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.

21 **ABSTRACT**

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

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

26 **DESIGN:** *In vitro* study.

27 **SETTING:** University hospital affiliated research laboratories.

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

31 **INTERVENTIONS:** None.

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

38 **RESULTS:** NGF/TrkA/p75NTR were expressed in the epithelium and stroma of the cul-de-
39 sac/uterosacral endometriosis, and were significantly elevated in women with deep dyspareunia
40 compared to women without deep dyspareunia. NGF immunoreactivity in the stroma was also
41 significantly associated with deep dyspareunia severity and with nerve bundle density. In

42 cultured ESCs, NGF significantly increased PTGS-2/COX-2 mRNA and protein levels as well as
43 PGE2 secretion, and these effects could be abolished by pretreatment of K252a.

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

48 **Key words:** NGF, TrkA, endometriosis, dyspareunia, prostaglandin E2

49

50 **INTRODUCTION**

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

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

68 Thus, additional factors must modulate whether cul-de-sac/uterosacral endometriosis causes
69 pain with intercourse or not. Possible factors include local inflammation and local neurogenesis
70 in the endometriotic lesions. Prostaglandin E2 (PGE2) production by prostaglandin-endoperoxide
71 synthase-2 (PTGS-2/COX-2) is known to a critical inflammatory factor in endometriosis (10). In
72 addition, we previously showed that in women with cul-de-sac/uterosacral endometriosis, women

73 with deep dyspareunia had an increase in nerve bundle density around the endometriotic lesions,
74 compared to women without deep dyspareunia (11).

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

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

89

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 perfomed 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.

114 **Antibodies and reagents**

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

121 **Immunohistochemistry**

122 Endometriosis samples were fixed in 4% formaldehyde and embedded in paraffin for
123 sectioning. Sections were deparaffinized in xylene, rehydrated through gradient ethanol, and
124 processed for wet heat-induced antigen retrieval in a steamer for 20 min with a modified citrate
125 buffer (pH 6.1; Dako, Mississauga, ON, Canada). Sections were incubated in 3% H_2O_2 in
126 phosphate-buffered saline (PBS) for 30 min at room temperature to quench endogenous
127 peroxidase, and then blocked with serum-free protein block for 1 hour at room temperature.
128 Sections were incubated with antibodies against NGF (1:20), TrkA (35ug/ml) and p75NTR (1:20)
129 overnight at 4°C. Immunoreactivity was detected using the horseradish peroxidase-linked
130 Envision™ system (Dako, Envision™ + Dual link) and 3,3′-diaminobenzidine chromogen
131 solution. Exposure time to 3,3′-diaminobenzidine chromogen solution for all slides were 5 min.
132 Slides were counterstained with Harris hematoxylin (Sigma-Aldrich) for 2 min, dehydrated
133 through graded ethanol to xylene, mounted in a xylene-based mounting medium, and observed
134 under a light microscope (Leica, Wetzlar, Germany).

135 Immunohistochemical scoring (Histoscore) was performed as previously described with minor
136 modifications (17-19). Briefly, all the specimens were scanned under a Leica light microscope at
137 200× magnification and all fields containing endometriosis glandular epithelial and stromal cells

138 were located. If no more than three fields containing endometriotic tissues were found in each
139 specimen, all fields were scored. If more than three fields containing endometriotic tissues were
140 found in each sample, three random fields were scored. The intensity of NGF, TrkA and
141 p75NTR immunostaining was classified into four categories (0 = negative, 1 = weak, 2 =
142 moderate, and 3 = strong). Immunostaining was scored in both the glandular epithelial cells and
143 the stromal cells. The percentage of cells in each cell population with negative, weak, moderate or
144 strong staining was noted. A Histoscore for each cell population in each field was calculated as
145 follows: Histoscore = 0 × percentage of negative staining cells + 1 × percentage of weak staining
146 cells + 2 × percentage of moderate staining cells + 3 × percentage of strong staining cells. The
147 Histoscore of each sample was calculated as the mean of the Histoscores from all the scored
148 fields.

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

150 The method of isolating and culture primary ESCs has been reported and adopted in our group
151 (20,21). Endometriotic samples were collected, washed with phosphate-buffered saline (PBS) and
152 transferred to the laboratory on ice. Fresh endometriotic samples were washed twice with PBS
153 and dissected to fine pieces using a blazer. The dissected tissue pieces were transferred to a 15ml
154 Falcon tube and digested with 1mg/ml type I collagenase (Thermo Fisher Scientific, Waltham,
155 MA) for 60-90 min. Debris and epithelial cells were removed by filtering the digested tissues
156 using 100 μm and 40 μm aperture sieves, respectively. Isolated stromal cells were resuspended in
157 DMEM/F12 medium containing 10% fetal bovine serum and cultured at 37 °C in a humidified
158 atmosphere containing 5% CO_2. The purity of ESCs was examined by separately immunostaining
159 for epithelial marker Cytokeratin 7 (clone OV-TL 12/30, EMDMillipore, Billerica, MA) and
160 stromal marker Vimentin (ab92547, Abcam). Only cultures that contained more than 99%
161 Vimentin positive cells were included in our study (data not shown).

162 **Experimental cell culture**

163 Primary endometriotic stromal cells (ESCs) were cultured in phenored-free Dulbecco's
164 Modified Eagle Medium/F12 medium 1:1 supplemented with 10% FBS and antibiotics (100 U/ml
165 penicillin and 100 μg/ml streptomycin). 2×10^5 cells were seeded in 60 mm diameter tissue culture
166 dishes 24h before serum starvation. Primary ESCs were incubated with vehicle control (water), or
167 human β-NGF (50mg/ml) for different times (1h to 24h). Additionally, the primary ESCs were
168 treated with varying concentrations of β-NGF (0, 1, 10, or 50 mg/ml) for 6h for mRNA collection
169 or 24h for protein collection. The Trk inhibitor K252a (200nM) was added 1h prior to treatment
170 with β-NGF (50mg/ml). The cells were maintained at 37°C in a humidified atmosphere with 5%
171 CO_2.

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

173 RNA was extracted using TRIzol reagent (Thermo Fisher Scientific) and 1 μg of total RNA
174 was reverse transcribed into first-strand cDNA with a mix of oligo-dT and random primers using
175 the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Thermo Fisher
176 Scientific). RT-qPCR was performed on an Applied Biosystems 7300 Real-Time PCR System
177 equipped with 96-well optical reaction plates. Each 20 μl reaction contained 1×SYBR Green PCR
178 Master Mix (Thermo Fisher Scientific), 25ng cDNA and 300 nM of each specific primer. The
179 primers used were: PTGS-2, 5'-CAA ATT GCT GGC AGG GTT GC-3' (forward), 5'-AGG GCT
180 TCA GCA TAA AGC GT-3' (reverse); GAPDH, 5'-ATG GAA ATC CCA TCA CCA TCTT-3'
181 (forward) and 5'-CGC CCC ACT TGA TTT TGG-3' (reverse). The amplification parameters
182 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.
183 Amplification efficiencies were validated by means of calibration curves, ensuring that the plot of
184 log input amount vs. ΔCq has a slope $< |0.1|$. Each experiment was repeated three times on
185 different cultures and each sample was assayed in duplicate. A mean value was used for the

186 determination of mRNA levels by the comparative Cq method with GAPDH as the reference

187 gene and using the formula $2^{-\Delta\Delta Cq}$.

188 **Western blot analysis**

189 Cells were washed twice in cold PBS prior to being lysed with Cell Extraction Buffer (Thermo

190 Fisher Scientific). Supernatants were collected following centrifugation at 15,000×g for 15 min

191 and protein concentrations were quantified using the DC protein assay (Bio-Rad Laboratories,

192 Hercules, CA). Equal amounts (30 µg) of protein were subjected to 8% SDS-polyacrylamide gel

193 electrophoresis and transferred to nitrocellulose membranes (GE Healthcare, Little Chalfont,

194 United Kingdom). Membranes were immunoblotted with primary antibodies against COX-2

195 (1:1000) or β-actin (0.2µg/ml) overnight at 4°C. Following incubation with horseradish

196 peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA),

197 immunoreactive bands were detected using enhanced chemiluminescence substrate (ECL,

198 Thermo Fisher Scientific) and CL-XPosure film (Thermo Fisher Scientific). Films were scanned

199 and quantified by densitometry using GeneTools software (Syngene, Frederick, MD) and all data

200 were normalized against β-actin.

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

202 Culture supernatants from ESCs were collected and centrifuged at 2000 rpm for 5 min to

203 remove debris. PGE2 ELISA was performed following the instruction of PGE2 Parameter Assay

204 Kit (R&D Systems, Minneapolis, MN). The assay was based on the competitive binding of PGE2

205 in the supernatants with horseradish peroxidase-conjugated PGE2 for limited binding sites on a

206 mouse monoclonal antibody. Each sample was assayed in duplicate and an average was used as

207 the PGE2 concentration in a given sample. The concentration of PGE2 in culture supernatants

208 from control sample was normalized to 1 and all values from treatment groups are relative values

209 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

241 endometriotic epithelium (P=0.027) and endometriotic stroma (P=0.049) from women with deep
242 dyspareunia compared to women without deep dyspareunia.

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

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

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

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

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

264 and 24h (Fig 3. C). Additionally, COX-2 protein levels were significantly increased at 24h after
265 treatment of 10ng/ml and 50ng/ml NGF, but not 1ng/ml NGF (Fig 3. D).

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

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

271 A pan Trk inhibitor K252a was used to investigate the involvement of Trk in NGF-induced
272 COX-2 and PGE2 secretion. Pre-incubation of 200nM K252a for 1h significantly attenuated
273 NGF-induced PTGS-2 mRNA levels (Fig 4. A) and COX-2 protein levels (Fig 4. B) at 12h and
274 24h after NGF treatment, respectively. Importantly, K252a at 200nM also abolished NGF-
275 stimulated PGE2 secretion in ESCs at 24h after NGF treatment (Fig 4. C).

276

277 **DISCUSSION**

278 In this blinded study, we reported on the expression of an important neurogenesis-related
279 factor, NGF, and its receptors TrkA and p75NTR in endometriosis of the cul-de-sac/uterosacrals.
280 We found that NGF/TrkA/p75NTR expression was significantly higher in women with deep
281 dyspareunia compared to women without deep dyspareunia. Moreover, we found evidence that
282 this relationship may be mediated by an increase in nerve bundle density (local neurogenesis) and
283 by NGF-induced PGE2 expression in cultured endometriotic stroma (local inflammation).

284 Strengths of the study include the rigorous phenotyping of the cases and controls. First, we
285 focused on endometriosis of the cul-de-sac/uterosacrals, which was previously shown to be the
286 anatomic location most associated with deep dyspareunia, and excluded advanced Stage (IV)
287 endometriosis where deep dyspareunia is likely related in part to adhesions. In addition, the
288 symptom of deep dyspareunia was reproduced objectively by the demonstration of tenderness on
289 endovaginal ultrasound-assisted palpation of the cul-de-sac/uterosacrals. On the other hand, in
290 women without deep dyspareunia, there was no tenderness on endovaginal ultrasound-assisted
291 palpation of the cul-de-sac/uterosacrals. We also had pain severity scores for deep dyspareunia
292 (0-10). Furthermore, the experiments were done blinded to the phenotypes. On the other hand, a
293 limitation is that the first component of the study was retrospective, and thus although
294 immunohistochemistry was feasible on the formalin-fixed paraffin-embedded tissues, we could
295 not assess RNA expression between the women with and without deep dyspareunia. Similarly,
296 the cultured endometriotic stromal cells were prospectively obtained from other women, and not
297 from the same women in the retrospective study.

298 NGF in peritoneal fluid from women with endometriosis has been shown to promote neurite
299 outgrowth using an *in vitro* chicken dorsal root ganglia culture model (22,23). Additionally, NGF
300 levels in peritoneal fluid was reported to be elevated in women with more severe dyspareunia

301 after an arbitrary cut-off point was selected to remove all the samples containing low NGF levels
302 (15). Though the above studies provided evidence about the potential involvement of NGF in
303 nerve growth and pain in endometriosis, our study is the first to show direct association between
304 NGF levels and local nerve density in endometriotic tissue from women with deep dyspareunia,
305 which we found most strongly for NGF expression in endometriotic stroma.

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

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

321 Though inhibition of NGF-induced COX-2 expression and PGE2 secretion by pre-treatment of
322 K252a may suggest the involvement of Trk receptors, the potential signaling and transcription
323 factors that mediates this effect is still largely unknown. For example, NGF has been found to
324 trigger MAP kinases and AKT phosphorylation though activating TrkA receptor in mouse

325 pheochromocytoma PC12 cells (28). Meanwhile, p75NTR has also been known to be involved in
326 NGF-induced NFκB signaling in neuroblastoma cells (29). NGF also stimulates transcription
327 factor AP-1 expression in PC12 cells (30). A previous study has also suggested that AP-1
328 transcriptionally regulates COX-2 (31). Thus, whether AP-1 transcription factor participates in
329 NGF-induced COX-2 expression may warrant further investigation.

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

336 Taken together, our results indicate that elevated NGF and its receptor levels are found in cul-
337 de-sac/uterosacral endometriotic tissues from women with deep dyspareunia. NGF expression in
338 endometriotic stromal cells was also correlated with nerve bundle density and the severity of deep
339 dyspareunia. Additionally, NGF elicits biological functions in endometrial stromal cells by
340 stimulating COX-2 and PGE2 production. All of above may suggest that NGF signaling may play
341 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 endeometriotic 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 endeometriotic 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 endoemtriotic 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).

475 **Table 1. Spearmen's Correlation between NGF/TrkA/p75NTR Histoscores and intensities**
476 **of deep dyspareunia as well as nerve bundle densities in endometriotic samples.**

Spearman's correlation	Correlation Coefficient	Significance (2-tailed)	N	Correlation Coefficient	Significance (2-tailed)	N
	Intensity of deep dyspareunia (0-10)			Nerve bundle density (bundles/high powered field)		
NGF Histoscore in endometriotic epithelium	.35	.06	30	.21	.27	30
NGF Histoscore in endometriotic stroma	.47**	.008	30	.56**	.001	30
TrkA Histoscore in endometriotic epithelium	.36*	.048	30	.29	.12	30
TrkA Histoscore in endometriotic stroma	.50**	.005	30	.29	.12	30
p75NTR Histoscore in endometriotic epithelium	.27	.15	30	.11	.57	30
P75NTR Histoscore in endometriotic stroma	.40*	.027	30	-.11	.55	30