Reproductive Sciences
2018, Vol. 25(7) 1045-1057

DOI: 10.1177/1933719117732161
journals.sagepub.com/home/rsx
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Role of Brain-Derived Neurotrophic Factor in Endometriosis Pain

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Abstract

Brain-derived neurotrophic factor (BDNF) is a regulator for the formation and maintenance of chronic pain in various chronic disorders and has been shown to increase in the serum of women with endometriosis. However, BDNF expression in the peritoneal fluid (PF) and ectopic lesions and its role in endometriosis pain remain unclear. Thus, this study aims to determine the BDNF concentrations in serum and PFs and BDNF expression levels in ectopic lesions and endometriotic stromal cells (ESCs) of women with endometriosis (n = 60). The obtained results were then compared with those of women without endometriosis (n = 38). Brain-derived neurotrophic factor concentrations in serum and PF, as well as the BDNF expression levels in ectopic lesions and endometriotic cells, were evaluated through enzyme-linked immunosorbent assay, immunohistochemical staining, quantitative real-time polymerase chain reaction, and Western blot analysis. As a result, BDNF concentrations in serum and PF were significantly higher in women with endometriosis with pain (2284.3 $\pm$ 51.5 pg/mL, n = 23; 58.8 $\pm$ 6.4 pg/mL, n = 16) than in women with endometriosis without pain (1999.8 $\pm$ 61.1 pg/mL, n = 37; 31.7 $\pm$ 2.9 pg/mL, n = 25; $P < .01$). Moreover, BDNF messenger RNA (mRNA) expression levels in ectopic lesions (8.97 $\pm$ 1.44, n = 29) were significantly higher than eutopic (0.97 $\pm$ 0.14, n = 16; $P < .01$) and control endometrium (1.23 $\pm$ 0.19, n = 18; $P < .01$) and were correlated with endometriosis pain ($P < .05$). Furthermore, increased BDNF mRNA and protein expression levels in ESCs induced by estradiol or interleukin 1β were removed using a phosphorylated extracellular-regulated protein kinase 1/2 inhibitor. These results suggest that BDNF may play an important role in the pathogenesis of endometriosis pain.

Keywords

endometriosis, brain-derived neurotrophic factor, pain, tyrosine receptor kinase B, extracellular-regulated protein kinase

Introduction

Endometriosis is a chronic inflammatory disease characterized by the presence of endometrial-like tissue outside the uterine cavity.[1] It affects approximately 10% of reproductive-age women and is a main cause of pelvic pain and infertility.[2] However, regardless of ethnicity, the prevalence and symptoms of endometriosis are related to personal traits and other factors such as age and economic status.[3-5] Increased inflammatory mediators such as interleukin (IL) 1β and 6, tumor necrosis factor α, and prostaglandins[6,7] are considered to sensitize sensory neurons by stimulating the sensory nerve fibers within the ectopic lesions[8,9] and thus trigger the pain signal cascade in women with endometriosis.[10] Endometriosis pain may be considered as a kind of inflammatory and neuropathic pain.[11]

Neurotrophins (NTs), including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT-3, and NT-4/5, play a critical role in endometriosis pain.[12,13] Peritoneal fluid (PF) from patients with endometriosis, which contains a high NGF level, can induce strong sensory and marginal sympathetic neurite outgrowth.[12,13] Increased BDNF concentrations in serum have been demonstrated in patients with endometriosis with central sensitivity syndrome.[14] However, endometriosis pain was not relieved when treated with melatonin even if the reduction of BDNF concentration in plasma occurred.[15] These findings suggest that BDNF may be involved in a specific kind of pain.

Brain-derived neurotrophic factor has been recognized as a regulator in the formation and maintenance of chronic pain in various chronic disorders, including osteoarthritis, rheumatoid arthritis, fibromyalgia, and facet joint distraction.[16-19] In acute and chronic models, BDNF, which is released from primary afferents, is involved in nociceptive signaling. Nociceptive signaling activates its affinity receptor tyrosine receptor kinase B (TrkB), thereby activates extracellular-regulated protein kinase

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1/2 (ERK1/2) via phosphorylation and finally contributes to spinal hyperexcitability.[20] Protein phosphorylation is the most crucial event in signal transduction in nociceptive sensitization, followed by nociceptor stimulation and messenger activation. Mitogen-activated protein kinases (MAPKs) are the main protein phospho-regulating effectors that mediate nociceptive sensitization.[21] In chronic constriction ligation models, MAPK inhibitors reverse the mechanical allodynia and heat the hyperalgesia by suppressing the BDNF expression in the dorsal root ganglion and the dorsal horn of the spinal cord.[22] Brain-derived neurotrophic factor plays an important role in nociceptive and neuropathic pain.

Recently, 3 studies have found that concentrations of BDNF in plasma increased in women with endometriosis and became higher than those of women without endometriosis[23-25] and decreased when treated with gonadotropin-releasing hormone agonist.[23] However, only Rocha et al[25] identified the correlation between BDNF concentrations in plasma and endometriosis pain. Moreover, the levels of BDNF and TrkB messenger RNA (mRNA), as well as protein expression levels, were found to increase in ectopic lesions when compared with eutopic endometrium of women with endometriosis[26,27] and were relative to the subtypes of endometriosis.[28] However, the correlation between BDNF expression levels in ectopic lesions and endometriosis pain was not proved. Thus, the involvement of BDNF in endometriosis pain is still under controversy.

On the other hand, it has been reported that BDNF *Val66Met* single-nucleotide polymorphism might result in the endometriosis-related infertility.[29,30] In a recent study, BDNF was reported to be regulated by estrogen, and BDNF-activated signal transducer and activator of transcription 3 (STAT3) signaling pathways were mediators of endometrial cell proliferation, while BDNF *Val66Met* polymorphism lost its function in regulation of endometrial cell growth.[31]

In the present study, we aimed to determine BDNF concentrations in serum and PF. We also aimed to determine BDNF mRNA expression levels in ectopic lesions in women with endometriosis and compared them with those in eutopic endometrium from women with endometriosis and control endometrium from nonendometriosis women. The correlations of BDNF expression levels in serum, PF, and ectopic lesions with endometriosis pain or infertility would then be identified. Endometriotic stromal cells (ESCs) were cultured in vitro and intervened with estradiol (E_2), IL-1β, and phosphrylated extracellular-regulated protein kinase 1/2 (p-E R K 1/2) inhibitor for the exploration of the possible mechanisms of BDNF in endometriosis pain.

Materials and Methods

Patients

A written informed consent was provided by each patient participating in this study, which was approved by the Human Ethics Committee of the Women's Hospital, School of Medicine, Zhejiang University (no. 20160110). A total of 98 Chinese Han women who were consecutively undergoing laparoscopic surgery at the Women's Hospital between January 2015 and June 2016 were recruited in this study. Ovarian endometrioma, pain, and infertility were among the indications for surgery in women with endometriosis (case group: n = 60, 35.3 ± 0.9 years). Out of the 60 women with endometriosis, 23 (38%) experienced pain and 7 (12%) experienced infertility. Other asymptomatic patients underwent surgery because of pelvic mass (ovarian endometriotic cyst). Endometriosis was graded according to the Revised American Fertility Society scoring system (I + II = 32, 53.3%; III + IV = 28, 46.7%). Uterine leiomyoma, pain, and infertility were among the indications for surgery in women without endometriosis (control group: n = 38, 35.6 ± 1.4 years). Out of the 38 women without endometriosis, 11 (29%) experienced pain and 4 (11%) experienced infertility. During the interview, each patient was asked about her personal history including gravidity, parity, abortion, menstrual status, drug therapy, and pain symptoms. The day of the menstrual cycle was verified by the histologic examination of the endometrium. The severity of pain was documented using a standardized questionnaire with a visual analog scale (VAS = 0, asymptomatic endometriosis; VAS = 1-3, minimal pain; VAS = 4-10, severe pain). None of the patients received sex hormone therapy 6 months before the surgery.

Collection of Samples

Peripheral blood samples (2 mL) were collected from all patients 1 day before the surgery. Then, samples of PF, ectopic lesions, and endometrial tissues were collected during the surgical procedure. The blood and PF samples were centrifuged at 1000*g* for 10 minutes. Approximately 200 μL of supernatants was collected into a 1.5-mL eppendorf tube and then frozen at −80°C until tested. Half of each endometrial tissue was frozen for mRNA analysis, and the other half was placed in formalin for immunohistochemical (IHC) staining. In addition, 6 ovarian ectopic lesions were collected in cold Dulbecco modified eagle medium/nutrient mixture F12 (Gibco, Grand Island, New York) in a sterile condition for primary ESC culture.

Determination of BDNF Concentrations in Serum and PF

The BDNF concentrations in serum (1:10 thawed) and PF were measured by Enzyme Linked Immunosorbent Assay (ELISA) using the BDNF ELISA kit (Promega, Madison, Wisconsin) according to the instructions indicated in the kit. Briefly, 96-well NUNC plates (Corning, Lowell, Massachusetts) were coated with anti-BDNF mAb. The absorbance of the BDNF concentration was measured at 450 nm using the Varioskan Flash (Thermo Fisher Scientific, Waltham, Massachusetts) after its incubation.

Immunohistochemical Staining and Assessment

Immunohistochemical staining and scoring were performed as previously described.[32] An anti-BDNF antibody (1:8000, ab108319; Abcam, Cambridge, Britain) or anti-TrkB antibody (1:350, ab81987; Abcam) was used to measure BDNF levels and TrkB protein expression in endometriotic lesions and

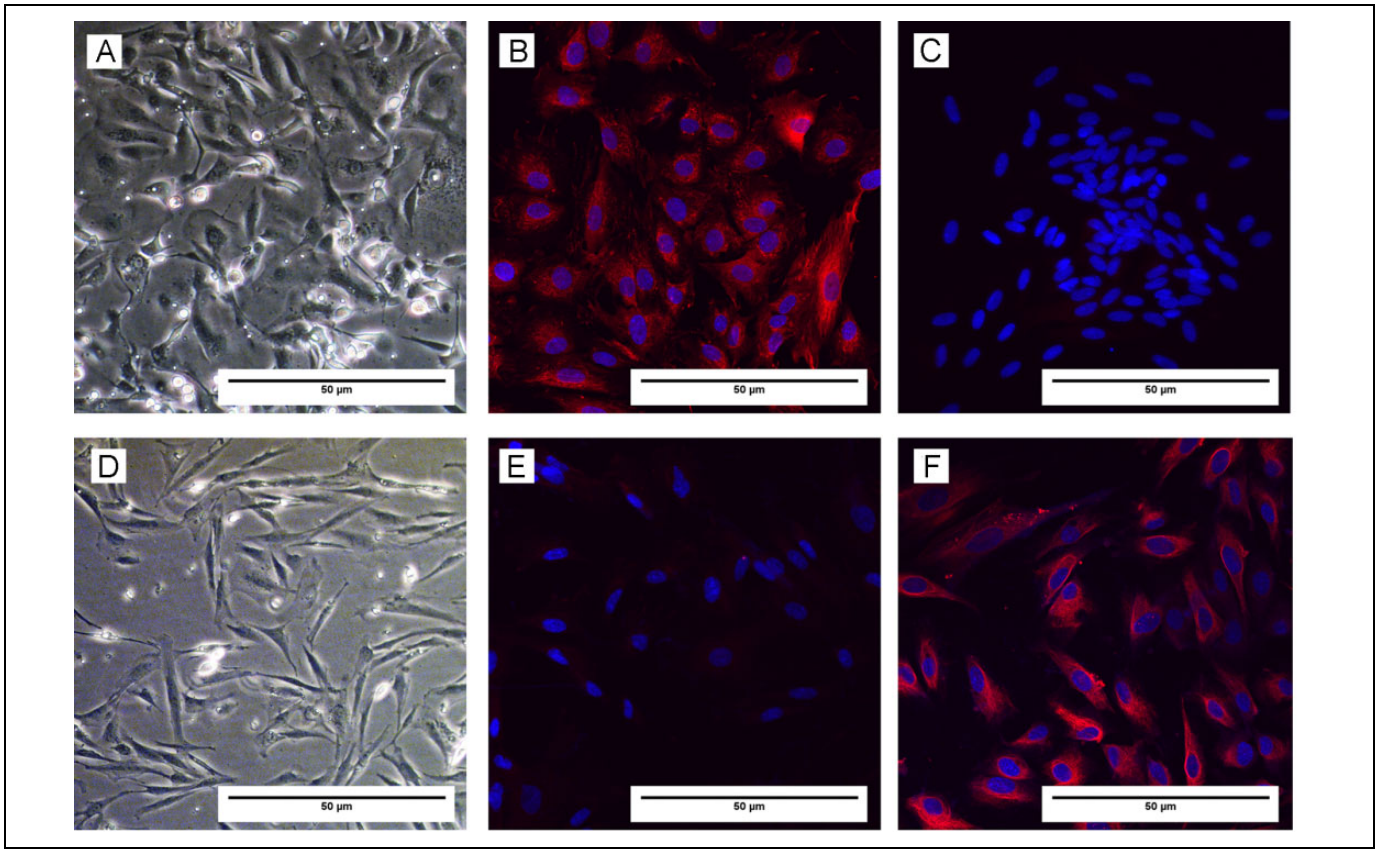

Figure 1. Morphology and identification of the primary endometriotic cells by using immunofluorescence analysis. The morphology of endometriotic epithelial cells (EPCs) (A) and endometriotic stromal cells (ESCs) (D) was observed under the phase-contrast microscope. The EPCs were positively immunostained with cytokeratin (B) but not vimentin (C). The ESCs were positively immunostained with vimentin (F) but not cytokeratin (E). The cell nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI). 400×, scale bar = 50 μm.

endometrial tissues. Envision-labeled polymer–alkaline phosphatase rabbit (Dako, Glostrup, Denmark) and diaminobenzidine (Dako) were used to visualize the antigen–antibody reaction, also known as chromogen. The sum of the percentage (0-3) and intensity scores (0-3) were represented as IHC scores. The average IHC score count of 5 different fields at a ×400 magnification was regarded as the molecule expression. Two blinded observers analyzed the slides.

Human Endometriotic Cell Culture and Intervention

Isolation of ESCs was performed as previously described.[33] Immunofluorescence analysis of cytokeratin (1:500, DC10; Dako, Glostrup, Denmark) and vimentin (1:200, V9; Dako) expression levels was used to identify the purification of the isolated endometriotic epithelial cells (EPCs) and ESCs (Figure 1). In this study, due to the difficult subcultivation of EPCs, only ESCs were selected. The cells were treated with IL-1β (10.0 ng/mL; Proteintech, Rosemont, Illinois) or E_2 (100 nM; Sigma-Aldrich, Louis, Missouri) and were then harvested after 15 minutes, 30 minutes, 1 hour, 2 hour, and 24 hours. Thereafter, the ESCs were preincubated with a p-ERK1/2 inhibitor (PD 98059, 20 μM; Calbiochem, Billerica, Massachusetts) for 45 minutes before IL-1β and E_2 treatment.

Reverse Transcription Quantitative Real-Time Polymerase Chain Reaction and Western Blot

TRIzol reagent (Takara, Shiga, Japan) and PrimeScript Reverse Transcription reagent kit (Takara) were used to extract the total RNA in endometrial tissues or ESCs through reverse transcription. For the real-time amplification monitoring, real-time polymerase chain reaction was performed using SYBR Premix Ex Taq kit (Takara). Specific primers used for amplification synthesized from Generay sequences are as follows: actin, 5′-ACTATCGGCAATGAGCGGTTC-3′ (forward) and 5′-AGAGCCACCA ATCCACACAGA-3′ (reverse); BDNF, 5′-CCATAAGGACGCGGACTTGTAC-3′ (forward) and 5′-GAGGAGGCTCCAAAGGCACTT-3′ (reverse). Average cycle threshold (Ct) value was calculated from the triplicate wells for each sample, and fold change was determined through $2^{-\Delta\Delta Ct}$ method.

Total degenerated protein was separated through 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then electrotransferred onto polyvinylidene difluoride membrane (Merck Millipore, Billerica, Massachusetts). Subsequently, the membrane was incubated with anti-BDNF antibody (1:500; Abcam), anti-p-ERK1/2 (1:2000, #4370; Cell Signaling Technology, Danvers, Massachusetts), anti-

Table 1. Patients' Characteristics.

Parameters	Endometriosis (n = 60)	Nonendometriosis (n = 38)
Age (years), mean ± SEM	35.3 ± 0.9	35.6 ± 1.4
Gravidity, median (range)	3 (0-6)	2 (0-5)
Parity, median (range)	1 (0-2)	1 (0-4)
Abortion, median (range)	2 (0-5)	1 (0-3)
Menstrual cycle phase, n (%)		
Proliferative	34 (57)	22 (58)
Secretory	26 (43)	16 (42)
Pain symptoms, n (%)	23 (38)	11 (29)
Infertility, n (%)	7 (12)	4 (11)

Abbreviation: SEM, standard error of the mean.

phosphorylated cyclic adenosine triphosphate response element–binding protein (p-CREB; 1:2000, #9198; Cell Signaling Technology), anti-tubulin (1:1000, ab6046; Abcam), or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:1000, Mab5465-100; Multi Sciences, Hangzhou, China). The membranes were further incubated with a secondary antibody (1:5000, ab97051/ab97023; Abcam) and were detected using an electrochemiluminescence detection kit (Biological Industries, Beit-Ha'emek, Israel). Relative protein levels were quantified on band volume with respect to GAPDH or tubulin as assessed by ImageJ (National Institutes of Health, Bethesda, Maryland).

Statistical Analysis

Data were analyzed using GraphPad Prism 5 (Graph Pad Software) and were presented as mean ± standard error of the mean (SEM). Statistical tests (χ^2 and Mann-Whitney *U* test) were performed to compare the frequency and median among groups. Multiple comparisons were first analyzed by 1-way analysis of variance, followed by Tukey tests. Unpaired Student *t* test was then performed to compare the mean values. The Spearman analysis was conducted to analyze the correlation among the groups. A *P* value of <.05 was considered statistically significant.

Results

Characteristics of the Patient

Table 1 summarizes the demographic characteristics of the patients. No statistical difference was observed between women with endometriosis and those without with respect to their age, gravidity, parity, abortion, pain frequency, infertility, or cycle stage ($P > .05$).

The BDNF Concentrations in Serum and PF in Women With and Without Endometriosis

The mean ± SEM concentrations of BDNF in serum ($P > .05$) or in PF ($P > .05$) in women with (in serum, 2108.9 ± 45.9 pg/mL, n = 60; in PF, 42.7 ± 3.7 pg/mL, n = 41) and without (in serum, 2085.1 ± 55.3 pg/mL, n = 38; in PF, 39.2 ± 3.2 pg/mL, n = 32) endometriosis were not significantly different. And among the serum or PF BDNF levels of women with early endometriosis (in serum, 2036.6 ± 72.2 pg/mL, n = 32; in PF, 36.2 ± 5.4 pg/mL, n = 13) and those with advanced endometriosis (in serum, 2191.5 ± 50.6 pg/mL, n = 28; in PF, 45.1 ± 4.7 pg/mL, n = 28), there were no significantly differences either ($P > .05$, Figure 2A; $P > .05$, Figure 2D). In women without endometriosis, BDNF concentrations in serum were significantly higher in the secretory phase (2326.3 ± 66.0 pg/mL, n = 16) than in the proliferative phase (1909.6 ± 59.7 pg/mL, n = 22; $P < .0001$; Figure 2B). But no similar situation occurred in serum BDNF levels in women with endometriosis (2191.6 ± 48.9 pg/mL, n = 26, vs 2045.6 ± 70.6 pg/mL, n = 34; $P > .05$; Figure 2B) or in PF BDNF levels in women with (47.0 ± 7.5 pg/mL, n = 14, vs 39.8 ± 4.0 pg/mL, n = 27; $P > .05$) or without (43.4 ± 5.5 pg/mL, n = 10, vs 37.2 ± 3.9 pg/mL, n = 22; $P > .05$) endometriosis (Figure 2E). Moreover, in women with endometriosis coexisting with endometriosis pain, both serum and PF BDNF levels (in serum, 2284.3 ± 51.5 pg/mL, n = 23; in PF, 58.8 ± 6.4 pg/mL, n = 16) were significantly higher than those without endometriosis pain (in serum, 1999.8 ± 61.1 pg/mL, n = 27; $P < .01$; in PF, 31.7 ± 2.9 pg/mL, n = 25; $P < .01$) and those in nonendometriosis women with (in serum, 2097.3 ± 67.2 pg/mL, n = 11; $P < .05$; in PF, 40.2 ± 4.4 pg/mL, n = 10; $P < .05$) or without (in serum, 2080.1 ± 73.6 pg/mL, n = 27; $P < .05$; in PF, 38.7 ± 4.3 pg/mL, n = 22; $P < .05$) pain (Figure 2C, F). No differences in serum or PF BDNF concentrations among women without endometriosis with pain and without pain were found ($P > .05$; Figure 2C, F).

Endometrial BDNF mRNA Levels in Women With and Without Endometriosis

In ectopic lesions of women with endometriosis, BDNF mRNA expression levels (fold expressions relative to control endometrium) were significantly higher (8.97 ± 1.44, n = 29) than those in eutopic endometrium (0.97 ± 0.14, n = 16; $P < .01$) and in control endometrium (1.23 ± 0.19, n = 18; $P < .01$; Figure 2G). Specifically, in this group, the expression levels were significantly higher in women with pain (12.74 ± 2.27, n = 14, vs 5.44 ± 1.29, n = 15; $P < .01$; Figure 2I) and did not correlate with the menstrual cycle phase (7.57 ± 2.36, n = 12, vs 9.95 ± 1.82, n = 17; $P > .05$; Figure 2H). In eutopic endometrium of women with endometriosis (1.46 ± 0.23, n = 6 vs 0.67 ± 0.11, n = 10; $P < .01$) and control endometrium (1.92 ± 0.48, n = 5 vs 0.96 ± 0.14, n = 13; $P < .05$), there were statistically significant differences between BDNF mRNA expression levels of the secretory and proliferative phase (Figure 2H).

Correlations Among BDNF Expression Levels in Serum, PF, and Endometriotic Lesions and Pain

Spearman analysis results showed that BDNF concentrations in serum ($r = .31$, n = 98, $P < .01$; Figure 3A) or PF ($r = .37$, n =

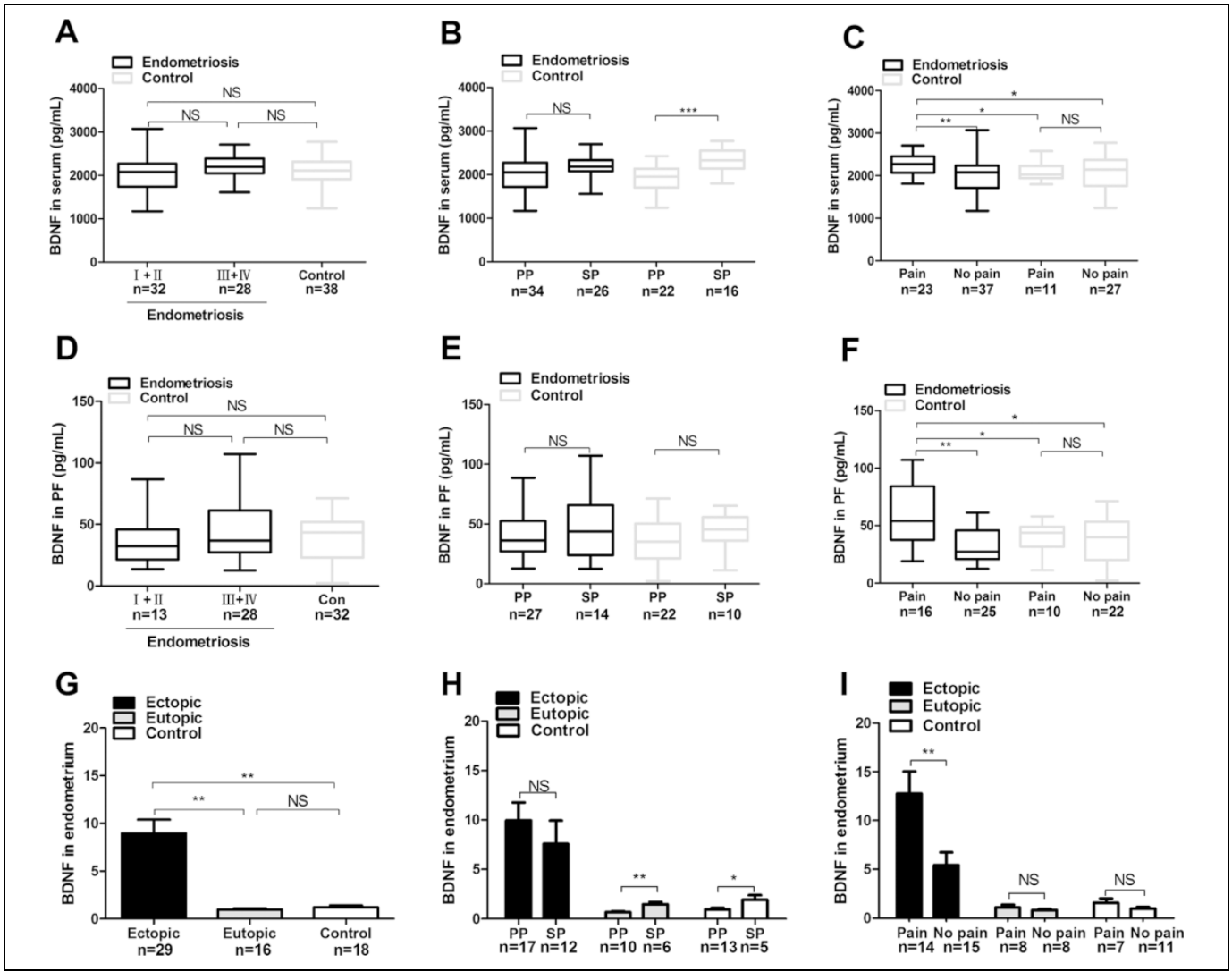

Figure 2. Increased levels of BDNF in serum, PF, and endometriotic lesions in women with endometriosis with pain. A-C, Serum BDNF concentrations were measured by ELISA and analyzed regardless of stage of disease (A), menstrual cycle phase (B), and endometriosis pain (C). D to F, The concentrations of BDNF in PF were measured and analyzed regardless of stage of disease (D), menstrual cycle phase (E), and endometriosis pain (F). G to I, The levels of BDNF mRNA expressions in ectopic lesions, eutopic, and control endometrium were tested by real-time polymerase chain reaction (PCR) and analyzed according to different classifications (G), menstrual cycle phase (H), and endometriosis pain (I). The number of primary cell cultures is indicated by n. BDNF indicates brain-derived neurotrophic factor; mRNA, messenger RNA; PF, peritoneal fluid; PP, proliferative phase; SP, secretory phase. *$P < .05$; **$P < .01$; ***$P < .0001$.

73, $P < .01$; Figure 3B), as well as the BDNF mRNA expression levels in ectopic lesions ($r = .52$, $P < .01$; Figure 3C), were positively correlated with the severity of pelvic pain. Moreover, when patients with endometriosis were divided into 3 groups by pain intensity: asymptomatic endometriosis (VAS = 0), minimal pain (VAS = 1-3), and severe pain (VAS = 4-10), compared to asymptomatic group, only in severe pain group, the BDNF levels in serum (2351.4 $\pm$ 61.1, n = 17 vs 1999.8 $\pm$ 61.1, n = 37; $P < .01$; Figure 3D), PF (63.9 $\pm$ 7.49, n = 11 vs 31.7 $\pm$ 2.9, n = 25; $P < .0001$, Figure 3E), and ectopic lesions (13.11 $\pm$ 2.96, n = 10 vs 5.44 $\pm$ 1.29, n = 15; $P < .05$; Figure 3F) were significantly higher. Furthermore, BDNF concentrations in serum ($r = .46$, n = 29, $P < .05$; Figure 3G) and PF ($r = .54$, n = 28, $P < .01$; Figure 3H) were positively correlated with BDNF mRNA expression levels in ectopic lesions. A significantly positive correlation between the serum BDNF concentrations and PF BDNF concentrations ($r = .37$, n = 41, $P < .05$; Figure 3I) was observed in women with endometriosis.

The BDNF Concentrations in Patients With Endometriosis With Infertility

Statistical analyses showed that in the endometriosis group, infertile patients had a lower level of BDNF concentrations in serum (1921.4 $\pm$ 153.5, n = 7 vs 2133.6 $\pm$ 47.4, n = 53; $P > .05$; Figure 4A), PF (37.8 $\pm$ 8.8, n = 6 vs 43.1 $\pm$ 4.1, n = 35; $P > .05$; Figure 4B), and mRNA in ectopic lesions

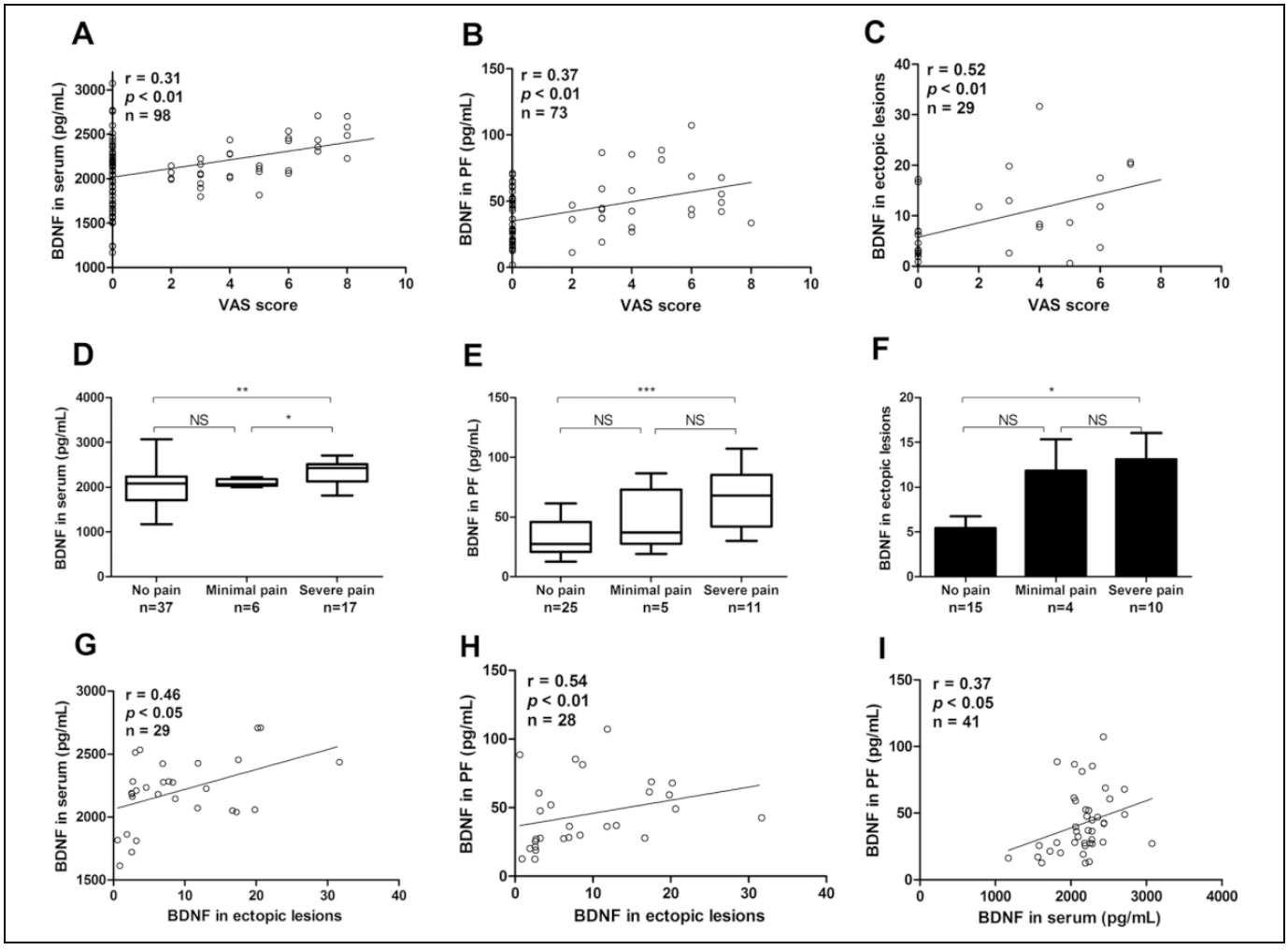

Figure 3. The correlations between brain-derived neurotrophic factor (BDNF) concentrations and the severity of endometriosis pain. A to C, The severity of pelvic pain quantified by visual analog scale (VAS) score was positively correlated with serum BDNF concentrations (A) and peritoneal fluid (PF) BDNF concentrations (B). In women with endometriosis with pain, VAS scores were significantly correlated with the levels of BDNF messenger RNA (mRNA) expressions in ectopic lesions (C). D to F, The BDNF expression levels in serum (D), PF (E), and ectopic lesions (F) were all significantly elevated in women with severe pain compared to asymptomatic women. G to I, Spearman analysis showed that in women with endometriosis, both the concentrations of BDNF in serum (G) and in PF (H) were positively significantly correlated with the levels of BDNF mRNA expression in ectopic lesions. In addition, there was a significant correlation between serum BDNF concentrations and PF BDNF concentrations in women with endometriosis (I).

(6.99 ± 1.41, n = 5 vs 9.88 ± 1.72, n = 24; $P > .05$; Figure 4C) compared to fertile patients with no statistical difference. Moreover, in the control group without endometriosis, infertile women also had a nonsignificant lower level of BDNF concentrations in serum (1956.3 ± 122.7, n = 4 vs 2100.2 ± 60.0, n = 34; $P > .05$; Figure 4A) and PF (26.7 ± 6.3, n = 4 vs 40.9 ± 3.5, n = 28; $P > .05$; Figure 4B).

Immunoreactivity of BDNF and TrkB in Endometrium in Women With and Without Endometriosis

Brain-derived neurotrophic factor was widely expressed in both ectopic endometrium and eutopic endometrium. In the ectopic endometrium, BDNF was mainly expressed in the glandular EPCs and also in stromal cells and interstitium. In the eutopic endometrium from patients with endometriosis and control endometrium, BDNF was mainly expressed in stromal cells and interstitium, but rarely in glandular EPCs (Figure 5A-F). And TrkB, receptor of BDNF, was mainly expressed in the endometrial EPCs but also observed in ESCs and interstitium (Figure 5G-L). The IHC scores of BDNF and TrkB expression in ectopic lesions (BDNF, 4.01 ± 0.17, n = 29; TrkB, 2.10 ± 0.18, n = 29) were significantly higher than those in eutopic (BDNF, 2.61 ± 0.29, n = 16, $P < .01$; TrkB, 1.50 ± 0.19, n = 16, $P < .05$) and control endometrium (BDNF, 2.04 ± 0.13, n = 18, $P < .01$; TrkB, 1.51 ± 0.15, n = 18, $P < .05$). In addition, the scores of BDNF and TrkB expression in the ectopic lesions were positively correlated with pain ($P < .01$, $P < .05$; Table 2).There was no significant difference between the BDNF and TrkB expression scores of eutopic and control

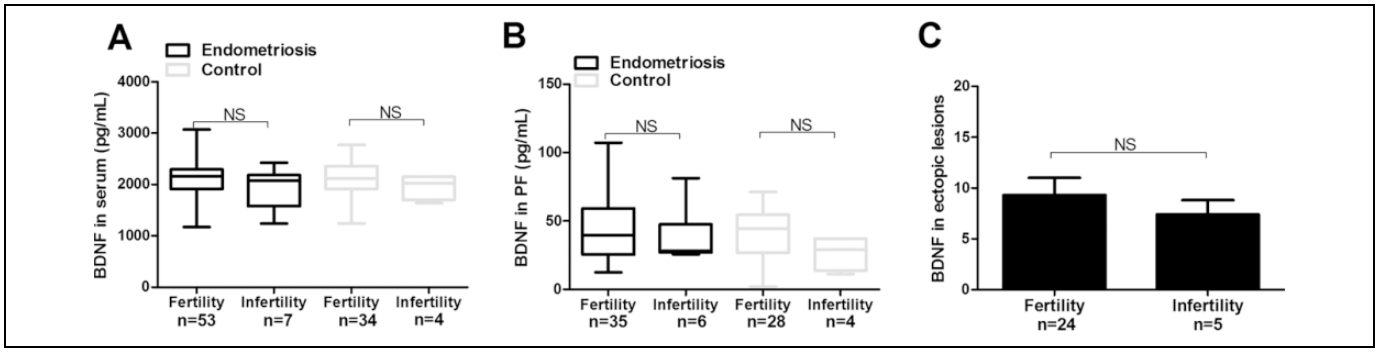

Figure 4. The correlations between brain-derived neurotrophic factor (BDNF) concentrations and infertility. No significant difference in BDNF expression levels in serum (A), peritoneal fluid (PF) (B), or ectopic lesions (C) were found between women with or without infertility.

endometrium ($P > .05$, $P > .05$). During the endometrial cycle phase, eutopic ($P < .01$, $P < .05$) and control endometrium ($P < .05$, $P < .05$), but not ectopic lesions ($P > .05$, $P > .05$), had significantly higher scores of BDNF and TrkB expression in the secretory phase than in the proliferative phase (Table 2).

Regulation of BDNF Expression in ESCs In Vitro by IL-1β, E_2, and p-ERK1/2 Inhibitor

Brain-derived neurotrophic factor mRNA expression levels in ESCs increased gradually after treatment with IL-1β and reached their peaks at 24 hours (2.42 ± 0.30; Figure 6A). When treated with E_2, these levels increased continuously and reached their peak at 2 hours (2.03 ± 0.22) and then decreased at 24 hours (0.93 ± 0.15; Figure 7A). Furthermore, BDNF mRNA expression levels in ESCs induced by IL-1β or E_2 had no increase (Figures 6E and 7E) when pretreated with PD98059.

Western blot analysis showed a specific band of 35 kDa for BDNF, 2 bands of 42/44 kDa for p-ERK1/2, and a specific band of 45 kDa for p-CREB. Similar to BDNF mRNA expression, BDNF protein expression levels in ESCs increased in a time-effect manner and reached their peaks at 24 hours (2.10 ± 0.13) and 2 hours (1.84 ± 0.07), respectively (Figures 6B and 7B) after the IL-1β or E_2 treatment. However, no increase in the BDNF protein levels in ESCs was observed when the ESCs were pretreated with PD98059 (Figures 6F and 7F). The protein levels of p-ERK1/2 in ESCs significantly increased after the IL-1β (Figure 6C) or E_2 (Figure 7C) treatment, but decreased when the ESCs were pretreated with PD98059 (Figures 6G and 7G). Meanwhile, p-CREB levels in ESCs significantly increased and reached their peaks at 30 minutes (21.10 ± 2.20, 9.90 ± 2.70) after the IL-1β and E_2 treatment (Figures 6D and 7D). Even after the ESCs were pretreated with PD98059, p-CREB expression levels in ESCs remained significantly higher at 15 minutes, 30 minutes, and 1 hour than the initial levels ($P < .05$) but were significantly lower when compared with those of ESCs being solely treated with IL-1β or E_2 (Figures 6H and 7H).

Discussion

Brain-derived neurotrophic factor is an NT that belongs to the family of growth factors and has been found to be extensively expressed not only in the central and peripheral nervous system but also in the female reproductive system.[34-36] In a normal ovary, BDNF and its specific receptor TrkB affect the development of follicle, corpus luteum, and embryo as well as the secretion of sex hormones.[37] Moreover, BDNF expression in normal endometrium is higher in the luteal phase than in the follicular phase.[38] In our study, BDNF concentrations in serum and BDNF mRNA and protein expression levels were all higher in the secretory phase than in the proliferative phase in the nonendometriosis women and eutopic endometrium and control endometrium, respectively, but not in women with endometriosis or in ectopic lesions. Furthermore, the BDNF mRNA and protein expression levels, as well as the TrkB protein expression in ectopic lesions, were all increased and were higher than those of eutopic or control endometrium. Thus, even though BDNF concentrations in serum or PF were similar in women with and without endometriosis and did not correlated with the severity of endometriosis, BDNF expression may still be involved in the pathogenesis of endometriosis.

Interestingly, the BDNF protein expression in serum, PF, and ectopic lesions, as well as the BDNF mRNA expression and TrkB protein expression in ectopic lesions, was correlated with endometriosis pain. It has been reported that in women with peritoneal endometriosis, NGF produced by peritoneal ectopic lesions were released into the peritoneal cavity, thereby resulting in a high level of NGF in PF.[13] Similarly, BDNF produced by ovarian ectopic lesions may also be released into the peritoneal cavity and peripheral blood, thus results in increased BDNF concentrations in serum and PF. Our results showed that serum and PF BDNF concentrations were remarkably correlated with the levels of BDNF mRNA expression in ectopic lesions. Therefore, BDNF expression in ectopic lesions may play a key role in triggering endometriosis pain (Figure 8).

Several studies found increased BDNF concentrations in plasma in women with endometriosis. Giannini et al[24] reported that plasma levels are significantly higher in patients with

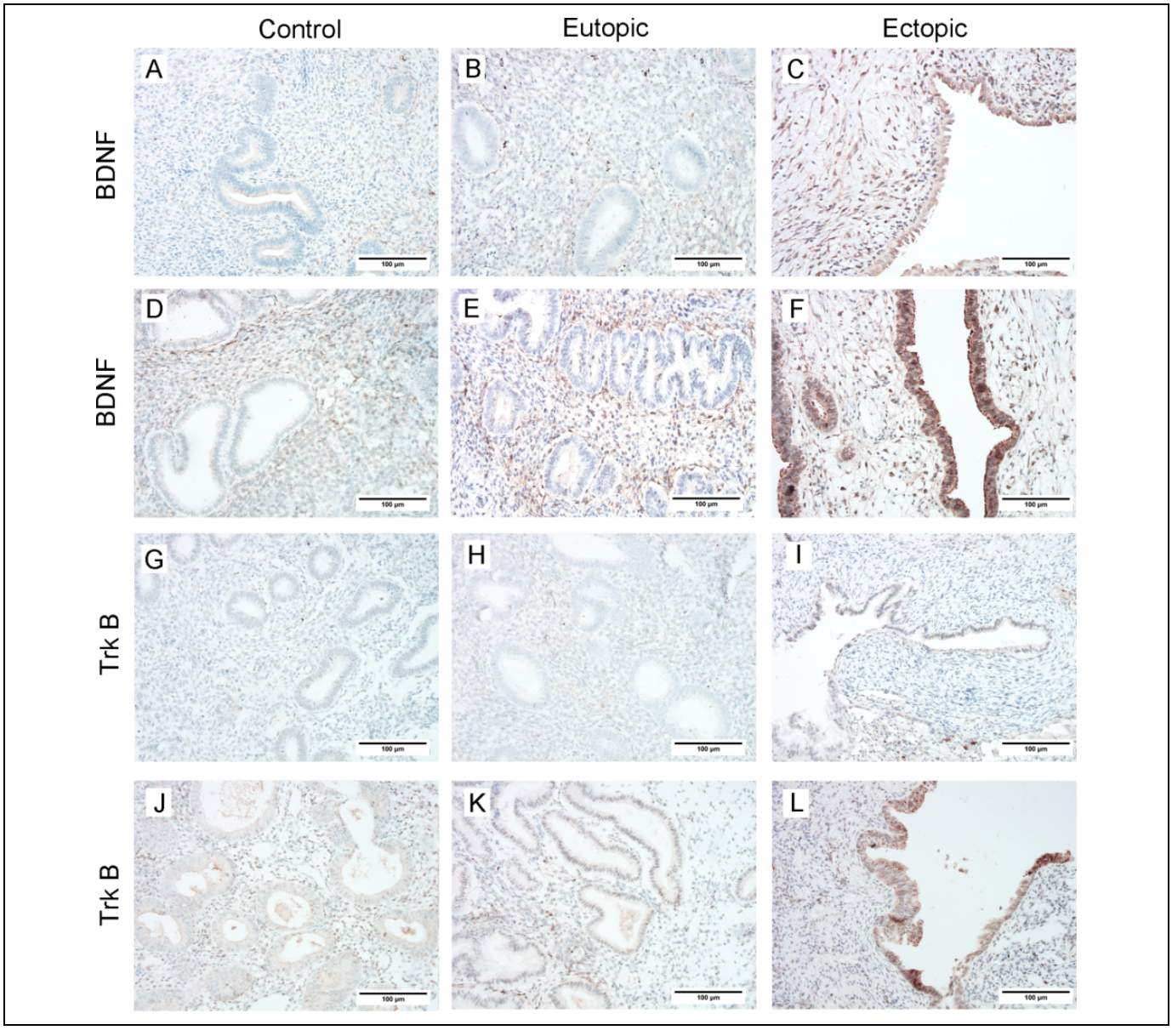

Figure 5. Brain-derived neurotrophic factor (BDNF) and tyrosine receptor kinase B (TrkB) immunoreactive staining in ectopic lesions as compared with eutopic endometrium and control endometrium. A to F, Immunohistochemical analysis showed that in control (A, D) and eutopic (B, E) endometrium, BDNF is mainly expressed in stromal cells and interstitium, but rarely in glandular epithelial cells, and correlated with the menstrual cycle phase (A, B, proliferative phase; D, E, secretory phase). However, BDNF was widely expressed in all endometrial tissues (C, F). In endometriotic lesions, BDNF is mainly expressed in glandular epithelial cells but also observed in stromal cells and interstitium and is significantly higher in ectopic lesions from women with pain (F) than that without pain (C). G to L, TrkB, affinity receptor of BDNF, is mainly expressed in endometrial glandular epithelial cells (G, J, control endometrium; H, K, eutopic endometrium; G, H, proliferative phase; J, K, secretory phase), but also observed in ectopic stromal cells and interstitium (I, L). Additionally, the levels of TrkB expression in ectopic lesions (L) from women with pain were significantly higher than those without pain (I). 400×, scale bar = 100 μm.

endometriosis than in patients without endometriosis and decreased significantly after surgery. However, only women with infertile endometriosis and fertile health were included in this study. Moreover, they did not consider the effect of pain. Wessels et al[23] found that the plasma concentrations of BDNF were significantly higher in women with endometriosis (especially at stages 1 and 2) than in women without endometriosis. Rocha et al[25] also reported that women with ovarian endometrioma have higher preoperative plasma BDNF concentrations compared with women with other benign ovarian tumors. However, while Rocha et al found the significant positive linear correlation between plasma BDNF levels and pelvic pain intensity, Wessels et al did not. The use of different samples may explain this phenomenon as Christian et al[39] reported that serum BDNF levels were associated with race. In our study, serum samples were used to measure peripheral blood

Table 2. Immunohistochemical Score of BDNF and TrkB in Endometrium in Women With and Without Endometriosis.[a]

Variables	IHC score	
	BDNF	TrkB
Ectopic endometrium (n = 29)	4.01 ± 0.17	2.10 ± 0.18
Proliferative (n = 15)	4.31 ± 0.26	2.39 ± 0.26
Secretory (n = 14)	3.70 ± 0.18	1.80 ± 0.23
With pain (n = 15)	4.55 ± 0.19	2.51 ± 0.22
Without pain (n = 14)	3.44 ± 0.18[b]	1.67 ± 0.24[c]
Eutopic endometrium (n = 16)	2.61 ± 0.29[d]	1.50 ± 0.19[e]
Proliferative (n = 10)	1.96 ± 0.22	1.18 ± 0.18
Secretory (n = 6)	3.70 ± 0.33[f]	2.03 ± 0.29[g]
With pain (n = 8)	2.78 ± 0.49	1.73 ± 0.29
Without pain (n = 8)	2.45 ± 0.31	1.28 ± 0.22
Control endometrium (n = 18)	2.04 ± 0.13[d]	1.51 ± 0.15[e]
Proliferative (n = 13)	1.89 ± 0.15	1.31 ± 0.15
Secretory (n = 5)	2.44 ± 0.15[g]	2.04 ± 0.29[g]
With pain (n = 7)	2.06 ± 0.14	1.46 ± 0.31
Without pain (n = 11)	2.04 ± 0.19	1.55 ± 0.16

Abbreviations: BDNF, brain-derived neurotrophic factor; TrkB, tyrosine receptor kinase B; IHC score, immunohistochemical score; SEM, standard error of the mean.
[a]Values are mean ± SEM.
[b]$P < .01$ (with pain vs without pain).
[c]$P < .05$ (with pain vs without pain).
[d]$P < .01$ (ectopic vs eutopic or control).
[e]$P < .05$ (ectopic vs eutopic or control).
[f]$P < .01$ (proliferative vs secretory).
[g]$P < .05$ (proliferative vs secretory).

BDNF levels, which may explain our average serum BDNF concentrations were approximately 2-fold of the aforementioned results and similar to the reports of Christian et al. More importantly, ovarian endometrioma patients with or without pelvic pain were all recruited in our study, thus increasing comparability between the case and control groups. Although differences between the serum or PF BDNF concentrations of the endometriosis group and controls were not found, serum and PF BDNF levels in women with endometriosis with pain were significantly higher than in endometriosis women without pain and control groups, which might explain both the reports of Rocha et al and Wessels et al.

As Buyuk and Seifer[29] have reported, there was a lower level of BDNF concentrations in follicular fluid in women with endometriotic infertility compared to those with male factor infertility. Moreover, Zhang et al[30] found that infertile patients with the BDNF *Met/Met* genotype had a poor in vitro fertilization outcome compared with the BDNF *Val/Val* genotype women, which might result from the decreased BDNF concentrations in follicular fluids. In this study, we found that infertile patients had a lower level of BDNF serum and PF concentrations in endometriosis or control group with nonsignificant difference, as well as BDNF mRNA levels in ectopic lesions. As both endometriosis and BDNF are racially characterized, more samples are needed for future experiments.

To investigate the possible mechanisms of BDNF involved in endometriosis pain, ESCs were cultured in vitro. The results

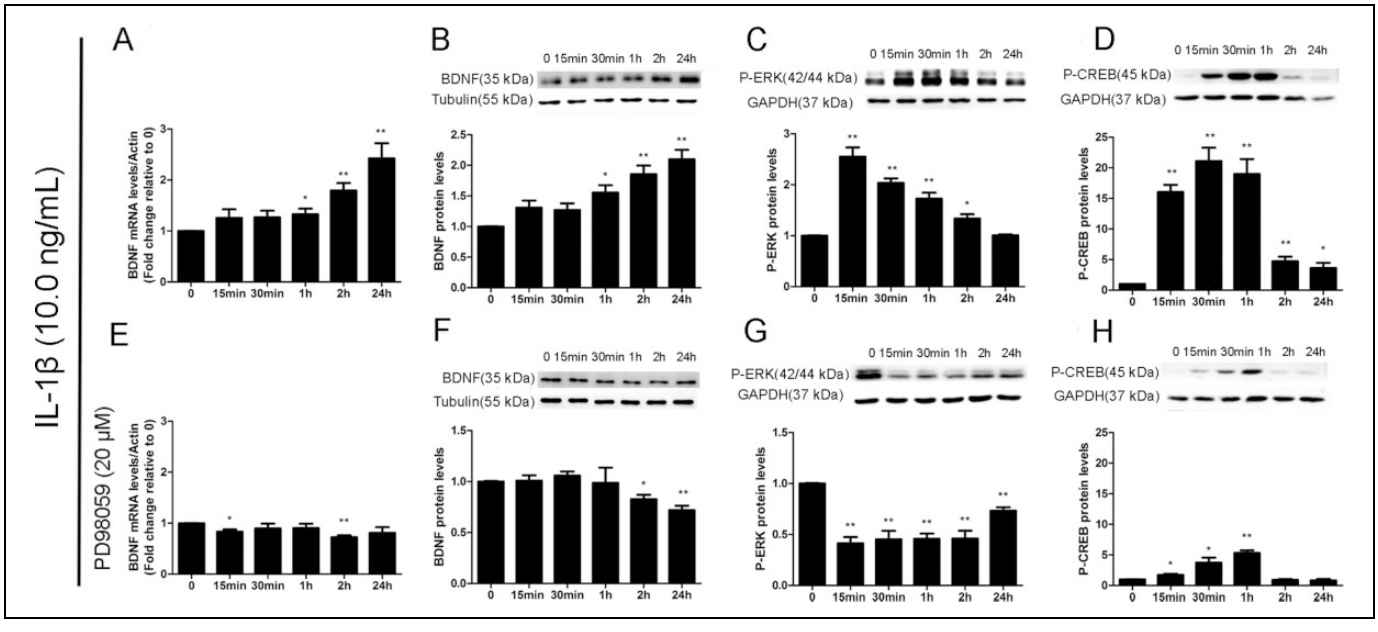

Figure 6. Increased brain-derived neurotrophic factor (BDNF) expression levels in endometriotic stromal cells (ESCs) induced by interleukin (IL) 1β via extracellular-regulated protein kinase 1/2 (ERK1/2)/cyclic AMP-response element–binding protein (CREB) pathway. The levels of BDNF messenger RNA (mRNA) expression in ESCs increased at 1 hour, 2 hours, and reached the peak at 24 hours after treated with IL-1β (A), which were totally blocked by PD98059 (E). The BDNF protein expression gradually increased from 1 hour to 24 hours (B) but did not increase when the ESCs were pretreated with PD98059 (F). The levels of p-ERK1/2 protein expression reached the peak at 15 minutes after IL-1β treatment, then gradually decreased (C), and were significantly inhibited by PD98059 (G). The levels of p-CREB protein expression increased at 15 minutes, reached the peak at 30 minutes compared to the initial levels (D), and were partly blocked by PD98059 (H) compared with those after treated with IL-1β alone. n = 3. Results are presented as the mean ± standard error of the mean (SEM). *$P < .05$; **$P < .01$.

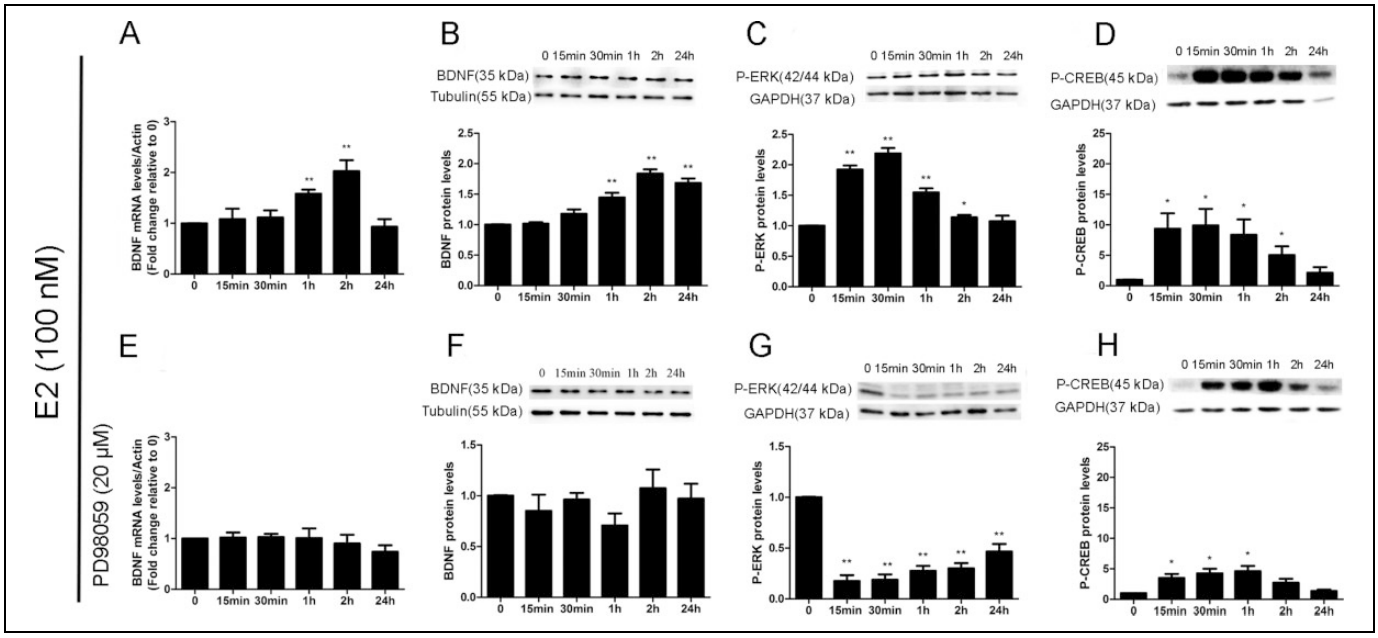

Figure 7. Increased brain-derived neurotrophic factor (BDNF) expression levels in endometriotic stromal cells (ESCs) induced by estradiol (E_2) via extracellular-regulated protein kinase 1/2 (ERK1/2)/cyclic AMP-response element–binding protein (CREB) pathway. The E_2 also elevated the levels of BDNF messenger RNA (mRNA) (A) and protein (B) with a time efficacy, which were totally prevented by PD98059 (E, F). Similar to interleukin (IL) 1β, E_2 enhanced the levels of p-ERK1/2 (C) and p-CREB (D) protein expression with a peak at 30 minutes. While the elevation of p-ERK1/2 expression levels induced by E_2 was totally inhibited by PD98059 (G), the levels of p-CREB protein expression also increased at 15 minutes, 30 minutes, and 1 hour after E_2 stimulation with presence of PD98059 compared to the initial levels (H) but were significantly lower as compared with those after treated with E_2 alone. n = 3. Results are presented as the mean ± standard error of the mean (SEM). *P < .05; **P < .01.

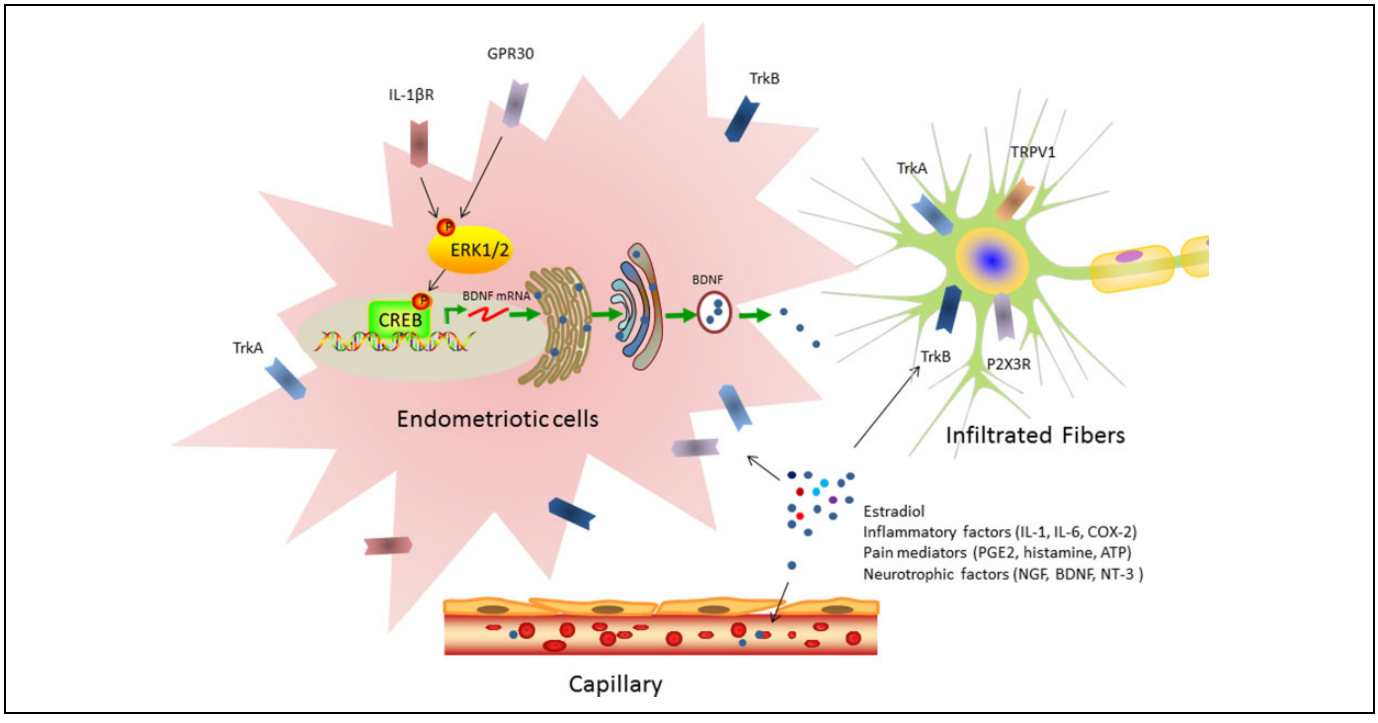

Figure 8. Schematic illustration of brain-derived neurotrophic factor (BDNF) induced in endometriosis pain. ATP indicates adenosine triphosphate; BDNF, brain-derived neurotrophic factor; COX-2, cyclooxygenase-2; CREB, cAMP-responsive element–binding protein; E_2, estradiol; ERK, extracellular-regulated protein kinase; GPR30, G protein-coupled estrogen receptor 30; IL-1, interleukin 1; IL-6, interleukin 6; IL-1βR, interleukin 1 beta receptor; NGF, nerve growth factor; NT-3, neurotrophin-3; P2XR3, P2X purinoceptor 3; PGE_2, prostaglandin E_2; TrkA, tyrosine receptor kinase A; TrkB, tyrosine receptor kinase B; TRPV1, transient receptor potential cation channel subfamily V member 1.

showed that BDNF mRNA expression in ESCs increased after the ESCs were treated with IL-1β or E_2 but did not increase when the ESCs were pretreated with p-ERK1/2 inhibitor. Western blot analysis showed that BDNF, p-ERK1/2, and p-CREB expressions levels in ESCs all increased when the ESCs were treated with IL-1β or E_2. In particular, p-ERK1/2 first peaked, followed by p-CREB, and finally BDNF. And these effects were blocked by p-ERK1/2 inhibitor. Thus, BDNF is involved in endometriosis pain via the MAPK signaling pathway, although the p-CREB inhibitor had not been used to intervene the ESCs in this study.

The CREB is the downstream molecule of the MAPK signaling pathway. Phosphorylated ERK1/2 indirectly promotes CREB phosphorylation through downstream signaling molecules. Brain-derived neurotrophic factor transcription is then promoted when p-CREB binds the promoter region of the BDNF gene.[40] In women with endometriosis, inflammatory mediators and estrogen concentrations in endometriotic lesions increase.[6,7,41,42] Furthermore, MAPK expression in endometriotic lesions increases,[43] suggesting that increased MAPK expression in endometriotic lesions may be induced by inflammatory mediators and estrogen, thereby resulting in an increased level of BDNF in endometriotic lesions.[44,45] Recently, Guo et al[46] found that estrogen had a positive effect on rats with migraine model through the BDNF/TrkB and ERK1/2/CREB axes. Moreover, Dong et al[31] lately reported that in endometrial cells, BDNF was regulated by E_2 and improved cell proliferations via ERK/STAT3 signaling pathway. As endometriosis is recognized as an estrogen-independent inflammatory disease, estrogen, inflammatory factor, and NTs play important roles in the occurrence and development of endometriosis and are closely related to the symptoms manifested by endometriosis.

Meanwhile, the Aδ and C sensory nerve fibers have been detected near endometriotic glands in ectopic lesions[47] and in the superficial functional layer of eutopic endometrium from women with endometriosis.[48] Neurotrophins in ectopic lesions play an essential role in the axonal growth and survival of infiltrated sensory nerve fibers.[12] Lentz et al[49] demonstrated that BDNF supported the axonal branching and growth of lathellipodia of sensory neurons in vitro. Furthermore, innervation in ectopic lesions induced the inception of endometriosis pain and subsequently contributed later to the maintenance and modulation of severity.[50,51] Therefore, BDNF production by endometriotic cells stimulates the sprouting of neurons and supports the growth and differentiation of new neurons. Growing and branching nerve fibers stimulated by pain mediators promote nociceptive sensitization and thus trigger the endometriosis pain signal cascade.[51]

In conclusion, our results show that the BDNF concentrations in serum and PF, as well as BDNF mRNA and protein expression levels and TrkB protein expressions in ectopic lesions, are all correlated with endometriosis pain. These results suggest that the involvement of BDNF in endometriosis pain signal transduction is due to the MAPK signaling pathway. However, these results require further validations from future studies.

Authors' Note

Shaojie Ding, Tianhong Zhu, and Yonghong Tian contributed equally to this study.

Acknowledgments

The authors would like to acknowledge the skillful work of Qi Cheng in the running of ELISA measurements and the assistance of immunohistochemical facility of Caiyun Zhou.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article. This study was supported by National Natural Science Foundation of China (grant nos 81270672, 81471433, 81471495, and 81671429), the Nature Science Foundation of Zhejiang Province (grant nosY2110181 and Y2110128), the Science and Technology Fund of Zhejiang Province (grant nos 2011C13028-1 and 2013C33149), and the Key Medical Science (Innovation) Project of Zhejiang Province.

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