Elevated Nerve Growth Factor and its Receptor Levels in Endometriotic Tissues are Associated with Deep Dyspareunia

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Capsule: We demonstrate the association of nerve growth factor (NGF) and its receptor levels with deep dyspareunia in women with endometriosis, and the stimulatory effect of NGF on prostaglandin E2 in endometriotic stromal cells.

Disclosure: The authors have nothing to disclose.
ABSTRACT

CONTEXT; Deep dyspareunia (pelvic pain with sexual intercourse) affects half of women with endometriosis, but the underlying mechanisms are unclear.

OBJECTIVE: To investigate whether nerve growth factor (NGF) and its receptor (TrkA/p75NTR) levels in endometriotic tissues are elevated in women with deep dyspareunia.

DESIGN: In vitro study.

SETTING: University hospital affiliated research laboratories.

PATIENTS: 34 women with endometriosis in the posterior pelvic compartment (cul-de-sac/uterosacrals) were included, either with (n=18) or without (n=16) deep dyspareunia symptoms which was confirmed by endovaginal ultrasound-assisted palpation on examination.

INTERVENTIONS: None.

MAIN OUTCOME MEASURES: Utilizing surgically excised cul-de-sac/uterosacral endometriosis, expression of NGF/TrkA/p75NTR was examined by immunohistochemistry and Histoscore. Cultured endometriotic stromal cells (ESCs, n=3) were incubated with/without NGF and/or Trk inhibitor K252a. Prostaglandin-endoperoxide synthase-2 (PGST-2/COX-2) mRNA and protein levels were examined by RT-qPCR and Western blot, respectively. Prostaglandin E2 (PGE2) secretion were examined by ELISA.

RESULTS: NGF/TrkA/p75NTR were expressed in the epithelium and stroma of the cul-de-sac/uterosacral endometriosis, and were significantly elevated in women with deep dyspareunia compared to women without deep dyspareunia. NGF immunoreactivity in the stroma was also significantly associated with deep dyspareunia severity and with nerve bundle density. In
cultured ESCs, NGF significantly increased PTGS-2/COX-2 mRNA and protein levels as well as PGE2 secretion, and these effects could be abolished by pretreatment of K252a.

CONCLUSIONS: Elevated NGF/TrkA/p75NTR levels may be associated with sexual pain (deep dyspareunia) in women with cul-de-sac/uterosacral endometriosis. This association may be mediated by an increase in nerve bundle density and by COX-2 and PGE2 stimulation via Trk receptor.

Key words: NGF, TrkA, endometriosis, dyspareunia, prostaglandin E2
INTRODUCTION

Endometriosis affects approximately 10% of women world-wide, and is a common cause of infertility and pelvic pain. It is an estrogen-dependent gynaecological disease that is characterized by the presence of endometrium-like tissue explants (epithelium and stroma) outside the uterus (1). The endometriosis explants are often found elsewhere in the pelvic cavity, such as on the ovaries or in the posterior compartment of the pelvis (the cul-de-sac peritoneal fold between the uterus and rectum, bordered by the uterosacral ligaments on either side) (2).

Endometriosis is a major cause of pelvic pain with sexual intercourse (deep dyspareunia), as deep dyspareunia is present in half of women with endometriosis (3,4). Deep dyspareunia has been shown to have a major detrimental impact on women’s quality-of-life (5,6). However, the mechanism of endometriosis-associated deep dyspareunia is still not well understood. Previous work has shown that endometriosis of the cul-de-sac/uterosacral is more likely to be associated with deep dyspareunia, compared to endometriosis of other pelvic sites (7,8). This finding is consistent with the anatomic location of the cul-de-sac/uterosacral at the top of the vagina between the uterus and rectum, which is likely to be contacted during intercourse. However, not all women with endometriosis of the cul-de-sac/uterosacral experience deep dyspareunia, and there can also be heterogeneity in the severity of pain with intercourse (8). Moreover, not all women with deep dyspareunia respond to the traditional hormonal therapies for endometriosis (9).

Thus, additional factors must modulate whether cul-de-sac/uterosacral endometriosis causes pain with intercourse or not. Possible factors include local inflammation and local neurogenesis in the endometriotic lesions. Prostaglandin E2 (PGE2) production by prostaglandin-endoperoxide synthase-2 (PTGS-2/COX-2) is known to a critical inflammatory factor in endometriosis (10). In addition, we previously showed that in women with cul-de-sac/uterosacral endometriosis, women
with deep dyspareunia had an increase in nerve bundle density around the endometriotic lesions, compared to women without deep dyspareunia (11).

Nerve growth factor (NGF) is an important endocrine regulator for neuronal growth, and plays essential roles in regulating neuronal survival and maturation (12). NGF is known to contribute to pain associated with variety of medical conditions such as rheumatoid arthritis and cancer (13). NGF, its high affinity receptor TrkA, and its low affinity receptor p75NTR, have been immunolocalized in endometriotic tissues (14). Additionally, increased NGF levels have been found in peritoneal fluid from women with endometriosis compared to women without endometriosis (15). However, whether NGF and its receptor levels in endometriotic tissues are associated with endometriosis-related pain, especially deep dyspareunia, is still unknown.

In this study, we investigated the role of nerve growth factor (NGF) and its receptors in local neurogenesis and inflammation. Specifically, we examined the location and expression of NGF, TrkA and p75NTR in endometriotic tissue from women with cul-de-sac/uterosacral endometriosis, either with or without deep dyspareunia. We also studied whether this relationship is mediated by an increase in local nerve bundle density, and whether NGF regulates PTGS-2/COX-2 expression and PGE2 secretion in cultured endometriotic stromal cells.
**MATERIALS AND METHODS**

**Study sample and sample collection**

This study was approved by the Research Ethics Board of the University of British Columbia, and involves a retrospective component (H11-02563) and prospectively obtained endometriosis tissues after informed consent (H11-00536 and H14-03040).

The retrospective component of the study has been described in our previous study (11). We obtained archived endometriosis specimens from laparoscopic excision of cul-de-sac/uterosacral endometriosis. These patients were divided into two groups: 1) Cases (n = 18) with cul-de-sac/uterosacral endometriosis who reported the presence of deep dyspareunia (severity rated 1-10/10), which was objectively reproduced by tenderness on palpation of the cul-de-sac/uterosacrals by endovaginal ultrasound-assisted pelvic exam prior to surgery (16); and 2) Controls (n = 16) with cul-de-sac/uterosacral endometriosis who reported the absence of deep dyspareunia (0/10) and who had no tenderness on palpation of the cul-de-sac/uterosacrals. We excluded any patients with Stage IV endometriosis associated with obliteration of the cul-de-sac/uterosacrals by severe endometriosis, as this sub-type of endometriosis is already well known to be associated with deep dyspareunia (8). Immunohistochemistry for NGF, TrkA and p75NTR was performed on this retrospective dataset. We also obtained the nerve bundle density from each case from our previous study (11), which was assessed by immunohistochemistry for the pan-neuronal marker protein gene product 9.5 (PGP9.5). All experiments were done blinded to the group (i.e. case or control).

In the prospective component of the study, surgically excised endometriosis was prospectively obtained for primary culture of endometrial stromal cells (ESCs) (n = 3), in order to assess the effect of NGF on PTGS-2/COX-2 expression and PGE2 secretion.
Antibodies and reagents

Rabbit polyclonal antibody against human NGF protein (#2046), rabbit monoclonal antibodies against human p75NTR protein (#8238, clone D4B3) and human COX-2 protein (#12282, clone D5H5), and human β-NGF protein (#5521) was purchased from Cell Signaling Technology (Danvers, MA). Rabbit monoclonal antibody against human TrkA (clone EP1058Y) was purchased from Abcam (Cambridge, MA). K252a, an inhibitor of Trk family of tyrosine protein kinases, was obtained from Sigma-aldrich (St. Louis, MO).

Immunohistochemistry

Endometriosis samples were fixed in 4% formaldehyde and embedded in paraffin for sectioning. Sections were deparaffinized in xylene, rehydrated through gradient ethanol, and processed for wet heat-induced antigen retrieval in a steamer for 20 min with a modified citrate buffer (pH 6.1; Dako, Mississauga, ON, Canada). Sections were incubated in 3% H₂O₂ in phosphate-buffered saline (PBS) for 30 min at room temperature to quench endogenous peroxidase, and then blocked with serum-free protein block for 1 hour at room temperature. Sections were incubated with antibodies against NGF (1:20), TrkA (35µg/ml) and p75NTR (1:20) overnight at 4°C. Immunoreactivity was detected using the horseradish peroxidase-linked Envision™ system (Dako, Envision™ + Dual link) and 3,3′-diaminobenzidine chromogen solution. Exposure time to 3,3′-diaminobenzidine chromogen solution for all slides were 5 min. Slides were counterstained with Harris hematoxylin (Sigma-Aldrich) for 2 min, dehydrated through graded ethanol to xylene, mounted in a xylene-based mounting medium, and observed under a light microscope (Leica, Wetzlar, Germany).

Immunohistochemical scoring (Histoscore) was performed as previously described with minor modifications (17-19). Briefly, all the specimens were scanned under a Leica light microscope at 200× magnification and all fields containing endometriosis glandular epithelial and stromal cells
were located. If no more than three fields containing endometriotic tissues were found in each specimen, all fields were scored. If more than three fields containing endometriotic tissues were found in each sample, three random fields were scored. The intensity of NGF, TrkA and p75NTR immunostaining was classified into four categories (0 = negative, 1 = weak, 2 = moderate, and 3 = strong). Immunostaining was scored in both the glandular epithelial cells and the stromal cells. The percentage of cells in each cell population with negative, weak, moderate or strong staining was noted. A Histoscore for each cell population in each field was calculated as follows: Histoscore = 0 × percentage of negative staining cells + 1 × percentage of weak staining cells + 2 × percentage of moderate staining cells + 3 × percentage of strong staining cells. The Histoscore of each sample was calculated as the mean of the Histoscores from all the scored fields.

**Primary culture of endometriosis stromal cells (ESCs)**

The method of isolating and culture primary ESCs has been reported and adopted in our group (20,21). Endometriotic samples were collected, washed with phosphate-buffered saline (PBS) and transferred to the laboratory on ice. Fresh endometriotic samples were washed twice with PBS and dissected to fine pieces using a blazer. The dissected tissue pieces were transferred to a 15ml Falcon tube and digested with 1mg/ml type I collagenase (Thermo Fisher Scientific, Waltham, MA) for 60-90 min. Debris and epithelial cells were removed by filtering the digested tissues using 100 µm and 40 µm aperture sieves, respectively. Isolated stromal cells were resuspended in DMEM/F12 medium containing 10% fetal bovine serum and cultured at 37 °C in a humidified atmosphere containing 5% CO₂. The purity of ESCs was examined by separately immunostaining for epithelial marker Cytokeratin 7 (clone OV-TL 12/30, EMDMillipore, Billerica, MA) and stromal marker Vimentin (ab92547, Abcam). Only cultures that contained more than 99% Vimentin positive cells were included in our study (data not shown).
**Experimental cell culture**

Primary endometriotic stromal cells (ESCs) were cultured in phenored-free Dulbecco's Modified Eagle Medium/F12 medium 1:1 supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). 2×10^5 cells were seeded in 60 mm diameter tissue culture dishes 24h before serum starvation. Primary ESCs were incubated with vehicle control (water), or human β-NGF (50mg/ml) for different times (1h to 24h). Additionally, the primary ESCs were treated with varying concentrations of β-NGF (0, 1, 10, or 50 mg/ml) for 6h for mRNA collection or 24h for protein collection. The Trk inhibitor K252a (200nM) was added 1h prior to treatment with β-NGF (50mg/ml). The cells were maintained at 37°C in a humidified atmosphere with 5% CO₂.

**RNA extraction and reverse transcription quantitative real-time PCR (RT-qPCR)**

RNA was extracted using TRIzol reagent (Thermo Fisher Scientific) and 1 μg of total RNA was reverse transcribed into first-strand cDNA with a mix of oligo-dT and random primers using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). RT-qPCR was performed on an Applied Biosystems 7300 Real-Time PCR System equipped with 96-well optical reaction plates. Each 20 μl reaction contained 1×SYBR Green PCR Master Mix (Thermo Fisher Scientific), 25ng cDNA and 300 nM of each specific primer. The primers used were: PTGS-2, 5'-CAA ATT GCT GGC AGG GTT GC -3' (forward), 5'-AGG GCT TCA TCA GCA TAA AGC GT-3' (reverse); GAPDH, 5' -ATG GAA ATC CCA TCA CCA TCTT -3' (forward) and 5'-CGC CCC ACT TGA TTT TGG -3' (reverse). The amplification parameters were 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Amplification efficiencies were validated by means of calibration curves, ensuring that the plot of log input amount vs. ΔCq has a slope < |0.1|. Each experiment was repeated three times on different cultures and each sample was assayed in duplicate. A mean value was used for the
determination of mRNA levels by the comparative Cq method with GAPDH as the reference gene and using the formula $2^{-\Delta \Delta C_q}$.

**Western blot analysis**

Cells were washed twice in cold PBS prior to being lysed with Cell Extraction Buffer (Thermo Fisher Scientific). Supernatants were collected following centrifugation at 15,000×g for 15 min and protein concentrations were quantified using the DC protein assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts (30 µg) of protein were subjected to 8% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (GE Healthcare, Little Chalfont, United Kingdom). Membranes were immunoblotted with primary antibodies against COX-2 (1:1000) or β-actin (0.2µg/ml) overnight at 4°C. Following incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), immunoreactive bands were detected using enhanced chemiluminescence substrate (ECL, Thermo Fisher Scientific) and CL-XPosure film (Thermo Fisher Scientific). Films were scanned and quantified by densitometry using GeneTools software (Syngene, Frederick, MD) and all data were normalized against β-actin.

**Prostaglandin E2 (PGE2) enzyme linked immunosorbent assay (ELISA)**

Culture supernatants from ESCs were collected and centrifuged at 2000 rpm for 5 min to remove debris. PGE2 ELISA was performed following the instruction of PGE2 Parameter Assay Kit (R&D Systems, Minneapolis, MN). The assay was based on the competitive binding of PGE2 in the supernatants with horseradish peroxidase-conjugated PGE2 for limited binding sites on a mouse monoclonal antibody. Each sample was assayed in duplicate and an average was used as the PGE2 concentration in a given sample. The concentration of PGE2 in culture supernatants from control sample was normalized to 1 and all values from treatment groups are relative values in comparison to control.
Statistical Analysis

Histoscore results are presented as the mean ± SD and were analyzed by non-parametric Mann-Whitney test. Spearman’s correlation of nerve bundle density and Histoscore was performed using SPSS software (IBM Corporation, Armonk, NY). Cell-based experiment results are presented as the mean ± SEM and were analyzed by Student’s t-test or one-way ANOVA followed by Tukey’s multiple comparison test using GraphPad Prism 5 software (GraphPad Software, San Diego, CA). Results are considered significant if P <0.05.
RESULTS

Immunolocalization of NGF, TrkA and p75NTR in endometriotic tissues from women with or without dyspareunia

First, we sought to examine the immunolocalization of NGF and its receptors TrkA and p75NTR in endometriotic tissues from women with deep dyspareunia (cases) or without deep dyspareunia (controls). NGF protein was immunolocalized in the cytoplasm of both endometriotic stroma and epithelium from women with deep dyspareunia (Fig 1. A and B) or without deep dyspareunia (Fig 1. C and D). Additionally, positive immunoreactivities of TrkA were observed in endometriotic stroma and epithelium from women with (Fig 1. E and F) or without (Fig 1. G and H) deep dyspareunia. Similarly, p75NTR protein was also immunolocalized in endometriotic stroma and epithelium from women with (Fig 1. I and J) and without (Fig 1. K and L) deep dyspareunia.

Elevated NGF, TrkA and p75NTR protein levels in endometriotic tissues from women with dyspareunia.

Next, the immunohistochemistry staining intensities of NGF, TrkA and p75NTR proteins in endometriotic stroma and epithelium from the 18 cases with deep dyspareunia and the 16 controls without deep dyspareunia were evaluated using Histoscore methods. Significantly elevated NGF immunoreactivity was observed in both endometriotic epithelium (Fig 2. A, P=0.004) and endometriotic stroma (Fig 2. B, P=0.007) from women with deep dyspareunia compared to the women without deep dyspareunia. Additionally, the immunoreactivity of TrkA from the women with deep dyspareunia was significantly increased in endometriotic epithelium (Fig 2. C, P=0.004) and endometriotic stroma (Fig 2. D, P<0.001) compared to women without deep dyspareunia. Similarly, significantly increased p75NTR immunostaining intensity was also observed in
endometriotic epithelium (P=0.027) and endometriotic stroma (P=0.049) from women with deep
dyspareunia compared to women without deep dyspareunia.

NGF expression in ESCs are correlated with nerve bundle density in endometriotic tissues
and severity of dyspareunia

In our previous study, nerve bundle density was assessed in this same cohort. (11). Utilizing
these existing data, we further investigated the correlation between NGF/TrkA/p75NTR
expression and nerve bundle densities in endometriotic tissues, with the nerve bundle density
corrected for the amount of endometriotic epithelium/stroma. As shown in Table 1, the NGF
Histoscore in endometriotic stroma was significantly correlated with higher nerve bundle density
(n=30, P<0.001).

We also compared NGF/TrkA/p75NTR expression to the severity of deep dyspareunia
reported by the women (0-10) (Table 1). NGF Histoscores in endometriotic stroma (n=30,
P=0.008), the TrkA Histoscores in endometriotic epithelium (n=30, P=0.048) and endometriotic
stroma (n=30, P=0.005), and the p75NTR Histoscores in endometriotic stroma (n=30, P=0.027)
were significantly correlated with more severe deep dyspareunia (Table 1).

NGF increases PTGS-2 (COX-2) expression and PGE2 secretion in ESCs.

We further investigated whether NGF could stimulate PTGS-2 (COX-2) expression and PGE2
expression in primary cultures for endometriotic stromal cells (ESCs). Primary ESCs were treated
with 50ng/ml NGF at different time points (0, 3, 6, 12 and 24h), and the mRNA levels of PTGS-2
was significantly elevated at 6, 12 and 24h compared to time-matched controls (Fig 3. A).
Treatment of primary ESCs with NGF at concentrations of 10 and 50ng/ml, but not at 1ng/ml,
significantly increased the mRNA levels of PTGS-2 at the 12h time point (Fig 3. B). At the
protein level, 50ng/ml NGF treatment significantly increased COX-2 expression in ESCs at 6, 12
and 24h (Fig 3. C). Additionally, COX-2 protein levels were significantly increased at 24h after treatment of 10ng/ml and 50ng/ml NGF, but not 1ng/ml NGF (Fig 3. D).

COX-2 plays important roles in the biosynthesis of PGE2. We further studied whether NGF could stimulate PGE2 secretion in cultured ESCs. NGF at a concentration of 50ng/ml could significantly increase the PGE2 protein levels in ESC conditioned medium at 24h after treatment (Fig 3.E).

**Trk inhibitor K252a abolishes NGF induced COX-2 expression and PGE2 secretion.**

A pan Trk inhibitor K252a was used to investigate the involvement of Trk in NGF-induced COX-2 and PGE2 secretion. Pre-incubation of 200nM K252a for 1h significantly attenuated NGF-induced PTGS-2 mRNA levels (Fig 4. A) and COX-2 protein levels (Fig 4. B) at 12h and 24h after NGF treatment, respectively. Importantly, K252a at 200nM also abolished NGF-stimulated PGE2 secretion in ESCs at 24h after NGF treatment (Fig 4. C).
In this blinded study, we reported on the expression of an important neurogenesis-related factor, NGF, and its receptors TrkA and p75NTR in endometriosis of the cul-de-sac/uterosacral. We found that NGF/TrkA/p75NTR expression was significantly higher in women with deep dyspareunia compared to women without deep dyspareunia. Moreover, we found evidence that this relationship may be mediated by an increase in nerve bundle density (local neurogenesis) and by NGF-induced PGE2 expression in cultured endometriotic stroma (local inflammation).

Strengths of the study include the rigorous phenotyping of the cases and controls. First, we focused on endometriosis of the cul-de-sac/uterosacral, which was previously shown to be the anatomic location most associated with deep dyspareunia, and excluded advanced Stage (IV) endometriosis where deep dyspareunia is likely related in part to adhesions. In addition, the symptom of deep dyspareunia was reproduced objectively by the demonstration of tenderness on endovaginal ultrasound-assisted palpation of the cul-de-sac/uterosacral. On the other hand, in women without deep dyspareunia, there was no tenderness on endovaginal ultrasound-assisted palpation of the cul-de-sac/uterosacral. We also had pain severity scores for deep dyspareunia (0-10). Furthermore, the experiments were done blinded to the phenotypes. On the other hand, a limitation is that the first component of the study was retrospective, and thus although immunohistochemistry was feasible on the formalin-fixed paraffin-embedded tissues, we could not assess RNA expression between the women with and without deep dyspareunia. Similarly, the cultured endometriotic stromal cells were prospectively obtained from other women, and not from the same women in the retrospective study.

NGF in peritoneal fluid from women with endometriosis has been shown to promote neurite outgrowth using an in vitro chicken dorsal root ganglia culture model (22,23). Additionally, NGF levels in peritoneal fluid was reported to be elevated in women with more severe dyspareunia.
after an arbitrary cut-off point was selected to remove all the samples containing low NGF levels (15). Though the above studies provided evidence about the potential involvement of NGF in nerve growth and pain in endometriosis, our study is the first to show direct association between NGF levels and local nerve density in endometriotic tissue from women with deep dyspareunia, which we found most strongly for NGF expression in endometriot stroma.

Noticeably, our finding of abundant immunoreactivity of NGF, TrkA and p75NTR in endometrial stroma and epithelium is mostly in consistent with an earlier study by Wang et.al (14). However, Wang et.al reported that p75NTR was expressed in endometriotic epithelium but not endometriotic stroma, and we have immunolocalized p75NTR to both tissue types. This difference may due to diverse sensitivity caused by distinct antibodies used in immunohistochemistry.

Our results also suggest that NGF may stimulate COX-2/PGE2 signaling, which is a critical component of inflammation-induced pain in endometriosis (1). To our knowledge, our study is the first to investigate the function of NGF in modulating gene expression in human endometriotic cells. In agreement with our results, a recent study observed that treatment with C2C12 muscle cells with NGF resulted in an increase of PGE2 secretion (24). In addition, an animal study also found that incubation with NGF significantly increased PGE2 secretion using an ex vivo rabbit uterus culture (25). Interestingly, PGE2 has been found to powerfully induce NGF secretion in mouse astrocytes cells and bone-marrow derived mast cells, suggesting PGE2 could be either upstream or downstream of NGF signals in different cells types (26,27).

Though inhibition of NGF-induced COX-2 expression and PGE2 secretion by pre-treatment of K252a may suggest the involvement of Trk receptors, the potential signaling and transcription factors that mediates this effect is still largely unknown. For example, NGF has been found to trigger MAP kinases and AKT phosphorylation though activating TrkA receptor in mouse
pheochromocytoma PC12 cells (28). Meanwhile, p75NTR has also been known to be involved in NGF-induced NFκB signaling in neuroblastoma cells (29). NGF also stimulates transcription factor AP-1 expression in PC12 cells (30). A previous study has also suggested that AP-1 transcriptionally regulates COX-2 (31). Thus, whether AP-1 transcription factor participates in NGF-induced COX-2 expression may warrant further investigation.

Our study supports future investigation of the role of NGF/TrkA/p75NTR inhibition in the treatment of endometriosis pain such as deep dyspareunia. Future studies will be performed to examine whether additional NGF-inhibition approaches, especially therapeutic drugs under development, could antagonize NGF-induced local neurogenesis and COX-2 expression and PGE2 secretion in endometriosis. One possibility is tanezumab, an monoclonal antibody against human NGF, which was found to improve pelvic pain in women with bladder-related pain (32).

Taken together, our results indicate that elevated NGF and its receptor levels are found in cul-de-sac/uterosacral endometriotic tissues from women with deep dyspareunia. NGF expression in endometriotic stromal cells was also correlated with nerve bundle density and the severity of deep dyspareunia. Additionally, NGF elicits biological functions in endometrial stromal cells by stimulating COX-2 and PGE2 production. All of above may suggest that NGF signaling may play important role in the pathogenesis of endometriosis-related pain such as deep dyspareunia.

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FIGURE AND TABLE LEGENDS

**Figure 1.** Immunolocalization of NGF, TrkA and p75NTR in endometriotic epithelium and stroma from women with or without deep dyspareunia. Representative images showing the immunolocalization of NGF (A, B, C and D), TrkA (E, F, G and H) and p75NTR (I, J, K and L) in endometriotic tissues. Specimens from women with or without dyspareunia were labeled as Cases (n=18) or Controls (n=16), respectively.

**Figure 2.** Comparison of NGF, TrkA and p75NTR Histoscores in endometriotic epithelium and stroma between women with or without deep dyspareunia. Dotplot representing the Histoscore of NGF (A and B), TrkA (C and D), and p75NTR (E and F) in endometriotic epithelium and stroma between Cases (women with deep dyspareunia, n=18) and Controls (women without deep dyspareunia, n=16). Results are presented as the mean ± SD and analysed by Mann-Whitney U-test.

**Figure 3.** NGF induces PTGS-2/COX-2 expression and PGE2 secretion in primary endometriotic stromal cells (ESCs). Primary ESCs were treated with vehicle control (Ctrl) or 50ng/ml human β-NGF for 1, 3, 6, 12 or 24h, after which PTGS-2/COX-2 mRNA (A) and protein (C) were examined by RT-qPCR and Western blot, respectively. Additionally, primary ESCs were treated with β-NGF at different concentrations (0, 1, 10 or 50 ng/ml) for 12h for PTGS-2/COX-2 mRNA (B) and 24h for PTGS-2/COX-2 protein (D). Moreover, condition medium were collected at 48h after treatment of primary ESCs with vehicle control or 50ng/ml human β-NGF, and PGE2 levels (E) in the condition medium were analysed by ELISA. GAPDH and actin were used to normalize RT-PCR and Western blot results, respectively. Results are presented as the mean ± SEM of three independent experiments (n=3, *=P<0.05, one-way ANOVA followed by Tukey’s test for A-D, Student’s t-test for E).

**Figure 4.** Trk inhibitor K252a attenuates NGF-induced PTGS-2/COX-2 expression and PGE2 secretion. Primary ESCs were treated with or without 50ng/ml β-NGF in the absence or presence of 200nM K252a. PTGS-2/COX-2 mRNA (A) at 12h and protein (B) at 24h were examined by RT-qPCR and Western blot, respectively. Secreted PGE2 protein levels (C) in condition medium were examined by ELISA at 48h after co-treatment of β-NGF and K252a. GAPDH and actin were used to normalize RT-PCR and Western blot results, respectively. Results are presented as the mean ± SEM of three independent experiments (n=3, *=P<0.05, one-way ANOVA followed by Tukey’s test).
Table 1. Spearmen’s Correlation between NGF/TrkA/p75NTR Histoscores and intensities of deep dyspareunia as well as nerve bundle densities in endometriotic samples.
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<td>P75NTR Histoscore in endometriotic stroma</td>
<td>.40*</td>
<td>.027</td>
<td>30</td>
<td>-.11</td>
<td>.55</td>
<td>30</td>
</tr>
<tr>
<td>Nerve bundle density (bundles/high powered field)</td>
<td></td>
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</tbody>
</table>