS), 1-10 µg/ml of LPS induced microglia morphology changes. 1 µg/ml of LPS was selected to stimulate microglia to evaluate the effect of BoNT-A. As showed in Figure 1B, both 1 U/ml and 5 U/ml BoNT-A reversed the effect of LPS and we chose 1 U/ml BoNTA for further study.

Immunofluorescence assay was performed to evaluate the effect of BoNT-A on microglial activation induced by LPS. Iba1 and OX42 expression was measured in our study. Compared with control, LPS induced up-regulation of Iba1 and OX42 expression which were considered as activated biomarkers of microglia (Figure 1C, 1D). BoNT-A significantly decreased expression of Iba1 and OX42 compared with that induced by LPS. LPS induced IL-1β, TNFα, IL-6 and PGE2 expression increase compared with control group (P<0.0001 for all comparisons) (Figure 1E-H) measured by ELISA. BoNT-A could significantly suppress the expression of IL-1β, TNFα, IL-6 and PGE2 induced by LPS compared with control despite not reverse the effect of LPS (P=0.0022, P<0.0001, P<0.0001, P=0.0001 respectively).

BoNT-A affects the expression of P2X7, CX3R1, AKT, P38MAPK and JNK in vitro

To investigate the mechanism of BoNT-A in microglia activation induced by LPS, we measured the mRNA expression of P2X7, CX3R1, AKT, P38MAPK and JNK by qPCR and protein expression using Western blotting. LPS induced significantly decreased mRNA expression of P2X7, CX3R1, AKT and P38MAPK while increased JNK compared with that in control group (P=0.0088, P<0.0001, P=0.0041, P=0.0018, P=0.0002, respectively) (Figure 2A-E). BoNT-A down-regulated mRNA expression of P2X7, CX3R1 and JNK compared with LPS group (P=0.0051, P=0.0029, P=0.0001, respectively). As for protein expression, LPS induced increased P2X7, CX3R1, AKT, p-P38MAPK and p-JNK expression (vs. control, P=0.0406, P<0.0001, P=0.0249, P<0.0001, respectively) and decreased p-AKT, P38MAPK and JNK compared with control group (P<0.0001 for all comparisons) (Figure 2F-N). BoNT-A down-regulated P2X7, CX3R1, p-P38MAPK and p-JNK expression (vs. control, P=0.0004, P=0.0001, P=0.0021, P<0.0001) and up-regulated p-AKT expression compared with that induced by LPS (P<0.0001).

Effects of BoNT-A on oxytocin-induced writhing test

Seven days after endometriosis model building surgery, oxytocin induced writhing in mice (Figure 3). BoNT-A significantly inhibited the oxytocin-induced writhing at both 0.5 U/dose and 1 U/dose (vs. model, P<0.0001 for all comparisons at all time points). The effect of BoNT-A on oxytocin-induced writhing was remarkable and time-dependent post administration.
BoNT-A inhibited microglial activation to release pain in endometriosis

**BoNT-A affected Iba-1 and OX42 expression in spinal cord**

As showed in Figure 4, Iba-1 and OX42 were immunofluorescence visualized in the spinal cord sections. We found Iba-1 and OX42 were abundantly expressed in spinal cord of endometriosis model mice and which was suppressed BoNT-A at the dose of 0.5 U and 1 U. The effect of BoNT-A on Iba-1 and OX42 expression in vivo was similar to that induced by LPS in vitro.

**The effect of BoNT-A on P2X7, CX3R1, AKT, P38MAPK and JNK expression in vivo**

We measured mRNA and protein expression of P2X7, CX3R1, AKT, P38MAPK and JNK in spinal cord by qPCR and western blotting. P2X7 and CX3R1 in spinal cord were also immunohistochemically stained (Figure 5A-E) while protein expression significantly increased in spinal cord of endometriosis model mice (Figure 5F-N). BoNT-A up-regulated P2X7, CX3R1, AKT, JNK and P38MAPK mRNA expression (vs. model; For 0.5U BoNT-A, P=0.013, P=0.0207, P=0.0368, P>0.05, respectively; For 1U BoNT-A, P=0.004, P=0.0032, P=0.0276, P=0.0109, P=0.0088, respectively) and down-regulated P2X7, CX3R1, AKT, p-AKT, P38MAPK, p-P38MAPK, JNK and p-JNK protein expression compared with endometriosis model group (For 0.5U BoNT-A, P=0.0001, P=0.0003, P=0.0002, P<0.0001; For 1U BoNT-A, P<0.0001, P=0.0001, P<0.0001, P<0.0001 for the last five comparisons, respectively). The reduction in expression of protein by BoNT-A was statistically significant.

**Discussion**

Endometriosis, usually associated with infertility, is a common clinical disease of gynecology of which the typical symptom is dysmenorrhea. Analgesics often show ineffective to the severe pain in this disease. The localized tissue inflammatory response stimulated by the bleeding of the lesion and the abnormal growth of the lesion causes the peripheral sensation and sensitization. Microglia of spinal cord was reported to play an important role in triggering the functional chronic visceral pain [5]. It is usually considered that sensitivity of neurons induces chronic pain. As reported, IL-1β, IL-6, TNF-α and PGE2 secreted by microglia accompanied with microglia activation could increase excitability of neurons and produce central sensitization, which induced neurons to produce a large amount of ATP, Substance P (SP) and glutamic acid to continue activating microglia [12-15]. P2 purine receptors are physiological...
BoNT-A inhibited microglial activation to release pain in endometriosis
BoNT-A inhibited microglial activation to release pain in endometriosis
receptors of ATP and other purines, these receptors contain seven ion channel type P2X receptors and 13 kinds of G protein coupled P2Y receptors. The P2X4 and P2X7 receptors are unique subtypes in the P2X family. P2Y12 and P2Y13 receptors are important members of the P2Y receptor family which express on the surface of microglia. It has been found that injury of peripheral nerve stimulates dorsal root ganglia (DRG) neurons to release ATP, it binds to P2X4 receptor of microglia in spinal cord to activate dorsal horn neurons, and then cause neuronal depolarization excitement and pain sensitivity [16]. P2X7 receptor could drive microglia activation and secreting neurotrophic substances and increase the expression of P2X7 receptor in spinal cord, which is consistent with the change of pain sensation [17, 18]. The expression of P2X12 receptor, OX42, Iba-1 and P38MAPK was increased in microglia in the spinal cord of rats underwent spinal nerve or sciatic nerve injury [19-21]. The expression of P2Y13 receptor was up-regulated in rats underwent pain induced by spinal nerve injury, and antagonist of P2Y13 receptor intramuscular injection could effectively relieve mechanical pain in rats with spinal nerve injury [21].

The expression of CXCL1 and CXCR2 was increased in DRG which was induced by neuritis [22] and CXCL1/CXCR2 in DRG could induce sensitivity to pain. CX3CL1/CX3CR1 regulated sensitivity to pain induced by nerve injury through the ERK5 signaling pathway [23]. Phosphatidylinositol-3-kinase (PI3K) was a molecule involving in intracellular signal transduction. Phosphorylated Akt (p-Akt) was often used as a marker for PI3K activation. PI3K/Akt signaling pathways were present in spinal microglia [16] and the expression increased in neuropathic pain and inflammatory pain models [17, 18]. Blocking the pathway could relieve the decrease in mechanical pain thresholds caused by nerve injury and inflammation.

BoNT-A mainly used for muscle spasms and dystonia treatment and had been found to be effective on pathological pain and primary headache recently. BoNT-A has been used in the treatment of migraine, musculoskeletal pain and refractory trigeminal neuralgia effectively [7, 8]. After being injected into the neural stem and its surroundings, BoNT-A had no neurological damage to them and could attenuate neuropathic pain in animal experiments through down-regulating the expression of the capsacin receptor (TRPV1) [9]. In present study, we evaluated the effect of BoNT-A on endometriosis pain in LPS-induced microglia activation model and animal model of endometriosis. BoNT-A was used to treat microglia activated by LPS and endometriosis mice after surgery. IL-1β, IL-6, TNF-α and PGE2 secreted by microglia were detected and mRNA expression of P2X7, CX3R1, AKT, P38MAPK and JNK was measured by qPCR (A-E) and western blotting (F-N). Immunohistochemistry method was used to further confirm the expression of P2X7 (O, left: ×100; right: ×400) and CX3R1 (P, left: ×100; right: ×400) in spinal cord of animals of endometriosis. Mean ± SD, n=5. *P<0.05, **P<0.01 versus control, #P<0.05, ##P<0.01 versus endometriosis model group (Model).

In conclusion, BoNT-A could inhibit activation of microglia induced by LPS in vitro and microglia in spinal cord in endometriosis mice. IL-1β, IL-6, TNF-α and PGE2 secreted by BV-2 were also
BoNT-A inhibited microglial activation to release pain in endometriosis

decreased by BoNT-A compared with LPS-stimulated group. BoNT-A relieved endometriosis pain in mice to cause decreased the number of writhing. BoNT-A down-regulated protein expression of P2X7, CX3R1, p-P38MAPK and p-JNK in BV-2 activated by LPS and activating microglial in spinal cord of mice underwent endometriosis pain. BoNT-A may offer a promising therapeutic approach for the treatment of pain in endometriosis.

Acknowledgements

This work was supported by Shanghai Science and Technology Commission: 201740098.

Disclosure of conflict of interest

None.

Address correspondence to: Yuanchang Xiong, Department of Anesthesiology, Changhai Hospital, Second Military Medical University, No. 168 Changhai Road, Yangpu District, Shanghai 200433, China. Tel: +86-13901911666; Fax: +86-21-31162428; E-mail: proychxiong@163.com

References


[20] Jin SX, Zhuang ZY, Woolf CJ and Ji RR. p38 mitogen-activated protein kinase is activated after a spinal nerve ligation in spinal cord mi-
BoNT-A inhibited microglial activation to release pain in endometriosis


