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ORIGINAL ARTICLE ***Reproductive endocrinology***

Estrogen induced changes in uterine brain-derived neurotrophic factor and its receptors

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STUDY QUESTION: Are brain-derived neurotrophic factor (BDNF) and its receptors, NTRK2, NGFR and SORT1, regulated by ovarian steroids in the uterus?

SUMMARY ANSWER: BDNF and its low affinity receptor, nerve growth factor receptor (NGFR), are regulated by estradiol in the uterus.

WHAT IS KNOWN ALREADY: Recent studies have revealed a central role for neurotrophins in placental development, endometrial stem cell neurogenesis, endometrial carcinoma and endometriosis. Complex signaling pathways involving BDNF and its receptors are regulated by ovarian hormones in the brain, however their expression and regulation in the uterus is poorly defined.

STUDY DESIGN, SIZE, DURATION: This experimental animal study involved a total of 80 mice.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Female C57BL/6 mice ($n = 50$) were monitored daily for estrous cycle stage, and uterine horns were collected. A second group of mice ($n = 30$) were ovariectomized and given estradiol, progesterone, estradiol + progesterone, or saline for 4 days. Uterine expression of BDNF and its receptors were quantified by real-time PCR and western blot, and localized using immunohistochemistry.

MAIN RESULTS AND THE ROLE OF CHANCE: During the estrous cycle, expression of BDNF, NTRK2 and SORT1 remained constant, while NGFR declined 11-fold from pro-estrus through to diestrus ($P = 0.005$). In ovariectomized mice, estradiol treatment increased uterine expression of mature BDNF greater than 6-fold ($P = 0.013$, 25 kDa; $P = 0.003$, 27 kDa), pro-BDNF 5-fold ($P = 0.041$, 37 kDa band; $P = 0.046$, 40 kDa band), and NGFR 5-fold ($P < 0.001$) when compared with other treatments. NTRK2 and SORT1 were unaffected by ovarian hormones. NGFR was primarily localized in epithelial cells in mice in diestrus or in ovariectomized mice treated with progesterone ($P \leq 0.001$; $P \leq 0.001$, respectively). In contrast, NGFR switched to a stromal localization in ovariectomized mice administered estradiol ($P = 0.002$).

LIMITATIONS, REASONS FOR CAUTION: This study was performed in one only species.

WIDER IMPLICATIONS OF THE FINDINGS: Results of this study demonstrate the uterine regulation of BDNF and NGFR by estradiol, and highlight the striking difference between hormone exposure during the estrous cycle and daily estradiol exposure after ovariectomy on neurotrophin expression in the uterus. The results also show the spatial regulation of NGFR in the uterus in response to ovarian hormones. Sustained estrogen exposure, as seen in estrogen-dependent disease, may alter the delicate neurotrophin balance and inappropriately activate potent BDNF-NTRK2 pathways which are capable of contributing to endometrial pathology.

STUDY FUNDING/COMPETING INTERESTS: This study was supported by the Canadian Institutes of Health Research (CIHR) (W.G.F.), a NSERC Discovery Grant (W.G.F.), and a Vanier Canada Graduate Scholarship-CIHR (J.M.W.). J.M.W. is a member of the CIHR sponsored Reproduction and Early Development in Health training program. The authors declare no conflicts of interest.

Key words: estrogen / BDNF / NGFR / NTRK2 / sortilin

Introduction

Although mainly recognized for their supportive function within the nervous system, brain-derived neurotrophic factor (BDNF) and its high affinity receptor neurotrophic tyrosine receptor kinase 2 (NTRK2) have been shown to participate in ovarian development (Dorfman *et al.*, 2011), follicular development (Kerr *et al.*, 2009) and oocyte survival (Dorfman *et al.*, 2014). The neurotrophins are also important in endometrial physiology where they participate in endometrial stem cell neurogenesis (Shoae-Hassani *et al.*, 2011) and normal placental development (Kawamura *et al.*, 2009, 2011; Non *et al.*, 2012). However, the overexpression of neurotrophins is associated with reproductive pathologies including premature ovarian failure (Dorfman *et al.*, 2014), endometrial cancer (Bao *et al.* 2013) and endometriosis (Borghese *et al.*, 2010; Browne *et al.*, 2012; Barcena de Arellano *et al.*, 2013).

The neurotrophins are small molecular weight proteins that act in the nervous system to promote neuronal development, differentiation, growth and maintenance (reviewed in Chao, 2003). The neurotrophin signaling network is complex. Neurotrophins can be translated as pro-proteins and cleaved into their active forms (Mowla *et al.*, 2001, Gray and Ellis, 2008) or they can induce signaling cascades in their pro-forms (Lee *et al.*, 2001; Koshimizu *et al.*, 2009). Generally, the two forms have opposing functions (reviewed in Chao and Bothwell, 2002; Teng *et al.* 2010). The neurotrophin family comprises four ligands, BDNF, nerve growth factor (NGF), neurotrophin 3 (NTF3) and neurotrophin 4 (NTF4), and four receptors: neurotrophic tyrosine receptor kinase (NTRK) 1, NTRK2, NTRK3, and the nerve growth factor receptor (NGFR) (reviewed in Chao, 2003; Reichardt, 2006). Although all four neurotrophins bind to NGFR with similar affinities (Chao, 2003; Reichardt, 2006), and their pro-protein forms have been shown to bind to this receptor as well (Lee *et al.*, 2001), they are more selective in binding the NTRKs. NGF binds to NTRK1, BDNF and NTF4 to NTRK2, and NTF3 to NTRK3, each with high affinity (reviewed in Chao, 2003). Another lesser known neurotrophin co-receptor, sortilin (SORT1), has been shown to interact with pro-neurotrophins in the brain and to control their release (reviewed in Nykjaer and Willnow 2012). SORT1 is also involved in intracellular trafficking, directing proteins to various fates: cell surface expression, secretion, endocytosis or transport within the cell (reviewed in Nykjaer and Willnow, 2012).

The interaction between BDNF and NTRK2 is not only capable of inducing neuronal development, differentiation, growth and maintenance, activation of the BDNF-NTRK2 pathway also induces angiogenesis (Kermani *et al.* 2005, Nakamura *et al.* 2006), proliferation (Tervonen *et al.*, 2006; Kawamura *et al.*, 2010), adhesion (Zhou *et al.*, 1997; Douma *et al.*, 2004; Geiger and Peeper, 2007) and resistance to apoptosis (Douma *et al.* 2004, Wang *et al.* 2005, Geiger and Peeper, 2007; Kawamura *et al.*, 2010; Li *et al.*, 2011). Each of these pathways is inextricably linked to reproduction, but the mechanisms regulating the uterine expression of neurotrophins remain unknown.

Both estrogen (Singh *et al.*, 1995; Gibbs, 1998, 1999, Jezierski and Sohrabji, 2000, 2001; Berchtold *et al.*, 2001; Liu *et al.*, 2001; Solum and Handa, 2002; Scharfman and Maclusky, 2005; Pan *et al.*, 2010; Tang and Wade, 2012) and progesterone (Kaur *et al.*, 2007; Jodhka *et al.*, 2009; Meyer *et al.*, 2012; Su *et al.*, 2012; Atif *et al.*, 2013) regulate BDNF and its receptors in the brain, and we propose that their uterine regulation occurs in a similar manner. The aims of this study are to determine whether uterine BDNF, NTRK2, NGFR and SORT1 are affected by: (i) the acute, naturally occurring hormone fluctuations of the estrous cycle, and (ii) daily exposure to the ovarian hormones in ovariectomized mice. Here, we contrast the relatively stable expression of BDNF and its receptors over the estrous cycle with the significant up-regulation of uterine BDNF and its low affinity receptor NGFR in response to prolonged exposure to estradiol. Additionally, we document for the first time the presence of NGFR and SORT1 in the uterus.

Materials and Methods

Ethical approval

All procedures were approved by the animal research ethics board, McMaster University, Hamilton, ON, Canada (AUP 12-04-13).

Mice

Sexually mature female C57BL/6 mice ($n = 80$) were purchased at 8 weeks of age from Charles River, and housed in a specific pathogen-free facility with a 12 h light/dark cycle, standard rodent chow, and water *ad libitum*.

Cycling mice

Mice ($n = 50$) were randomly selected for estrous cycle monitoring. Animals were acclimated to vaginal lavage using sterile saline and a curved eye-dropper for a 2-week period. Lavage was dried on a glass slide, and stained with a rapid Giemsa (Sigma-Aldrich, Oakville, ON, Canada) protocol. Briefly, slides were fixed in methanol for 5 min, air dried, and stained with Giemsa for 5 min. Estrous cycle stage was assessed on a daily basis by vaginal cytology (Wood *et al.*, 2007; Caligioni, 2009; Byers *et al.*, 2012). Animals were euthanized at each stage of the estrous cycle (pro-estrus $n = 8$; estrus $n = 18$; metestrus $n = 9$; diestrus $n = 15$) by anesthetic overdose (isoflurane, Pharmaceutical Partners of Canada, Inc., Richmond Hill, ON, Canada). Uterine horns were immediately removed and stored at -80°C.

Ovariectomy and hormone replacement

In the second experiment, sexually mature female mice ($n = 30$) were ovariectomized, and allowed to recover for 2 weeks. Mice were randomly assigned to treatment groups as outlined in Fig. 1, using previously established methods and doses (Domino and Hurd, 2004; Gillgrass *et al.*, 2005; Salgado *et al.*, 2009, 2011). All groups except the OVX group were primed for 3 days with 5 μg of 17-β estradiol (EMD Millipore, Billerica, MA, USA) by subcutaneous injection. After 2 days of rest, animals were given 5 μg of estradiol, 500 μg of progesterone, 5 μg of estradiol plus 500 μg of progesterone (EMD Millipore), or saline by subcutaneous injection for 4 days. Animals were euthanized, and uterine horns were collected as described above.

RNA and protein extraction

RNA and protein were extracted simultaneously from one uterine horn using the RNA/Protein Purification Plus kit (Norgen Biotek Corp., Thorold, ON, Canada). Approximately 30 mg of uterine horn was cut and sonicated in 300 μl lysis buffer on ice for 30 s, three times. RNA was extracted following the manufacturer's protocol and quantified by Nanodrop (Thermo Scientific, Wilmington, DE, USA). cDNA was prepared using the iScript cDNA Synthesis Kit (Bio-Rad, Mississauga, ON, Canada). Protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad).

Real-time PCR

Real-time PCR primers (Table I) were designed against the coding region of genes (to capture all isoforms) using sequences from NCBI Nucleotide,

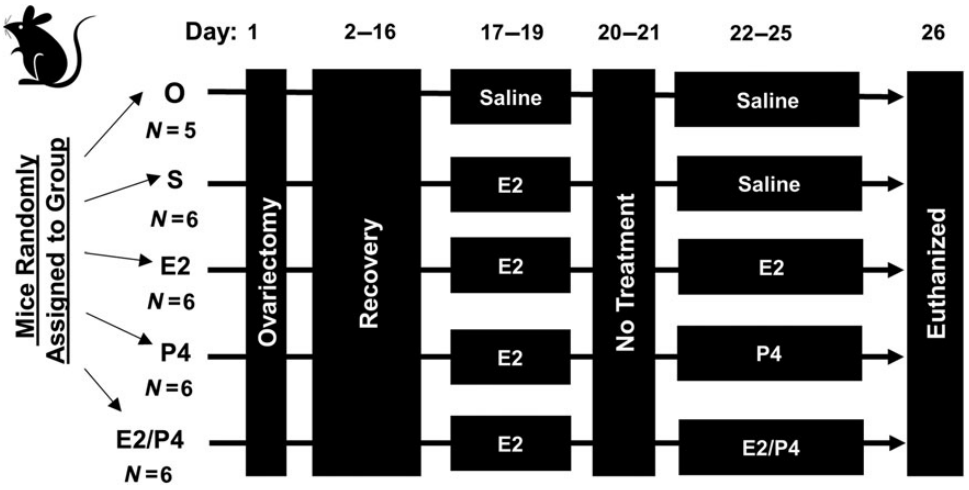


Figure 1 Experimental design for the ovariectomy and hormone replaced mice. Female mice ($n = 30$) were ovariectomized, and allowed to recover for 2 weeks. Mice were randomly assigned to one of five treatment groups (O, S, E2, P4, or E2/P4), and received daily subcutaneous injections of 5 μg of 17-β estradiol (E2), 500 μg of progesterone (P4), 5 μg of estradiol plus 500 μg of progesterone (E2/P4), or saline (S) as a vehicle according to the regimen and timing outlined. O: ovariectomized control group (no hormone exposure).

Table I Real-time PCR primers and information.

	Primers	Anneal (°C)	Melt Peak (°C)	Cycles	Accession number
BDNF	F: GCCCAACGAAGAAAACCATA R: TCAGTTGGCCTTTGGATACC	56	87	55	KF982302
NTRK2	F: CGAGGTTGGAACCTAACAGC R: TTACCCGTCAGGATCAGGTC	62	82	60	KF982303
NGFR	F: GAAGCTGCTCAATGGTGACA R: CACAGAGATGCTCGGTTCTG	58	90	55	KF982304
SORT1	F: TATGCCCCGAATTCCTAGTG R: CCACCTCACATGCAATGTTC	56	87	55	KF982305
GAPDH	F: TGTTCCTACCCCCAATGTGT R: ATGTAGGCCATGAGGTCCAC	56	85	55	KF982306

and Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Primers were purchased from IDT Technologies (Coralville, IA, USA). PCR product was sequenced (Laboratory Services, University of Guelph, Guelph, ON, Canada), and BLASTed to confirm its identity. Sequences were submitted to NCBI's GenBank and are listed in Table I. Plate-based real-time PCR was performed in duplicate (95°C 5 min, denaturation: 95°C 10 s; annealing: see Table I 20 s; elongation: 72°C 15 s; melting curve: 65–97 2.5°C/s) using the Roche LightCycler 480 (Roche Diagnostics, Laval, QC, Canada) and the SYBR Green I Master Mix (Roche). Relative quantification was performed with *Gapdh* as a reference gene using the Roche LightCycler software, which calculates an efficiency corrected normalized ratio of target gene to *Gapdh* using a mathematical algorithm developed by Roche. Bar graphs represent the group mean plus standard error of measurement (SEM).

For real-time PCR, *Gapdh* was used as a reference gene. Before relative quantification, a one-way ANOVA was used to determine if significant differences existed in crossing points between groups. No significant differences in *Gapdh* were observed between estrous cycle phases ($P = 0.179$) nor between groups of the mice receiving hormone supplementation ($P = 0.271$, data not shown).

Western blot

Total uterine protein (20 μg) was run on a 4–20% gradient gel (Thermo Scientific, Burlington, ON, Canada) under reducing conditions at 150 V for 50 min, and transferred to PVDF (VWR International, Mississauga, ON, Canada) at 40 V for 90 min. Skim milk/TBS-T (5%) was used to block for 1 h at room temperature. Blots were incubated with primary antibody (Table II) overnight at 4°C. Anti-Rabbit-ECL secondary (GE, Mississauga, ON, Canada) was applied for 1 h at 1:5000; then blots were incubated with enhanced chemiluminescence (ECL) substrate (Thermo-Scientific) for 5 min. X-ray film (Thermo-Scientific) was used for imaging; exposure times are listed in Table II. Blots were stripped using Restore Western Blot stripping buffer (Thermo-Scientific), and rinsed in TBS prior to incubation with another primary antibody. Densitometry was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). β-Tubulin was employed as

Table II Western blot information.

	Primary antibody concentration	Source	Exposure length (minutes)
BDNF	1:1000	Abcam (ab6201)	60
NTRK2	1:200	Abcam (ab18987)	60
NGFR	1:2000	Abcam (ab8874)	2
SORT1	1:2000	Abcam (ab16640)	3
β-tubulin	1:5000	Abcam (ab6046)	1

the reference gene for the western blots. No differences in β-tubulin were observed in cycling mice ($P = 0.086$) nor in ovariectomized mice receiving hormone supplementations ($P = 0.327$, data not shown).

Immunohistochemistry

One uterine horn was fixed in 10% formaldehyde, processed, and embedded in paraffin. Uterine cross sections were cut at 4 μm, deparaffinized, and stained for BDNF (ab9794, Abcam, Cambridge, MA, USA, 1:200), NTRK2 (ab56652, Abcam, 1:200), NGFR (ab8874, Abcam, 1:100), and SORT1 (ab16640, Abcam, 1:500) using 1% BSA in PBS as a diluent. In lieu of primary antibody, negative sections were incubated with the blocking solution in the Rabbit Vectastain ABC kit (Vector Labs, Burlington, ON, Canada). The ABC kit was used as per manufacturer's protocol, and DAB was employed as a chromogen (including negative sections). Images were captured with an Infinity camera (Lumenera Corp., Ottawa, ON, Canada) and Olympus IX81 microscope (Olympus, Richmond Hill, ON, Canada).

Quantification of NGFR staining

Four random images of uterine cross sections per mouse were obtained from mice in all cycle phases and treatment groups ($n = 3$ per phase or group). Luminal epithelial, glandular epithelial and stromal cells were counted (100 cells per type) and the percent staining positive for NGFR was calculated.

Data and statistical analysis

Within our data, there were values non-detectable by real-time PCR or western blot. There are several methods to handle non-detectable data points including: assigning these data a value of 0, the limit of detection for the assay, the square root of the limit of detection, or a random number between the limit of detection and zero (Newton and Rudel, 2007; Fievet and Della Vedova, 2010; Ballenberger *et al.*, 2012; Boyer *et al.*, 2013). We assigned a random number between the limit of detection for the gene or protein of interest and zero using the random number generator in the SigmaStat software package (SigmaStat 3.5 Systat Software, Inc., Chicago, IL, USA) because this method will randomly skew the data toward or away from zero, rather than always skewing it in the same direction. Statistical outliers in the data were identified by Grubb's test (http://graphpad.com/quickcalcs/Grubbs1.cfm) for $N > 6$, and the Dixon's Q test for a single outlier, for smaller sample sizes (http://contchart.com/outliers.aspx). Outliers were removed prior to analysis. Any other sample omissions were due to technical error. The number of non-detects, outliers, and omissions are in Supplementary Fig. S1. Real-time PCR and western blot data were compared by one-way ANOVA (SigmaStat 3.5 Systat Software, Inc., Chicago, IL, USA) and Tukey *post hoc* test. Data that were not normally distributed were analyzed by ANOVA on rank's and Dunn's *post hoc* test performed. A P-value of <0.05 was considered significant. Bars on the graphs represent the mean plus the standard error of measurement (SEM). Uterine localization of NGFR was compared by *t*-test.

Results

BDNF expression in the cycling mouse uterus

Bdnf transcripts were decreased ($P = 0.031$) in metestrus compared with estrus (Fig. 2A). When BDNF expression was assessed by western blot, four bands (~25, 27, 37, and 40 kDa) were observed in 37 of 39 uteri (Fig. 2B and D). No differences in the 25, 27, 37 or 40 kDa BDNF bands ($P = 0.425$, 0.263, 0.137, 0.107 respectively; Fig. 2B and D) were observed over the estrous cycle.

BDNF receptor expression in the cycling mouse uterus

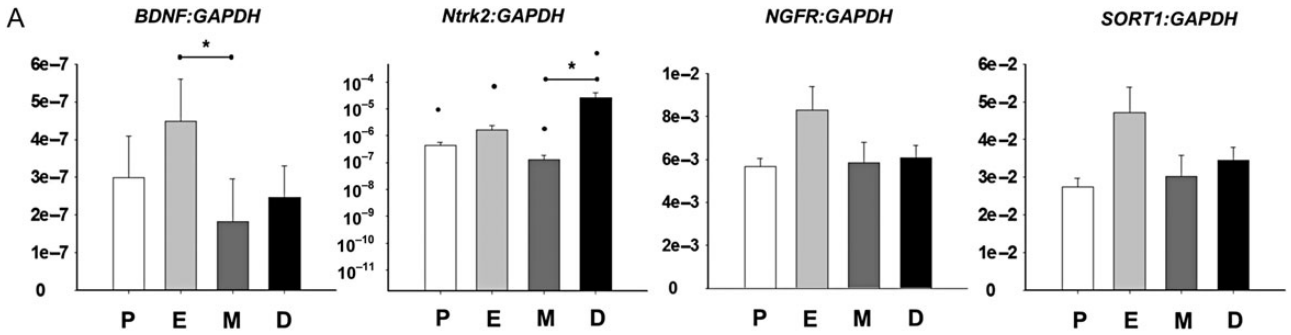
Ntrk2 transcripts were elevated in diestrus compared with metestrus ($P = 0.017$; Fig. 2A). NTRK2 55 kDa protein (Fig. 2B and D), an isoform we previously demonstrated in the human uterus and mouse brain using another NTRK2 antibody (Wessels *et al.*, 2014), remained stable over the estrous cycle ($P = 0.691$). The long (140 kDa) and truncated (95 kDa) forms of NTRK2 were below the limit of detection, even after an hour exposure. *Ngfr* transcripts were unaltered across the estrous cycle ($P = 0.221$; Fig. 2A). However NGFR protein decreased over the estrous cycle with levels at diestrus being significantly lower ($P = 0.005$) than those at pro-estrus or estrus (Fig. 2B–D). Transcripts and protein for SORT1 were unaffected by estrous cycle stage ($P = 0.104$, $P = 0.130$; Fig. 2A, B and D).

Uterine localization of BDNF and its receptors in the cycling mouse uterus

BDNF and NTRK2 were co-localized in the luminal epithelium, glandular epithelium, stroma and smooth muscle in the cycling mouse uterus (representative images, Fig. 3). NGFR was also present in all uterine cell types (Figs 3 and 4), but its expression in the luminal epithelium was dependent on whether there was a dominance of estrogen (pro-estrus, estrus, metestrus) or progesterone (diestrus) (Fig. 4A and B). NGFR expression increased ($P < 0.001$) in the luminal epithelium at diestrus when compared with other cycle stages (Fig. 4A). NGFR expression was absent in the internal layer of smooth muscle in the myometrium, but present in the external layer (Fig. 3). SORT1 remained consistently expressed in the luminal and glandular epithelium (Fig. 3).

Hormonal regulation of BDNF in the mouse uterus

Estrogen and progesterone increased *Bdnf* transcripts above ovariectomized controls, estrogen treated, and progesterone treated alone ($P = 0.002$; Fig. 5A). Treatment with estradiol significantly increased all quantified isoforms of BDNF in the mouse uterus (Fig. 5B–D). The 25 kDa band increased 6-fold above estrogen primed mice given saline ($P = 0.013$), and the 27 kDa band increased >7-fold ($P = 0.003$) above those given saline or progesterone. Estrogen treatment also significantly increased ($P = 0.041$) the 37 kDa form of BDNF above mice receiving saline. Additionally, estrogen treatment enhanced the 40 kDa band ($P = 0.046$) when compared with those treated with progesterone only.



B

Densitometry Values (% β-tubulin)		Proestrus	Estrus	Metestrus	Diestrus	P value
BDNF	25 kDa	70.5 ± 24.0	39.2 ± 26.6	49.1 ± 11.6	31.2 ± 7.43	0.425
	27 kDa	28.9 ± 9.44	71.9 ± 22.0	54.0 ± 19.1	31.3 ± 9.77	0.263
	37 kDa	39.0 ± 14.0	20.7 ± 11.5	34.6 ± 10.5	10.7 ± 2.07	0.137
	40 kDa	143.2 ± 44.5	95.2 ± 34.9	122.2 ± 16.9	35.7 ± 7.33	0.107
NTRK2	55 kDa	74.1 ± 10.3	67.7 ± 12.3	89.0 ± 22.4	65.5 ± 12.3	0.691
NGFR	75 kDa	111.1 ± 15.3^{A}	103.2 ± 24.8^{A}	63.8 ± 27.5^{A,B}	6.61 ± 2.20^{B}	***0.005***
SORT1	100 kDa	126.8 ± 36.1	91.4 ± 24.3	172.3 ± 69.5	147.1 ± 16.1	0.130

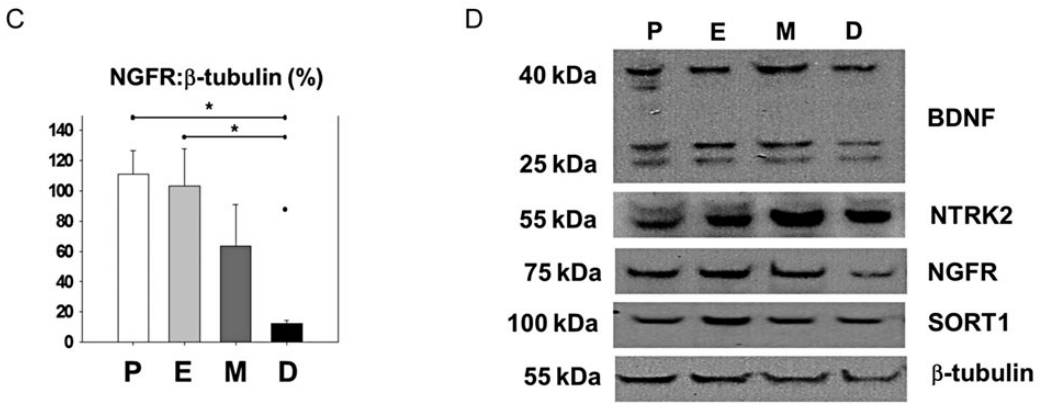


Figure 2 BDNF and its receptors in the cycling mouse uterus. Quantification of *Bdnf* ($n = 8, 17, 8, 14$), *Ntrk2* ($n = 7, 17, 8, 14$), *Ngfr* ($n = 8, 18, 9, 15$), and *Sort1* ($n = 8, 18, 9, 15$) transcripts using *Gapdh* as a reference gene (**A**). Densitometry values for BDNF ($n = 8, 11, 9, 10$), NTRK2 ($n = 7, 12, 9, 10$), NGFR ($n = 7, 12, 8, 9$) and SORT1 ($n = 8, 12, 9, 10$), expressed as a % loading control using β-tubulin (**B**). Graph of the statistically significant differences in NGFR expression over the estrous cycle (**C**). Representative western blot images showing immunoreactive bands for BDNF, NTRK2, NGFR, SORT1 and β-tubulin which was used as the reference gene for densitometry (**D**). Data are presented as mean ± standard error. Statistically significant differences are denoted by an asterisk (*) above the graph, or by different superscripts in the table (B). Outliers were not included in statistical analysis, but are denoted by a dot on the graph if they fell within its range. E: estrus, D: diestrus, M: metestrus, P: pro-estrus.

Hormonal regulation of BDNF receptors in the mouse uterus

No significant change in uterine *Ntrk2* transcripts was identified ($P = 0.066$, Fig. 5A). The 55 kDa band was not changed by hormonal treatment ($P = 0.788$; Fig. 5B and D). The full-length (140 kDa) and truncated (95 kDa) NTRK2 receptors were not quantifiable by western blot, after a 1 h exposure. No differences in *Ngfr* transcripts in the uterus were observed in the ovariectomized mice supplemented with exogenous

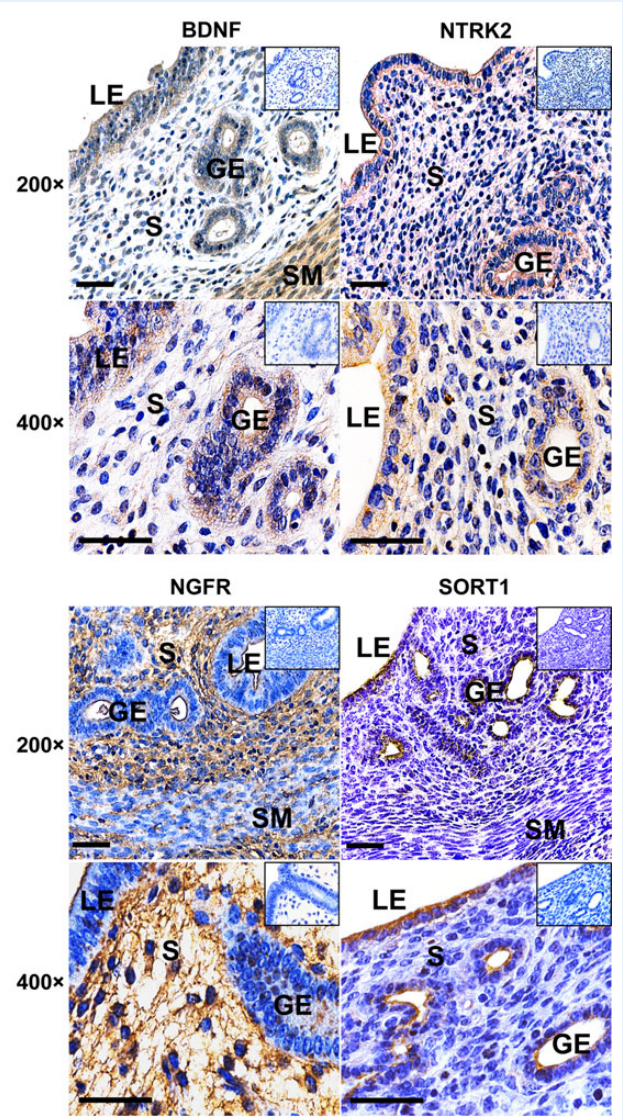


Figure 3 BDNF and receptor localization in the cycling mouse uterus. BDNF, and NTRK2 were co-localized in the luminal epithelium, glandular epithelium, stroma, and smooth muscle in the cycling mouse uterus. Expression of NGFR was also present in all uterine cell types but its localization was dependent on whether there was a dominance of estrogen (pro-estrus) or progesterone (diestrus) (see Fig. 4). SORT1 remained consistently expressed in the luminal and glandular epithelium. Inset images: negative control sections incubated with blocking serum in lieu of primary antibody. Original magnification: ×200, ×400. Scale bar = 50 μm. $n = 8$ (pro-estrus), 18 (estrus), 9 (metestrus), 15 (diestrus). GE: glandular epithelium, LE: luminal epithelium, S: stroma, SM: smooth muscle. E: estrus, D: diestrus, M: metestrus, P: pro-estrus.

hormones ($P = 0.131$; Fig. 5A). NGFR expression in the uterus was significantly increased ($P < 0.001$) by estradiol treatment when compared with saline and P4 treated animals (Fig. 5B–D). Estrogen and progesterone co-treatment increased *Sort1* transcripts in the uterus above mice treated with estrogen alone, or saline ($P = 0.007$; Fig. 5A). This difference in SORT1 was not observed at the protein level ($P = 0.503$; Fig. 5B and D).

Uterine localization of BDNF and its receptors in the hormone replacement mouse uterus

BDNF and NTRK2 were located in the luminal epithelium, glandular epithelium, stroma and smooth muscle in the mouse uterus of all treatment groups (representative images from mice treated with estradiol in Fig. 6). Mice treated with estradiol had enhanced BDNF expression, particularly in stromal cells. NGFR was found in all uterine cell types (Figs 4 and 6) but, as in cycling mice, its localization was dependent on whether mice were exposed to estrogen or progesterone (representative images from mice treated with estradiol in Fig. 6). NGFR expression was increased in the stromal cells of ovariectomized mice given estrogen ($P = 0.002$) when compared with mice given progesterone or the ovariectomized controls (group O), and its expression switched to the luminal ($P < 0.001$) and glandular epithelium ($P \leq 0.001$) in mice given progesterone (Fig. 4C). SORT1 was located on the apical side of the glandular epithelium, and occasionally, the luminal epithelium (representative images from mice treated with progesterone in Fig. 6).

Discussion

Emerging evidence suggests an important role for BDNF in uterine physiology and pathology. Herein we show that BDNF and its low affinity receptor NGFR are regulated by estradiol in the uterus. We contrast the expression of uterine BDNF and its receptors during the 4-day estrous cycle with expression in response to daily estradiol exposure during hormone replacement, as summarized in Fig. 7.

Estrogen regulates BDNF expression in the uterus

In ovariectomized mice, daily estrogen significantly increased all of the BDNF isoforms quantified. BDNF can be a monomer (13 kDa), dimer (26 kDa), or pro- (42 kDa) protein, and can undergo post-translational modifications (Mowla *et al.*, 2001; Teng *et al.*, 2005; Pruunsild *et al.*, 2007; Matsumoto *et al.*, 2008; Koshimizu et al., 2009). Stability studies suggest BDNF dimers are stable, even under reducing blot conditions (Radziejewski *et al.*, 1992; Kolbeck *et al.*, 1994; Pan *et al.*, 1998). Thus, the 25, 27, 37 and 40 kDa bands are likely dimerized and pro-BDNF, with and without post-translational modification. Although progesterone affects BDNF expression in the brain (Kaur *et al.*, 2007; Jodhka *et al.*, 2009; Meyer *et al.*, 2012; Su *et al.*, 2012; Atif *et al.*, 2013) and nervous system (Gonzalez *et al.*, 2004, 2005; De Nicola *et al.*, 2006; Gonzalez Deniselle *et al.*, 2007; Cekic *et al.*, 2012), and BDNF is expressed in luteinized granulosa cells (Dominguez *et al.*, 2011), progesterone did not alter uterine BDNF. Our results concur with Coughlan *et al.* (2009) where progesterone did not alter BDNF expression in response to neuronal injury. As Jodhka *et al.* (2009) reported that progesterone was capable of increasing BDNF in the brain but medroxyprogesterone was not, we speculate that the form of progesterone employed affects induction of BDNF.

While this is the first report of estrogen-induced BDNF expression in the uterus, previous studies in the brain support a role for estrogen in BDNF regulation (Toran-Allerand *et al.*, 1992; Miranda *et al.*, 1993; Singh *et al.*, 1995; Sohrabji *et al.*, 1995; Gibbs, 1998, 1999; Jezierski and Sohrabji, 2000, 2001; Berchtold *et al.*, 2001; Liu *et al.*, 2001; Solum and Handa, 2002; Scharfman and Maclusky, 2005; Pan *et al.*, 2010; Tang and Wade, 2012). Additionally, circulating levels of BDNF strongly

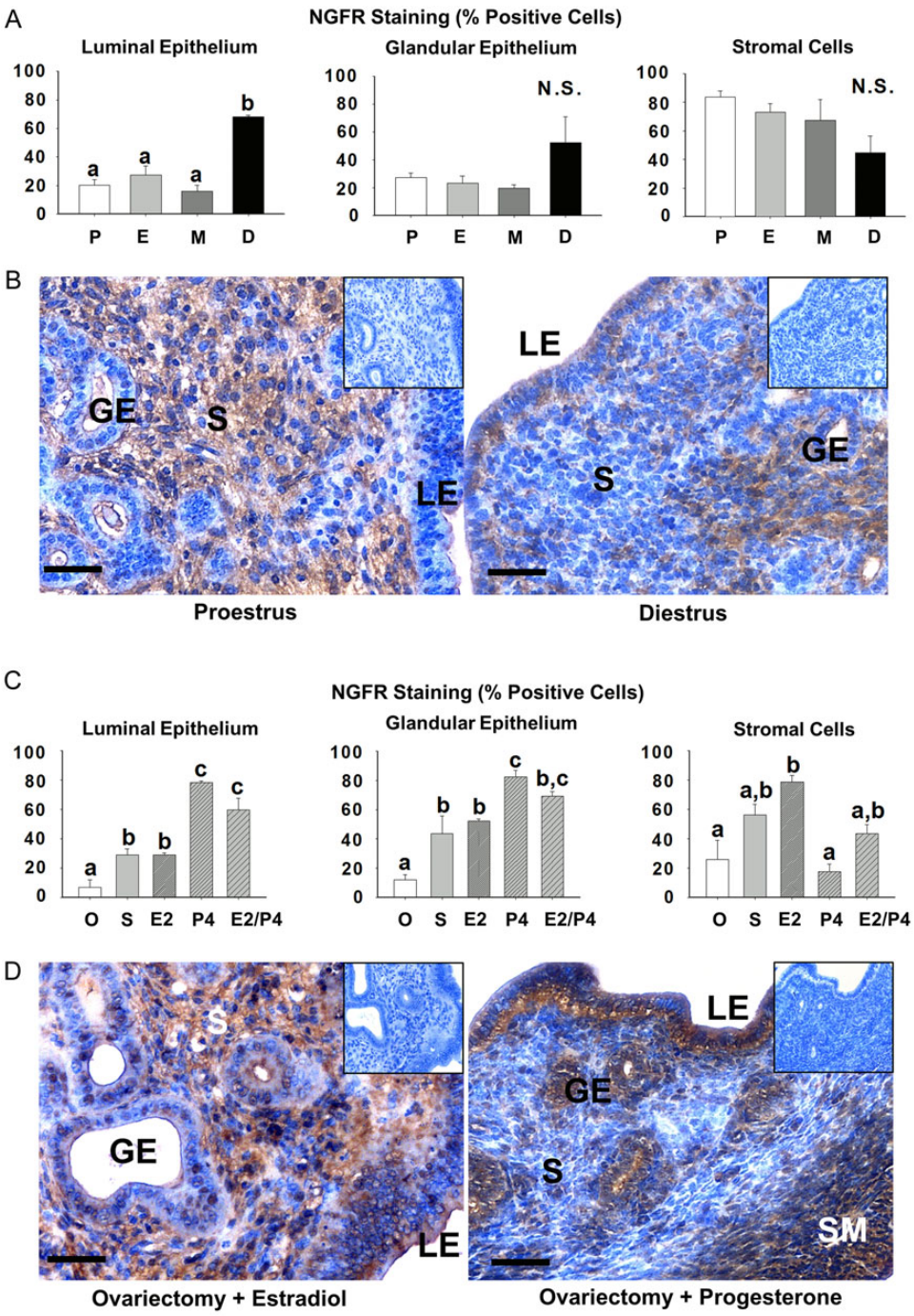
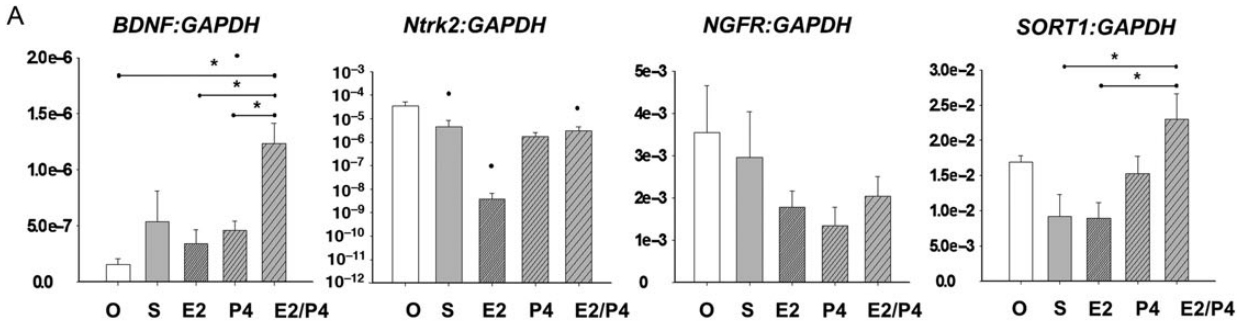


Figure 4 NGFR localization in response to estrogen versus progesterone. In cycling mice, NGFR expression was increased in the luminal epithelium at diestrus ($P < 0.001$) compared with the other cycle phases (**A**, **B**). In ovariectomized mice, the administration of estradiol increased NGFR expression in the stromal cells ($P < 0.001$) when compared with animals given progesterone. The pattern of expression switched to the luminal ($P < 0.001$) and glandular epithelium ($P = 0.002$) when mice were given progesterone (**C**, **D**). Data are presented as mean ± standard error. Statistically significant differences are denoted by different superscripts above the bars. Inset images: negative control sections incubated with blocking serum in lieu of primary antibody. Original magnification: ×200. Scale bar = 50 μm. $n = 3$ per group. E: estrus, D: diestrus, M: metestrus, P: pro-estrus. GE: glandular epithelium, LE: luminal epithelium, S: stroma, SM: smooth muscle. O, S, E2, P4, E2/P4: treatment groups according to Fig. 1. N.S.: not statistically significant.

correlate with estradiol (Pluchino *et al.*, 2009), and fluctuate over the menstrual cycle in women (Begliuomini *et al.*, 2007), and BDNF can be induced by estrogen in the rat uterus (Krizsan-Agbas *et al.*, 2003). Here we have shown that daily estrogen exposure after ovariectomy significantly increases uterine BDNF, but the hormonal fluctuations of the murine estrous cycle do not.

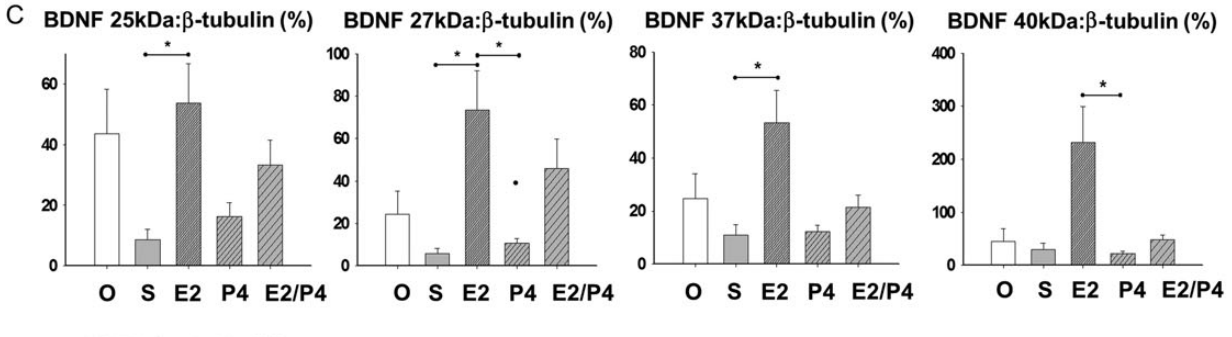
A

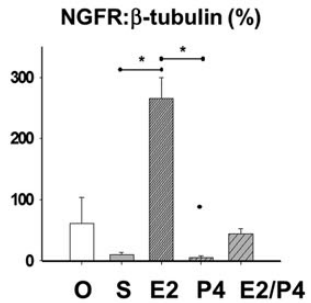


B

Densitometry Values (% β-tubulin)		OVX	Saline	Estradiol	Progesterone	Estradiol + Progesterone	P value
	E2 primed	–	+	+	+	+	N/A
BDNF	25 kDa	$43.7 \pm 14.5^{A,B}$	8.48 ± 3.41^{B}	53.4 ± 13.1^{A}	$16.1 \pm 4.61^{A,B}$	$33.3 \pm 8.14^{A,B}$	***0.013***
	27 kDa	$29.9 \pm 11.8^{A,B}$	5.87 ± 2.40^{A}	73.4 ± 18.5^{B}	10.5 ± 2.46^{A}	$46.0 \pm 13.7^{A,B}$	***0.003***
	37 kDa	$24.7 \pm 9.36^{A,B}$	10.9 ± 4.00^{A}	53.4 ± 12.3^{B}	12.3 ± 2.42^{A}	21.5 ± 4.52^{A}	***0.041***
	40 kDa	$44.8 \pm 23.5^{A,B}$	$29.8 \pm 11.9^{A,B}$	231.7 ± 67.3^{A}	21.7 ± 4.22^{B}	$47.7 \pm 9.52^{A,B}$	***0.046***
NTRK2	55 kDa	51.6 ± 23.1	64.0 ± 17.8	86.0 ± 15.0	28.8 ± 11.7	80.5 ± 6.36	0.788
NGFR	75 kDa	$61.4 \pm 42.4^{A,B}$	9.85 ± 3.71^{A}	265.0 ± 34.5^{B}	5.39 ± 2.48^{A}	$44.0 \pm 8.29^{A,B}$	***<0.001***
SORT1	100 kDa	216.3 ± 28.0	172.4 ± 37.9	134.6 ± 35.4	154.7 ± 31.7	174.3 ± 19.4	0.503

C





D

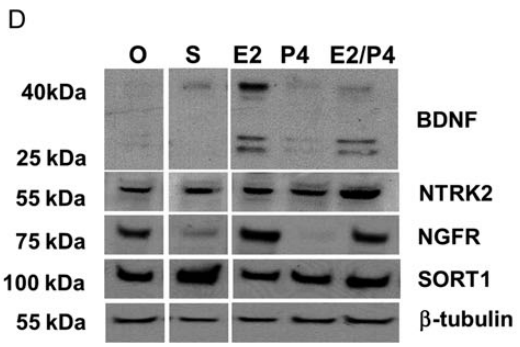


Figure 5 Hormonal regulation of BDNF and its receptors in the mouse uterus. Ovariectomized mice were assigned to treatment groups as outlined in Fig. 1. Quantification of *Bdnf* ($n = 5, 6, 6, 5, 6$), *Ntrk2* ($n = 5, 5, 5, 6, 5$), *Ngfr* ($n = 5, 6, 6, 6, 6$) and *Sort1* ($n = 4, 6, 6, 6, 6$) transcripts using *Gapdh* as a reference gene (**A**). Densitometry values for BDNF ($n = 5, 6, 6, 6, 6$), NTRK2 ($n = 5, 5, 6, 6, 6$), NGFR ($n = 4, 6, 6, 5, 6$) and SORT1 ($n = 5, 6, 6, 6, 6$), expressed as a % loading control using β-tubulin (**B**). Graph of the statistically significant differences in BDNF and NGFR expression in response to exogenous hormones (**C**). Representative western blot images showing immunoreactive bands for BDNF, NTRK2, NGFR, SORT1 and β-tubulin which was used as the reference gene for densitometry. Each gene quantified by densitometry was run on the same membrane; the images have been cropped to reflect the experimental groupings in the graphs (**D**). Data are presented as mean ± standard error. Statistically significant differences are denoted by an asterisk (*) above the graph, or by different superscripts in the table (B). Outliers were not included in statistical analysis, but are denoted by a dot on the graph if they fell within its range. O, S, E2, P4, E2/P4: treatment groups according to Fig. 1. OVX: ovariectomy.

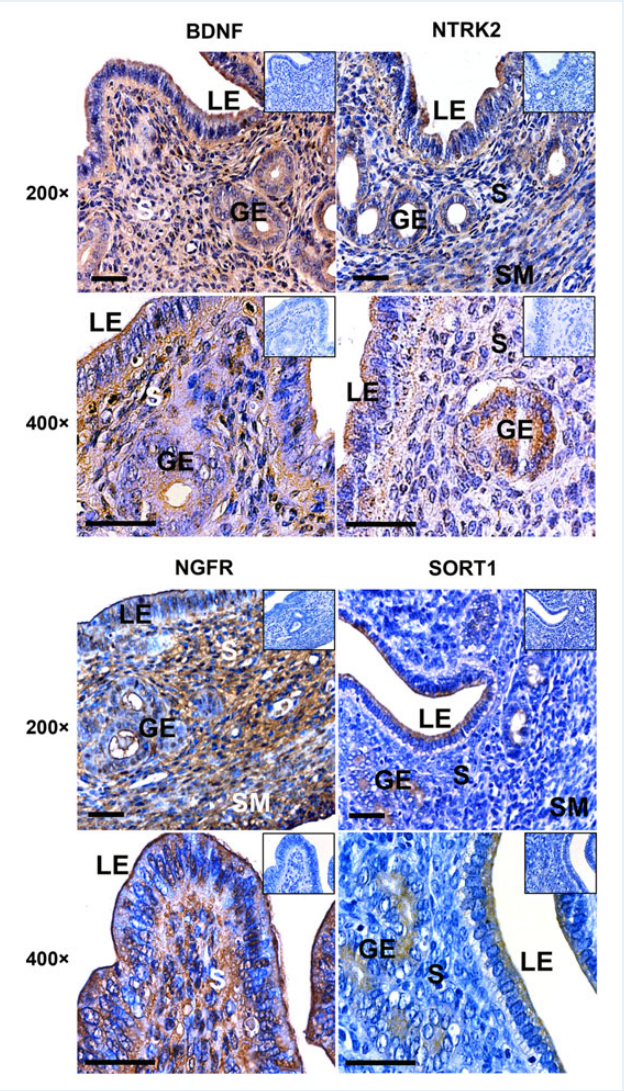


Figure 6 BDNF and receptor localization in the hormone replacement mouse uterus. Uterine localization of BDNF and its receptors in ovariectomized mice given hormone supplementation according to Fig. 1. BDNF, and NTRK2 were located in the luminal epithelium, glandular epithelium, stroma, and smooth muscle in the mouse uterus of all treatment groups (representative images from mice treated with estradiol). NGFR was found in all uterine cell types, but as in the cycling mice its localization was dependent on whether mice were exposed to estrogen or progesterone (see Fig. 4) (representative images from mice treated with estradiol). SORT1 was located on the apical side of the glandular epithelium, and the luminal epithelium in the uteri of mice in all treatment groups (representative images from mice treated with progesterone). Inset images: negative control sections incubated with blocking serum in lieu of primary antibody. Original magnification: ×200, ×400. Scale bar = 50 μm. GE: glandular epithelium, LE: luminal epithelium, S: stroma, SM: smooth muscle.

Estrogen regulates BDNF receptors in the uterus

The uterine expression of NGFR decreased over the estrous cycle, and increased in response to estrogen supplementation, while no hormonal regulation of NTRK2 or SORT1 was observed. We postulate that estrogen stabilizes NGFR or increases its half-life, as *Ngfr* transcripts are not affected by estradiol. Alternately, estrogen may enhance translation of transcripts, without increasing their quantity (signal amplification). The precise mechanism of estradiol action is unclear, but is likely via indirect regulation of the NGFR protein. Further, NGFR was spatially regulated in the uterus; expression shifted from stromal to epithelial cells when ovariectomized animals were given estrogen versus progesterone.

Regulation of BDNF receptors by estradiol and progesterone in the brain, nervous system (Gibbs and Pfaff, 1992; Sohrabji *et al.*, 1994a,b; Jezierski and Sohrabji, 2001; Brito *et al.*, 2004; Hasan *et al.*, 2005; De Nicola *et al.*, 2006; Spencer *et al.*, 2008; Anesetti *et al.*, 2009; Pan *et al.*, 2010; Cekic *et al.*, 2012; Tang and Wade, 2012) and ovary (Lara *et al.*, 2000) have been reported. Interestingly, in Hasan *et al.* (2005), acute estrogen exposure in sympathetic neurons did not affect NGFR expression, but chronic exposure did. Here we have shown that uterine NGFR expression decreases over the estrous cycle, and increases in response to daily estrogen exposure after ovariectomy, while other BDNF receptors remain stable. We have also demonstrated the spatial regulation of NGFR in response to ovarian hormones.

BDNF and receptor expression in ovary intact cycling mice when compared with ovariectomized and estradiol replaced mice

In mice, the estrous cycle likely occurs too quickly to significantly affect uterine neurotrophins. Although transcripts for *Bdnf* and *Ntrk2* varied over the estrous cycle, BDNF, NTRK2, and SORT1 expression remained stable and NGFR declined from pro-estrus through diestrus. This decline would increase the local bioavailability of BDNF and signaling through the BDNF-NTRK2 pathways in the uterus during the latter part of the cycle. Thus, under physiological conditions the neurotrophic *milieu* of the uterus is controlled by NGFR. However, when mice were exposed to daily high dose estrogen, which models the chronic estrogen present in endometriotic lesions in women with endometriosis (Noble *et al.*, 1996; Huhtinen *et al.*, 2012) or other estrogen-dependent diseases, the exposure had profoundly different effects on the uterine expression of BDNF and its receptors. Estradiol treatment significantly increased the uterine expression of mature BDNF (>6-fold), pro-BDNF (>5-fold) and NGFR (5-fold) when compared with the other treatments. While neither NTRK2 nor SORT1 were affected by ovarian hormones, continued daily exposure to estradiol increased mature BDNF which would lead to the induction of the BDNF-NTRK2 pathways, without affecting NTRK2 levels.

The neurotrophins are a complex network, and regulation of BDNF and NGFR by estrogen in the uterus can impact many BDNF pathways including angiogenesis (Kermani *et al.*, 2005; Nakamura *et al.*, 2006), cellular proliferation (Tervonen *et al.*, 2006; Kawamura *et al.*, 2010), adhesion (Zhou *et al.*, 1997; Douma *et al.*, 2004; Geiger and Peeper, 2007) and resistance to apoptosis (Douma *et al.*, 2004; Wang *et al.*, 2005; Geiger and Peeper, 2007; Kawamura *et al.*, 2010; Li *et al.*, 2011). Here, we also demonstrated the effect of estrogen on pro-BDNF in the uterus. The precise function of each BDNF isoform is only beginning to be elucidated, but generally pro-BDNF counteracts the effects of mature BDNF, providing another level of regulation for the powerful pathways activated by BDNF. We have shown a temporal effect to the hormonal regulation of NGFR in the cycling uterus, and highlighted the

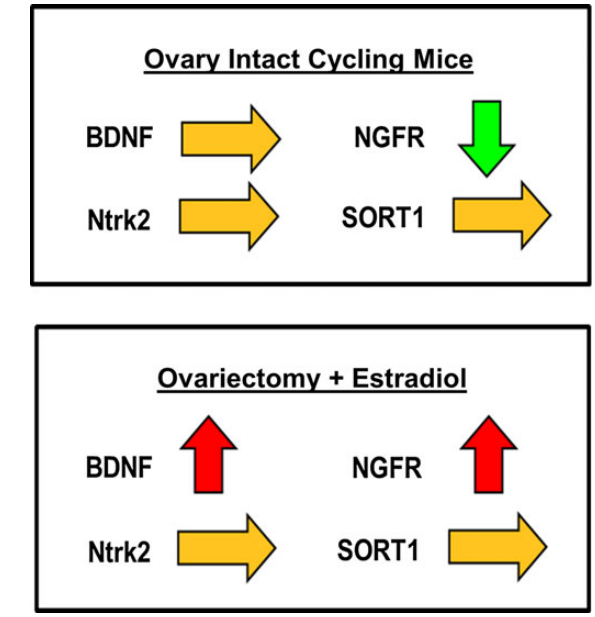


Figure 7 Contrasting uterine BDNF and receptor expression in cycling mice versus ovariectomy + estradiol replacement. A summary of the uterine expression of BDNF, NTRK2, NGFR and SORT1 in the uterus under physiological conditions (hormone exposure during the estrous cycle) when compared with mice undergoing daily estradiol replacement according to the regimen in Fig. 1 (E2 group). Green arrow: NGFR significantly decreases over the estrous cycle, under physiological conditions. Red arrow: BDNF and NGFR are significantly increased by daily estrogen exposure in ovariectomized mice. Yellow arrow: no change over the estrous cycle or treatment groups.

differential spatial localization of NGFR in response to ovarian hormones. The neurotrophins are involved in reproductive pathologies (Borghese *et al.*, 2010; Browne *et al.*, 2012; Bao *et al.*, 2013; Barcena de Arellano *et al.*, 2013), and physiological processes (Kawamura *et al.*, 2009, 2011; Kerr *et al.*, 2009; Dorfman *et al.*, 2011, 2014; Shoae-Hassani *et al.*, 2011; Non *et al.*, 2012). Although little is known about the functions of BDNF and its receptors within the reproductive system, they are poised to participate in many aspects of reproductive physiology and pathology. The results of this study implicate estrogen in the uterine up-regulation of BDNF and NGFR, and highlight the differing effect of hormone exposure during the estrous cycle versus estradiol replacement after ovariectomy on neurotrophin expression. Sustained estrogen exposure, as seen in estrogen-dependent disease, may tip the neurotrophin balance and inappropriately activate pathways important in the disease pathophysiology.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

All authors contributed to the study concept and design; drafted and critically revised the manuscript; and provided final approval of the version to be published. J.M.W. acquired, analyzed and interpreted the data.

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Conflict of interest

The authors do not have any conflicts of interest to declare.

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