# Expression of BDNF and TrKB in Endometriosis and Dysmenorrhea

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**Research**

# Abstract

**Background:** Brain-derived neurotrophic factor (BDNF) has been recognized as a regulator in the formation and maintenance of chronic pain in various chronic disorders. BDNF together with its high-affinity tyrosine kinase type B (TrkB) receptor were found to be extensively expressed in mammalian female reproductive system. However, BDNF and TrkB expression in different stages of endometriosis, and the correlation between their expression in ectopic lesions and endometriosis pain remains unclear.

**Methods:** This study enrolled 78 women underwent laparoscopic surgery. 62 women diagnosed as ovarian endometrioma, were recruited in the study group.16 women diagnosed as ovarian benign tumors were in the control group. The message RNA (mRNA) level of BDNF and TrKB was detected by real-time PCR, while the protein level was detected by immunohistochemical staining for eutopic and ectopic endometrium in both groups. Dysmenorrhea was assessed by the visual analogue scale (VAS) before the surgery.

**Results:** Immunohistochemical analysis revealed that the expression of BDNF and TrKB in ovarian endometriotic lesions was the highest, followed by those in eutopic and normal endometrium ($P$<0.05). There was significant difference among each stage of endometriosis for the expression, which increased with the severity of stages. The results of RT-qPCR and immunohistochemistry were consistent with each other. Furthermore, The correlation between the mRNA expression of BDNF, TrKB in eutopic endometrium, and dysmenorrhea VAS score revealed that r=0.52 and 0.56, respectively (P<0.05). And when it came to BDNF, TrKB in eutopic endometrium, the correlation (r) was 0.82 (P<0.05).

**Conclusions:** BDNF and TrKB may play essential roles in promoting disease progression during the development of endometriosis, and are closely related to dysmenorrhea caused by endometriosis.

# Introduction

Endometriosis is one of the most common chronic gynecological conditions, characterized by the presence of endometrial-like tissue which undergoes proliferation, bleeding and regeneration outside the uterine cavity. This disease has become one of the most important causes of infertility and pelvic pain, affecting 6–10% of women of reproductive age (1). There are several and not fully confirmed theories that describe endometriosis pathogenesis. However, the exact etiology of endometriosis remains uncertain. Recent study showed that the endometriotic lesions are caused by repeated tissue injury and repair(TIAR).TIAR is considered an evolutionarily conserved system independent of steroidal precursors that are usually produced in endocrine glands such as the adrenal gland and gonads. Increased inflammatory mediators such as interleukin (IL) 1β and 6, tumor necrosis factor α, and prostaglandins are considered to sensitize sensory neurons by stimulating the sensory nerve fibers within the ectopic lesions and thus trigger the pain signal cascade in women with endometriosis.Endometriosis pain may be considered as a kind of inflammatory and neuropathic pain.(2).

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of secreted growth factors, classically involved in the development, growth and function of both central and peripheral neurons. Brain-derived neurotrophic factor plays an important role in nociceptive and neuropathic pain. BDNF 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. BDNF together with its high-affinity tyrosine kinase type B (TrkB) receptor were found to be extensively expressed in mammalian female reproductive system.Increased BDNF concentrations in serum have been demonstrated in patients with endometriosis with central sensitivity syndrome.However,there are few reports whether there are differences in the expression of BDNF and TrKB in different stages of endometriosis, and the correlation between BDNF and TrKB expression levels in ectopic lesions and endometriosis pain remains unclear.

In the present study, we aimed to detect the expression of BDNF and TrKB in eutopic endometrium and ovarian endometriotic lesions of patients with endometriosis, and analyzed the expression differences in patients with and without endometriosis. We then examined the expression of BDNF and TrKB in different stages of endometriosis. We also discussed whether there are correlations between BDNF and TrKB and the severity of endometriosis and dysmenorrhea, which may provide a theoretical basis for etiological mechanism of endometriosis and treatment of endometriosis-correlated pain.

# Methods

# Patients and specimens

Patients diagnosed with endometriomas and ovarian benign tumors, which were referred from May 2017 to July 2018. The ethical committee of the Beijing Obstetrics and Gynecology Hospital approved the experiments. Written informed consent was obtained from each patient before sampling. The study group acquired 62 patients who underwent laparoscopic surgery for ovarian endometrioma and diagnosed with histological pathology at Beijing Obstetrics and Gynecology Hospital. Endometriosis was surgically and histologically diagnosed as Stage I-IV according to the revised American Fertility Society (r-AFS) classification scheme (3). The patients were staged according to the criteria where 6 were in the stage II, 3 in proliferative phase and 3 in secretory phase, the mean ages were 33.70 ± 5.80; 19 were in the stage III, 9 in proliferative phase and 10 in secretory phase, the mean ages were 33.20 ± 6.20; 21 were in the stage IV, 9 in proliferative phase and 12 in secretory phase, the mean ages were 32.50 ± 6.40; the difference of the ages between these patients was not significant statistically ($P > 0.05$). Patients were excluded who hadn't a history of a sexual life, had hormonal medicine within 3 months before surgery, with adenomyosis, polycystic ovary syndrome (PCOS), pelvic inflammatory disease, endometrial lesions, genital dysplasia, malignant tumors of any organ, pregnancy and neurological diseases.

16 patients in reproductive age with laparoscopic surgery for ovarian benign tumors and diagnosed with histological pathology as well as no endometriosis were recruited as control group; 8 cases were in the proliferative phase and 8 in the secretary phase; the mean ages were 33.40 ± 7.00. The difference of the

ages between this group and endometriosis group was not statistically significant (*P* > 0.05). Excluded criteria was as same as the endometriosis group. The basic characteristics of the two groups of patients are shown in Table 1. Eutopic endometrial and ovarian endometriotic lesions were taken from endometriosis group, and normal endometrium were from control group, which was about 1cm×1cm×0.5cm.

Table 1. Overview of the demographics and other characteristics of the recruited patients

| Parameter | Endometriosis N = 46 | Control N = 16 | *P* |
|---|---|---|---|
| Age, mean (SD) | 32.97 (7.23) | 33.40(7) | 0.74 |
| Gravidity, median (range) | 2 (0, 5) | 2 (1, 5) | 0.76 |
| Parity, median (range) | 1 (0, 5) | 1 (0, 2) | 0.59 |
| Haemoglobin, mean (SD) | 102.8 (27) | 120.9 (15.2) | 0.01 |
| CA125, mean (SD) | 55.8 (5.2) | 13.32 (6.24) | 0.995 |
| PBAS, mean (SD) | 158.7 (67.6) | 112.9 (25.9) | 0.12 |
| Menstrual cycle (Proliferative phase) n (%) | 21 (45.7%) | 8 (50%) | 0.98 |
| Abbreviations: PBAS, Pictorial Blood Loss Assessment Chart Score. | | | |

Differences among groups were analysed by Student's t-test and the Mann–Whitney U test.

# Pain (dysmenorrhea) evaluation

A VAS (0-10 score, 0 = no pain and 10 = maximum pain) was used before surgery to evaluate the severity of dysmenorrhea of patients with endometriosis. The VAS scores were grouped into three levels: mild (ranging from 0 to 3), moderate (ranging from 4 to 6), and severe (ranging from 7 to 10).

# Immunohistochemistry and Quantification by H-score

All samples were fixed with 10-mM PBS-buffered 10% formalin, embedded in paraffin, and cut into sections of 4-μm thickness. For heat-induced epitope retrieval, deparaffinized sections in 0.01 mmol/L citrate buffer were heated for 20min at 95°C using a microwave oven. Immunohistochemical staining was carried out according tothe avidin-biotin immunoperoxidase method using the UltraSensitiveTM S-P kit (Maixin corporation, China) Endogenous peroxidase activity was blocked for 10min with 50μl peroxidase blocker from UltraSensitive[TM] S-P kit, followed by washing with PBS for 3 times, 2min each

time. The sections were incubated at 4 °C overnight with the following primary antibodies: anti- BDNF (1:50 dilution, ab108319; Abcam, Cambridge, MA, USA), anti- TrKB (1:100 dilution, ab108319; Abcam, Cambridge, MA, USA)The sections were then rinsed and incubated with biotinylated secondary antibodies for 10 min. After washing with PBS, the sections were further incubated with horseradish peroxidaseconjugated streptavidin for 5 min and finally treated with diaminobenzidine in 0.01% $H_2O_2$ for 5 min. The slides were counterstained with Meyer's Hematoxylin, and the stained sections were observed under a microscope (Axio Imager 2, Zeiss, Oberkochen, Germany).The immunoreactive intensity of the BDNF and TrKB - stained cells was quantified by a modified method of histogram scoring (H-score) as described previously (4). The staining intensity was graded as 0-no staining, 1-weak,2-moderate, and 3-strong. A total percentage score (% of cells with staining intensity ≥1; i.e., the sum of the percentage of cells with intensities 1, 2, and 3) was used to semiquantitatively evaluate tissue expression of BDNF and TrKB. The H-score was calculated using the following formula: Hscore = [(% at 0) ×0] + [(% at 1+)×1] + [(% at 2+) ×2] + [(%at 3+) ×3].

## Statistical analysis

Statistical analysis was done by Statistical Product and Service Solutions (SPSS) 22.0. Quantified data was expressed as mean±standard deviation. Comparison between groups was done with independent-sample t-test. Analysis of variance (ANOVA) comparing the means of more than two groups. The correlations among BDNF and TrKB expression, and the severity of dysmenorrhoea were assessed using Spearman's correlation. *P* values of <0.05 were considered significant.

## Results

## BDNF and TrKB protein expression levels in endometriosis and control groups

BDNF and TrKB were stained in normal endometrium, eutopic endometrium, and ectopic endometrium. In both proliferative and secretory phases, BNDF had the highest protein expression level in ectopic endometrial tissue, followed by eutopic and normal endometrium (*P*<0.05). So did the expression of TrKB (P<0.05). As shown in Fig. 1D and 2D. However, when comparing the expression of BDNF and TrKB between proliferative and secretory phases, there was no statistical difference in both ectopic and the normal endometrium (*P*>0.05).

## Expression of BDNF, TrKB in different stages of endometriosis

Afterwards, the expression of BDNF in eutopic and ectopic endometrium in each stage was analyzed. In patients with endometriosis stage II, the expression of BDNF in the ectopic endometrium was significantly

higher than that in the eutopic endometrium ($P$<0.05). In patients with endometriosis stages III and IV, the expression of BDNF in the ectopic endometrium was also higher than that in the eutopic endometrium ($P$<0.05). As shown in Fig. 2.

Farther, the analysis of the expression of BDNF in each stage of the ectopic endometrium showed that the expression of stage IV was significantly higher than that of stage III and II. The expression in phase III is also significantly higher than in phase II ($P$<0.05). However, the expression of BDNF in phase III had no significant difference from that in phase II ($P$>0.05). Meanwhile, in the analysis of the BDNF expression in the eutopic endometrium in each stage, it was found that as the stage increased, its expression gradually increased ($P$<0.05).

Similarly, in the analysis of TrKB expression in each stage, we found the same pattern. In patients with endometriosis stage II, stage III and stage IV, TrKB expression was found to be higher in ectopic endometrium than in eutopic endometrium ($P$<0.05), as shown in Fig.2. In eutopic and ectopic endometrium of patients with endometriosis, TrKB expression increased with the increase of stage. There were significant differences in TrKB expression in each phase of the eutopic endometrium ($P$<0.05). While in the analysis TrKB expression in ectopic endometrium, there were significant differences between each stage ($P$<0.05), but not for the comparison between stage III and stage II ($P$>0.05).

## The correlation between mRNA expression of BDNF, TrKB in endometriosis group and dysmenorrhea VAS score

To analysis the correlation among BDNF, TrKB and dysmenorrhea, the mRNA expression of BDNF, TrKB in endometriosis group were explored, as can be seen in the figure 3. Spearman rank correlation was conducted to analyze the correlation between the mRNA expression of BDNF in eutopic endometrium of endometriosis group and dysmenorrhea VAS score and revealed that $r$=0.52, demonstrating there was a moderate positive correlation between the expression of BDNF in eutopic endometrium and dysmenorrhea VAS score ($P$<0.05); but there was no correlation between the expression of BDNF in ovarian endometriotic lesions and dysmenorrhea VAS score ($P$>0.05).

Spearman rank correlation was also conducted to analyze the correlation between the expression of TrKB in eutopic endometrium of endometriosis group and dysmenorrhea VAS score and revealed that $r$=0.56, meaning there was a moderate positive correlation between the expression of TrKB in eutopic endometrium and dysmenorrhea VAS score ($P$<0.05); but there was no correlation between the expression of TrKB in ovarian endometriotic lesions and dysmenorrhea VAS score ($P$>0.05).

The correlation between BDNF and TrKB were calculate by Pearson analysis in both eutopic and ectopic endometrium, which were revealed that r=0.82 and 0.66, respectively ($P$<0.05).

## Discussion

The excessive expression of BDNF and TrKB in malignant tumors will promote the development of tumor. Though endometriosis is a benign disease, the extensive lesions, strong invasiveness and reoccurrence make its biological behavior have many similarities to malignant tumors. The most characteristic symptom is pelvic pain, including dysmenorrhea and chronic pelvic pain(5). BDNF plays an important role in the visceral pain and high sensitivity (6). Therefore, it needs to be further discussed whether there is a correlation between BDNF, TrKB and the development of endometriosis and endometriosis-related dysmenorrhea.

BDNF was belonged to the neurotrophin (NT) family, which is a small molecular polypeptides growth factor. BDNF exert their functions through engagement of Trk tyrosine kinase receptors, which can active activate downstream pathways to enhance cell survival and growth. The most predominant receptor is TrkB.

There are many theories on pathogenesis of endometriosis, but the menstrual blood reflux theory proposed by Sampson still occupies the leading position (5). A research found that BDNF was detected in menstrual blood and endometrium in reproductive female with quantitative analysis showing that the concentration of BDNF in menstrual blood is higher than in plasma (7), which indicated BDNF may play a role in female reproductive function. The expression of TrKB increased in eutopic endometrium of patients with endometriosis and TrKB was considered to be one of the most potent growth factors for inhibiting anoikis (8). Binding BDNF to TrKB, or activating PI3K pathway can inhibit proapoptotic protein activation and activate antiapoptotic protein Bcl-2 (9); or activating MAPK signaling pathway can influence transcription factor and activate Bcl-2 (10); co-inhibit shedding endometrium from anoikis. Our study found that there was a significant difference in the expression of BDNF and TrKB between endometriosis endometrium and non-endometriosis endometrium, which is consistent with literature reported (11, 12). Moreover, this study showed that the expression of BDNF and TrKB in ovarian endometriotic lesions was higher than that in eutopic endometrium, which is similar to Borghese et al. who found that the expression of BDNF in ovarian endometriotic lesions was higher than that in eutopic endometrium. This suggest that BDNF and TrKB may be involved in the pathogenesis of endometriosis.

Previous studies have found that in mice, estrogen exposure after ovariectomy significantly upregulated BDNF, but the hormonal fluctuations of the murine estrous cycle did not (13); detecting the plasma BDNF concentration of endometriosis patients by ELISA found that menstrual period did not affect the expression BDNF, either (14); other researches believed that the expression of TrKB in eutopic endometrium has no correlation with menstrual period (15). Our results suggested that there was no significant difference statistically of the expression of BDNF and TrKB between proliferative and secretary phases in both ovarian endometriotic lesions and eutopic endometrium of endometriosis patients.

The study also found that there was a significant difference in degree of adhesion and infiltration depth between different stages of endometriosis, and the degree of adhesion and infiltration depth also increased with the increase of stages. Results of our study showed that the expression of BDNF and TrKB in ovarian ectopic lesions and eutopic endometrium increased with the increase of r-AFS stage,

speculating that BDNF and TrKB may be correlated with severity of endometriotic lesions. The expression of BDNF and TrKB increased with the increase of stages and resulted in neogenesis of ectopic lesions and aggravated progression of the original lesions continuously, through inhibiting anoikis, promoting cell invasion, proliferation and angiogenesis and so on. Meanwhile, the study demonstrated that ectopic lesions can synthesize BDNF continuously (16, 17), thus forming a vicious cycle and further leading to the aggravation of the disease.

Consistent with previous studies, We found that the expressions of BDNF and TrKB in ovarian endometriotic lesions was no correlation with dysmenorrhea VAS score (18). Meanwhile, We also found that there was a moderate positive correlation between the expression of BDNF, TrKB in eutopic endometrium and dysmenorrhea VAS score. Therefore we can speculate the characteristics of eutopic endometrium play a key role in endometriosis dysmenorrhea and the effect caused by molecular biological changes of eutopic endometrium on the genesis of endometriosis dysmenorrhea may be larger than that caused by ovarian endometriotic lesions, speculating that BDNF and TrKB may be correlated with endometriosis dysmenorrhea. The significant increase of BDNF in endometriosis and abnormal distribution of different types of nerve fibers may be the causes leading to endometriosis pain. The joint effect of BDNF, vascular growth factor and immune inflammation factor are involved in peripheral nerve sensitization caused by chronic activation of nerve endings. Pelvic visceral pain can transfer to brainstem via vagus nerve, or to sacral dorsal commissural nucleus via pelvic nerve, where existing the high expression of $K^+$-$Cl^-$cotransporter2 (KCC2). The high expression of KCC2 or the decrease of ion transportation activity will destroy the stability of $Cl^-$ in nerve cells, causing γ-aminobutyric acid (GABA) nerve deinhibition, thus leading to hyperpathia (19).

This study further validated the effect of BDNF/TrKB signaling pathway on the process of endometriosis and analyzed its expression changes, combined with the degree of dysmenorrhea, in different parts of the endometrium at different stages. BDNF is produced by BDNF precursor (proBDNF) cleavage intercellularly or by secreting to extracellular space followed by protease cleavage. The low-affinity receptor of BDNF, p75 receptor, has a high affinity for proBDNF, but a low affinity for BDNF. Binding proBDNF to P75 receptor can activate the pro-apoptotic signaling pathway. Binding BDNF to its high-affinity receptor TrKB can activate MAPK pathway, PI3K/AKT pathway and PLCγ pathway, exerting various effects of inhibiting anoikis (20, 21), autophagocytic defect (22, 23), angiogenesis (3, 24), cell invasion and proliferation (20, 22, 25) and highly expressing in various malignant tumors (26), which, has been the focus of tumor researches recently (Figure 5A).

Pelvic pain is the most typical clinical symptom in endometriosis, but the exact mechanism remains unclear yet. At present three main mechanisms causing pain include: the close connection between lesions and surrounding nerves, specific inflammatory environment and the effect of central nerve system in pain (27). As an important member of NT family, BDNF exerts a key effect in growth, differentiation and survival of nerve fibers (26), and in visceral pain and high sensitivity (19). A research found that there were no nerve cells in endometriotic lesions originally, but BDNF and NGF can promote the generation of nerve tissue in endometriotic lesions (28) and the expression level of BDNF and NGF is correlated with

pain degree of endometriosis patients (29). However, another study showed that the expression level of BDNF and NGF was no significant correlation with the degree of pain (30). The manifestations of endometriosis pain in different types and locations are varying and the mechanisms are different between peritoneal endometriosis and deep infiltrating endometriosis (DIE), the former may be correlated with the stimulation of cytokines and growth factor generated by lesions, while the latter is correlated with stimulation of various factors, as well as the deep lesions as reported in previous study (31) (Fig. 5 B).

Whereas our previous study was exploratory with small sample size, and lack of r-AFS stage I patients when collecting samples and the little number of r-AFS stage II due to preliminary exploratory research. Further, we will expand the sample size, and systematically explore the mechanism of Neurotrophin / Trk signaling pathway and its effect on endometrium and nerve fiber cells.

## List Of Abbreviations

Brain-derived neurotrophic factor (BDNF); Tyrosine receptor kinase B (TrKB); message RNA (mRNA); visual analogue scale (VAS); neurotrophin (NT); revised American Fertility Society (r-AFS); polycystic ovary syndrome (PCOS); histogram scoring (H-score); Analysis of variance (ANOVA).

## Declarations

### Ethics approval and consent to participate

We confirm that the study was approved by the local research and ethics committee of Beijing Obstetrics and Gynecology Hospital (No.2016-KY-01) and certify that the study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

### Consent for publication

No objection

### Availability of data and materials

All analysis results are displayed at the results. For specific experimental data, please contact the corresponding author.

### Competing interests

The authors declare that there is no conflict of interest regarding the publication of this article

### Funding

## Authors' contributions

SW performed the laboratory experiments, analyzed all the data and write the manuscript, BHL writhe and edit the manuscript, HD developed the project and edited the manuscript, WH, XL, YW, and ZCG edit the manuscript. All authors read and approved the final manuscript.

## Acknowledgements

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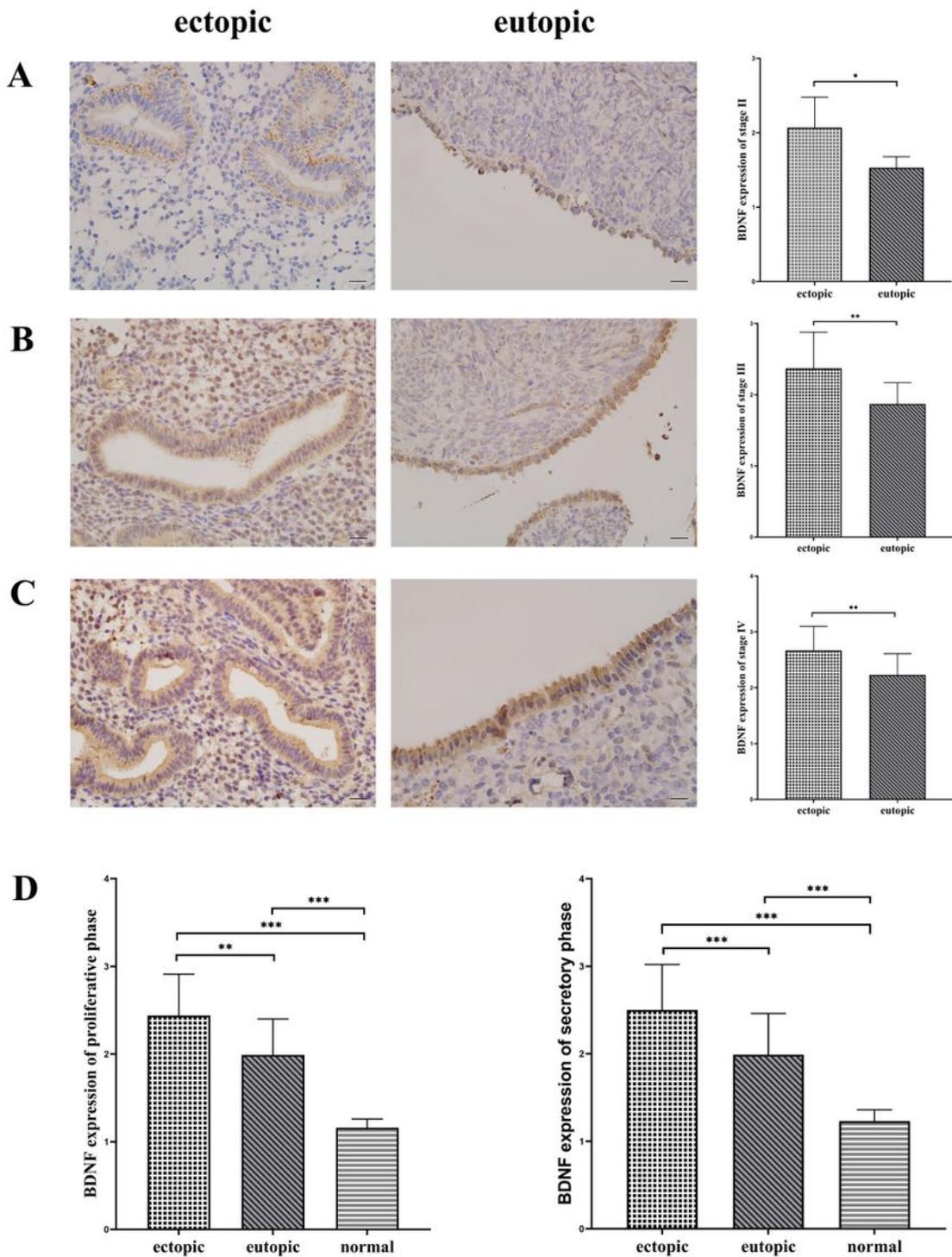
# Figures

**Figure 1**

Immunohistochemical staining of BDNF. (immunohistochemical staining, ×400, scale 20μm). The expression of BDNF in eutopic and ovarian ectopic endometrium of endometriosis group was positive, which was indicated by yellow, brown and tan particulates, mainly discovered in cytoplasm of epithelial cells or glandular epithelial cells. (A) Ectopic and eutopic endometrium staining and H-score of BDNF in stage II. (B) Ectopic and eutopic endometrium staining and H-SCORE values of BDNF in stage III. (C)

Ectopic and eutopic endometrium staining and H-SCORE values of BDNF in stage IV. (D) H-SCORE values of BDNF expression of both proliferative (left) and secretory (right) phases for ectopic and eutopic endometrium in endometriosis group and normal endometrium in control group. *, P<0.05;∗∗, P<0.01;∗∗∗, P<0.001.
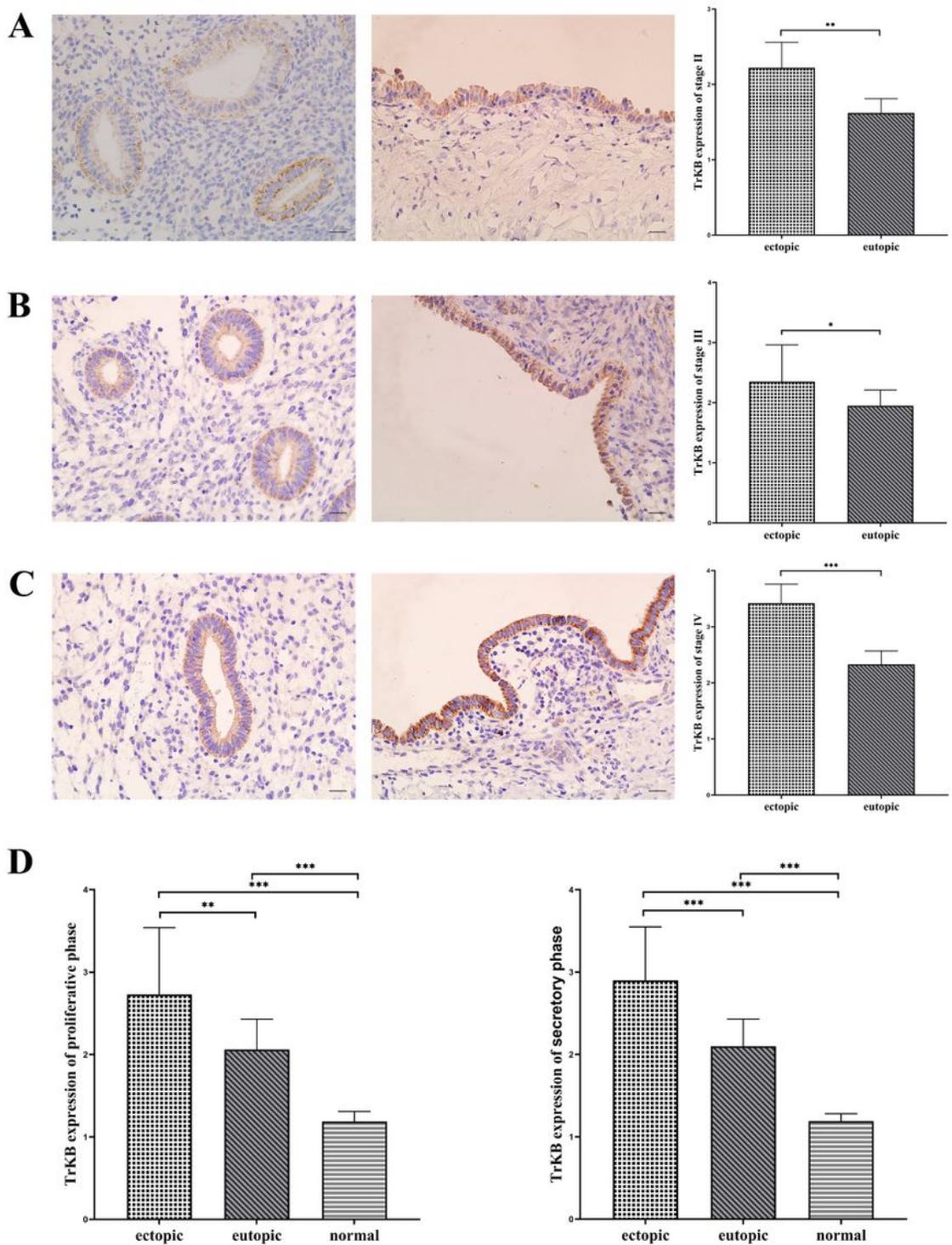


Figure 2

Immunohistochemical staining of BDNF. (immunohistochemical staining, ×400, scale 20μm). The expression of BDNF in eutopic and ovarian ectopic endometrium of endometriosis group was positive, which was indicated by yellow, brown and tan particulates, mainly discovered in cytoplasm of epithelial cells or glandular epithelial cells. (A) Ectopic and eutopic endometrium staining and H-SCORE values of BDNF in stage II. (B) Ectopic and eutopic endometrium staining and H-SCORE values of BDNF in stage III. (C) Ectopic and eutopic endometrium staining and H-SCORE values of BDNF in stage IV. (D) H-SCORE values of BDNF expression of both proliferative (left) and secretory (right) phases for ectopic and eutopic endometrium in endometriosis group and normal endometrium in control group. *, $P<0.05$;✱✱, $P<0.01$;✱✱✱, $P<0.001$.
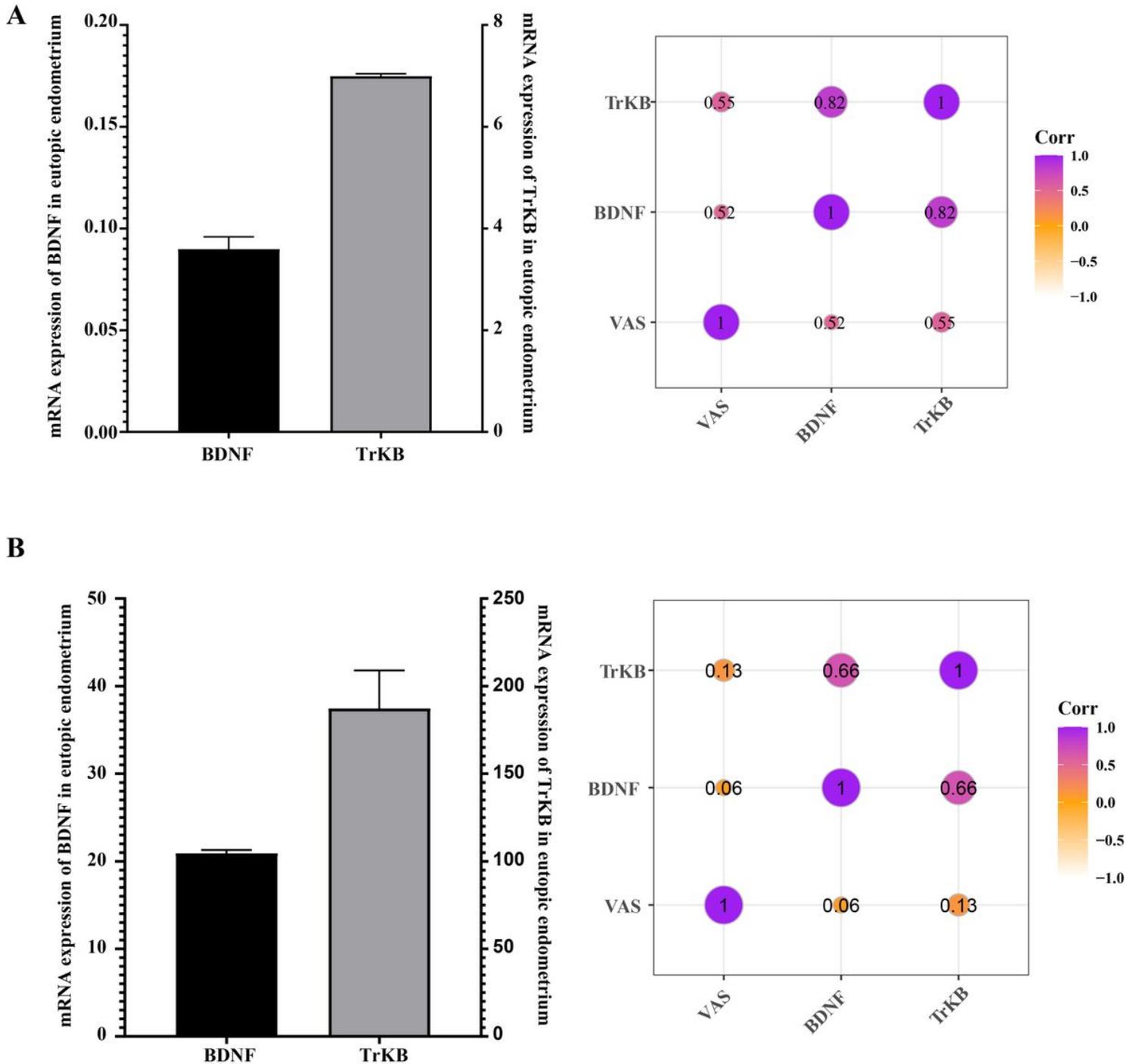


**Figure 3**

Correlation between mRNA expression of BDNF, TrKB in endometriosis group and dysmenorrhea VAS score. A) mRNA expression of BDNF, TrKB in eutopic endometrium and the correlation among BDNF, TrKB and dysmenorrhea. B) mRNA expression of BDNF, TrKB in ectopic endometrium and the correlation among BDNF, TrKB and dysmenorrhea.
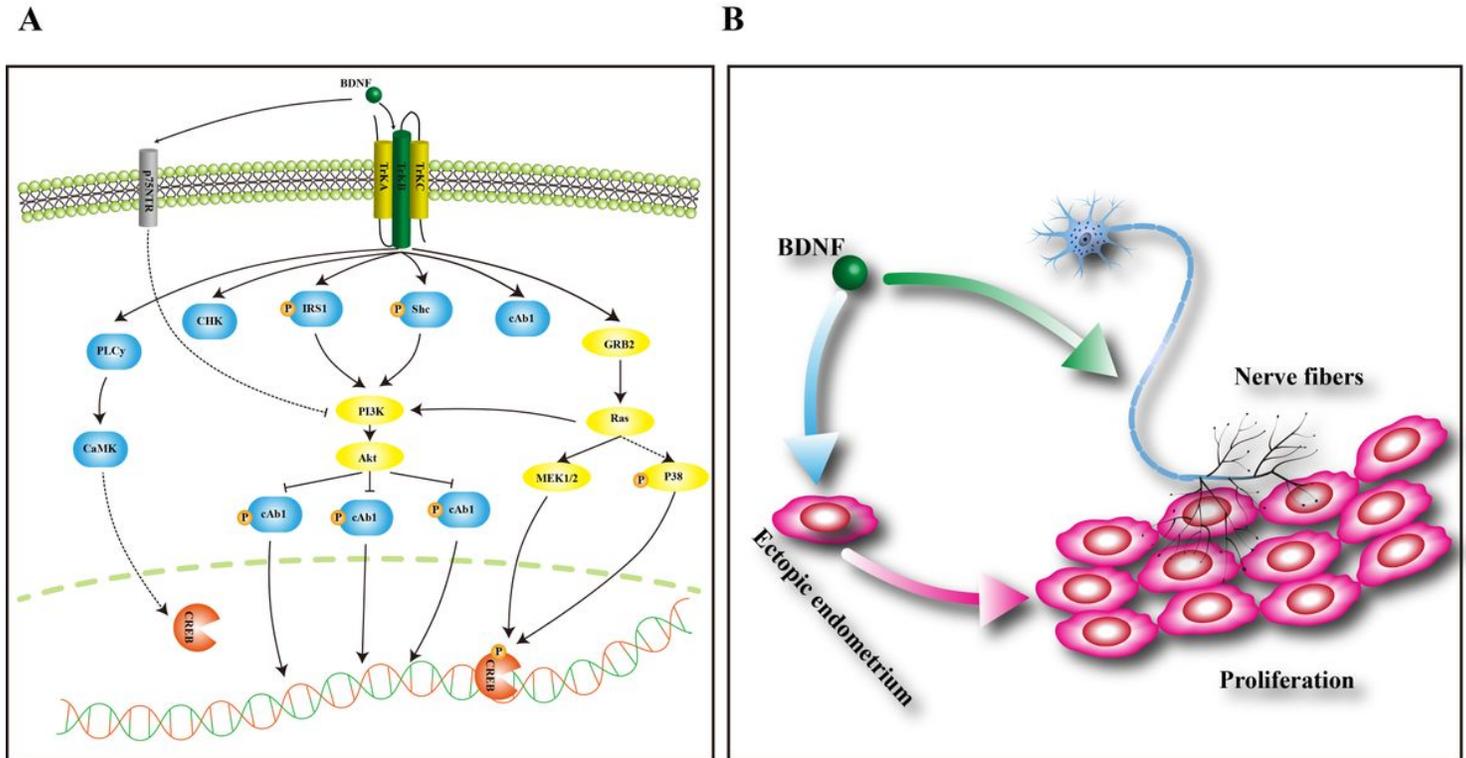


**Figure 4**

Diagram of the molecular mechanisms of BDNF. A) BDNF exert their functions through engagement of TrkB or p75. Neurotrophin/Trk signaling is regulated by connecting a variety of intracellular signaling cascades, which include MAPK pathway, PI-3 kinase pathway, and PLC pathway, transmitting positive signals like enhanced survival and growth. On the other hand, p75 transmits both positive and nagative signals. B) BDNF may be synthesized in ectopic endometrium which can promote the growth of nerve fibers and endometrium cells.