Supplemental Material for

Sustained adrenergic signaling promotes intratumoral innervation through BDNF induction

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Supplementary Material includes:

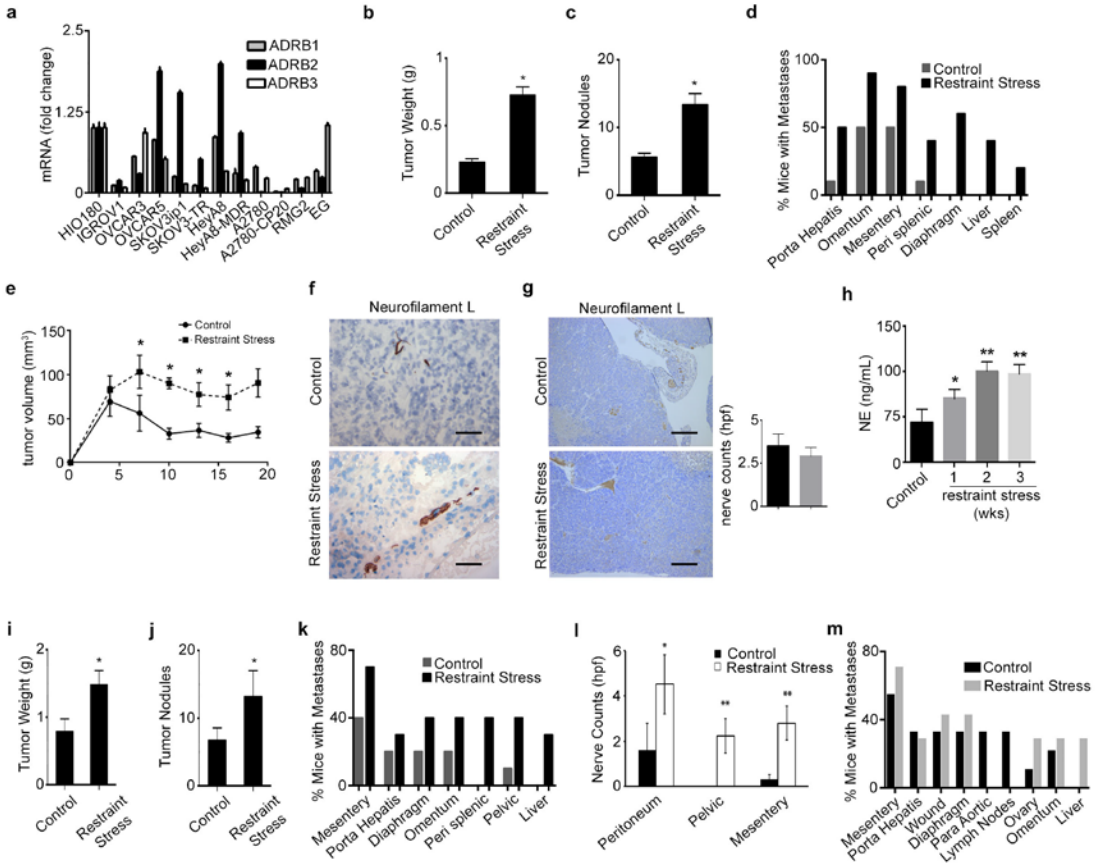

Figure S1. Sustained adrenergic signaling increases nerve counts in tumors. ADRB1, ADRB2 and ADRB3 **(a)** mRNA expression in a panel of ovarian cancer cells. **(b)** Tumor weight, **(c)** nodules and **(d)** metastases locations in mice inoculated *ip* with SKOV3ip1 cells and subjected to daily restraint stress. **(e)** Restraint stress induced tumor growth of OVCA432 model. Neurofilament expression in orthotopic **(f)** HeyA8 or **(g)** ADRB-null A2780 tumors from control mice and mice exposed to restraint stress. Scale bar represents 50 μM in f and100 μM in g. **(h)** Tumoral NE levels in mice subjected to restraint stress for one, two or three weeks. **(i)** Tumor weight, **(j)** nodules, **(k)** metastases locations and **(l)** nerve counts in mice injected with SKOV3ip1 cells directly into the ovary and subjected

to daily restraint stress. Metastases location in mice injected with SKOV3ip1 directly into the ovary and subjected to daily restraint stress **(m)**.

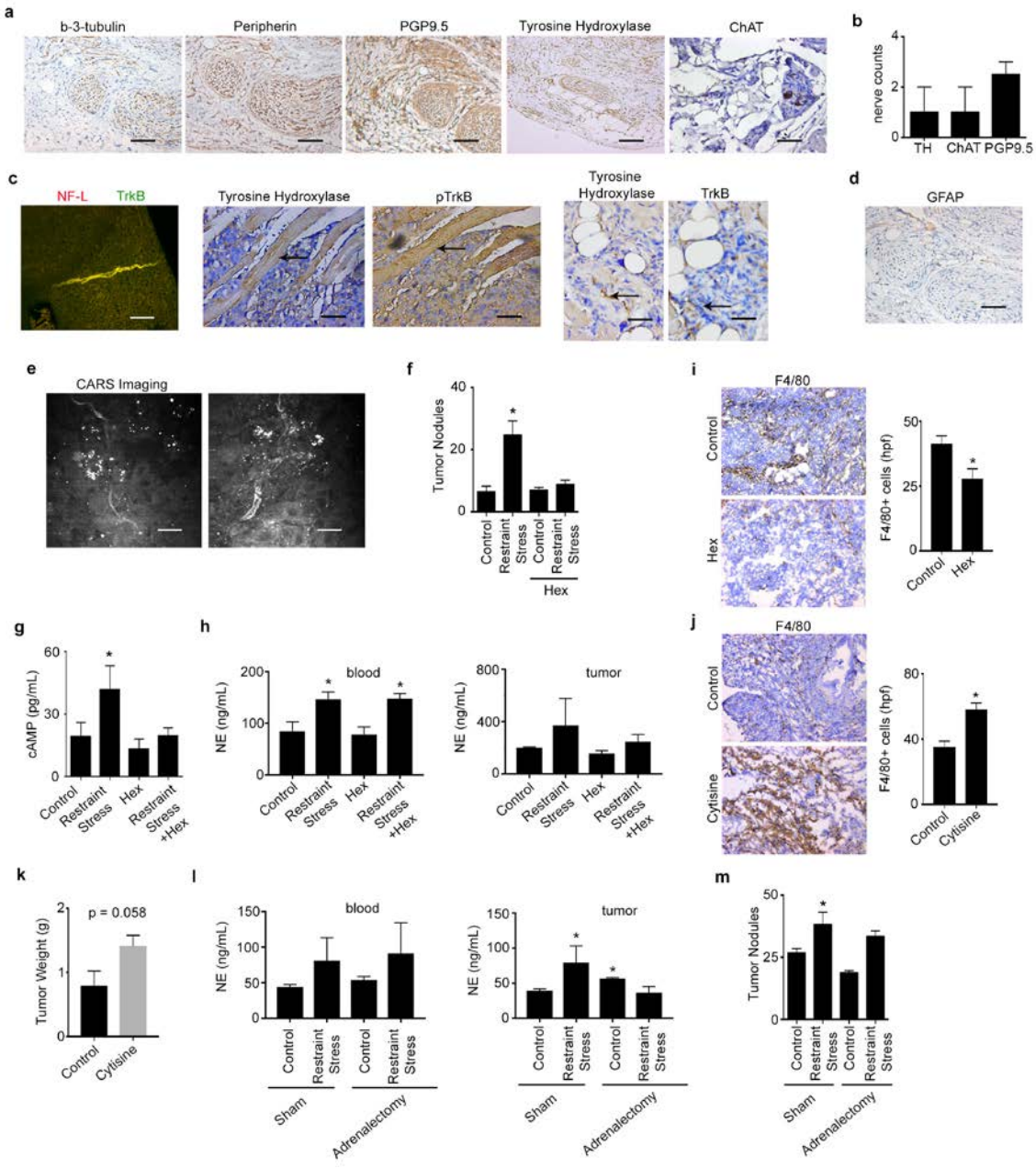

Figure S2. Characterization of tumoral innervation. Representative images depicting **(a)** beta-3-tubulin, peripherin, pgp9.5 (Scale bar represents 50 μM), tyrosine hydroxylase (Scale bar represents 100 μM) and ChAT (Scale bar represents 25 μM). **(b)** Quantification of pgp9.5, tyrosine hydroxylase and choline acetyltransferase positive nerves. **(c)** Representative images depicting TrkB,

pTrkB and Tyrosine Hydroxylase positive nerves in tumors. **(d)** GFAP expression in tumor associated nerves. Scale bar represents 50 μM. **(e)** *Ex-vivo* images of intratumoral nerves by coherent anti-Stokes Raman scattering microscopy. Scale bar represents 50 μM. **(f)** Number of tumor nodules in SKOV3ip1 control mice and mice treated with hexamethonium bromide and exposed to restraint stress. **(g)** cAMP or **(h)** NE tumoral levels from mice exposed to restraint stress and treated with hexamethonium bromide. Quantification of F4/80+ macrophages per high power field in hexamethonium bromide **(i)** or cytisine **(j)** treated tumor bearing mice. **(k)** Tumor weight from cytisine treated mice. **(l-m)** NE levels and number of tumor nodules in adrenalectomized and sham surgery mice exposed to restraint stress. Means and standard error of the mean are shown; n = 10 mice per group, except for tumor and blood NE analyses, F4/80 infiltration and k (n = 2-5 mice per group). $^{*}P < 0.05$.

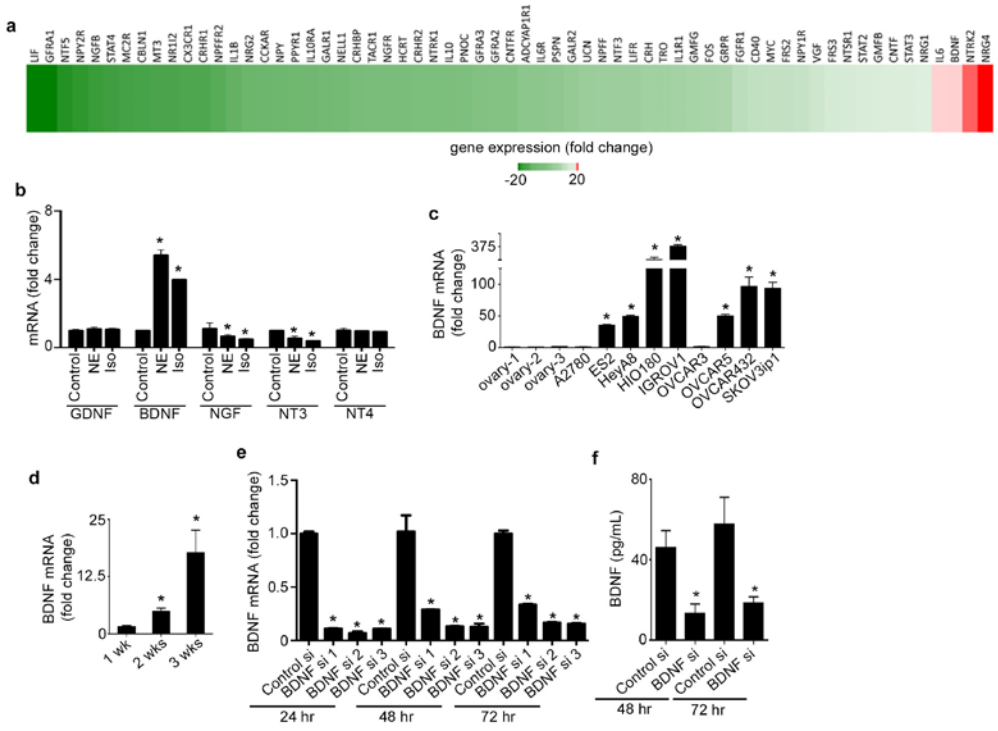

Figure S3. NE induces BDNF expression. (a) Neurotrophic factor array generated from SKOV3ip1 cells treated with NE. **(b)** qRT-PCR results for GDNF, BDNF, NGF, NT3 and NT4 levels after NE or Iso treatment. **(c)** BDNF mRNA levels in a panel of ovarian cancer cells and normal ovarian samples. **(d)** BDNF mRNA levels in tumor bearing mice subjected to one, two or three weeks of daily restraint stress. **(e-f)** qRT-PCR validating 3 unique BDNF siRNA sequences and BDNF protein silencing.

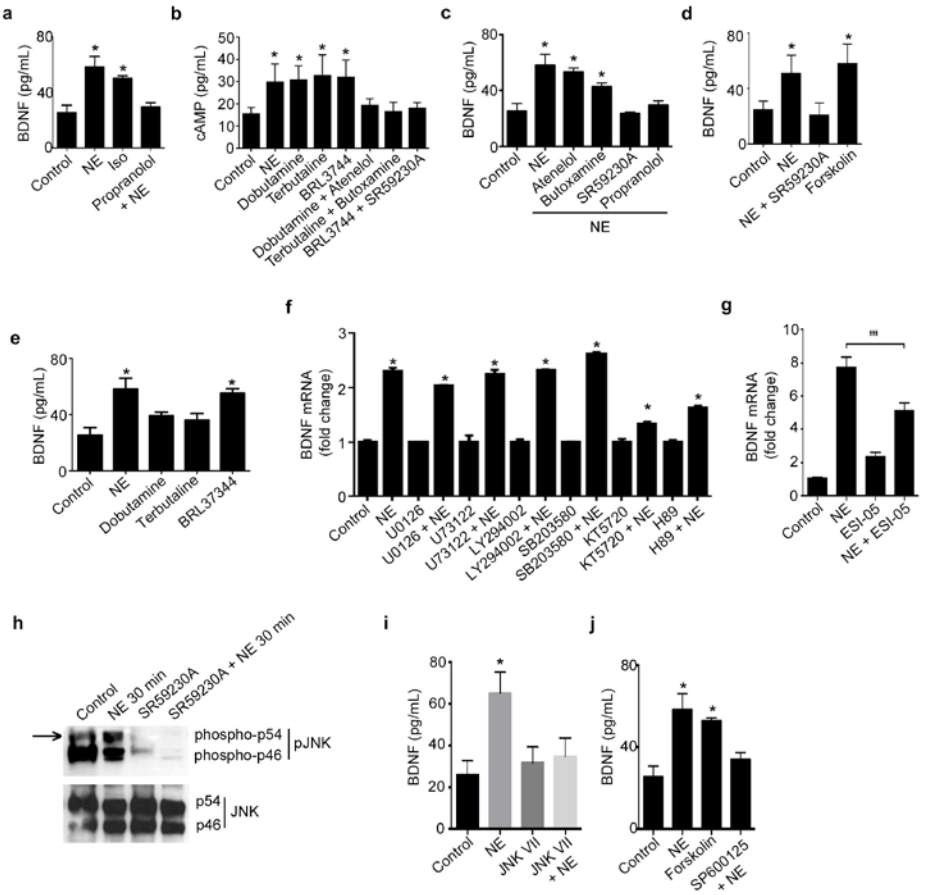

Figure S4. NE-induced BDNF expression is mediated by ADRB3/Epac/Jnk. **(a)** BDNF protein levels in the supernatant of HeyA8 cells treated with NE, Iso or propranolol. **(b)** Validation of ADRB agonists and antagonist efficacy by measuring cAMP levels. **(c-d)** BDNF protein levels in the supernatant of HeyA8 cells treated with NE and specific ADRB inhibitors atenolol (ADRB1), butoxamine (ADRB2), SR59230A (ADRB3) and propranolol (non-specific ADRB). **(e)** BDNF protein levels in the supernatant of HeyA8 cells treated with NE and specific ADRB agonists dobutamine (ADRB1), terbutaline (ADRB2) and BRL37344 (ADRB3). **(f)** BDNF mRNA levels of HeyA8 cells treated with U0126 (MEK), U73122 (PLC), LY294002 (PI3K), SB203580 (P38), KT5720/H89 (PKA) or ESI-05 (Epac)

antagonists. **(h)** Western blot analyses depicting pJnk levels after NE treatment and/or SR59230A (ADRB3 antagonist) in HeyA8 cells. **(i-j)** BDNF protein levels in the supernatant of HeyA8 cells treated with NE, forskolin, SP600125/Jnk VII (Jnk inhibitor). Means and standard error of the mean are shown; $*P < 0.05$.

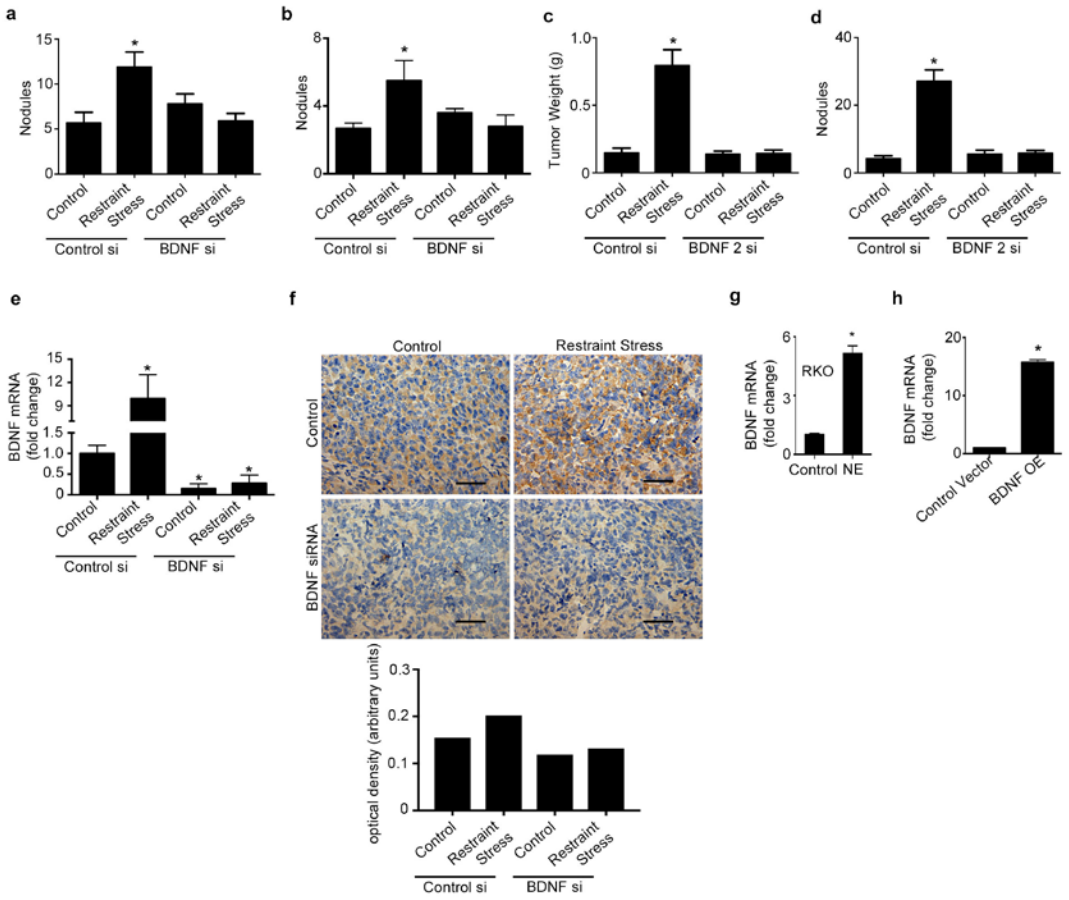

Figure S5. BDNF increases nerve counts. (a) Number of tumor nodules in SKOV3ip1 mice treated with control or BDNF siRNA in the presence or absence of restraint stress. **(b)** Number of tumor nodules in HeyA8 mice treated with control or BDNF siRNA in the presence or absence of restraint stress. **(c-d)** Tumor weight and number of nodules in SKOV3ip1 mice treated with control or a second sequence of BDNF siRNA in the presence or absence of restraint stress. **(e)** Relative expression of BDNF gene in each group in the SKOV3ip1 model. **(f)** Representative BDNF protein expression in tumors from each group in the SKOV3ip1 model and image quantification. Scale bar represents 50 μM. **(g)** BDNF mRNA levels in NE treated RKO cells. **(h)** mRNA levels of BDNF in SKOV3ip1-

BDNF-OE cells. Means and standard error of the mean are shown; n = 10 mice per group for a-b and n = 5 mice per group for c-d. *$P < 0.05$.

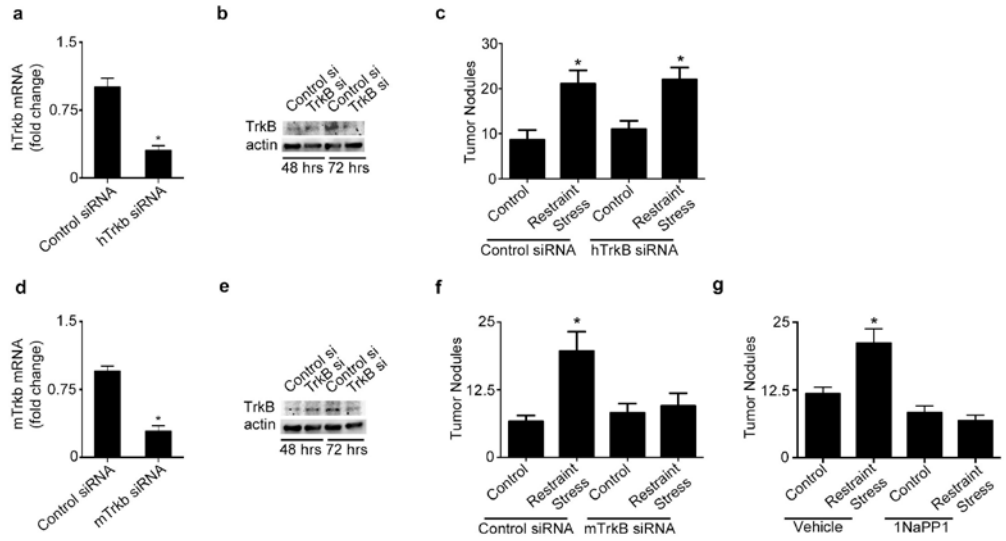

Figure S6. Adrenergic-mediated mTrkB activation leads to increased *in vivo* tumor nodule counts. (a) hTrkb mRNA or **(b)** protein levels after siRNA induced silencing. **(c)** Number of tumor nodules in SKOV3ip1 mice treated with control or hTrkB siRNA-DOPC. **(d)** mTrkb mRNA or **(e)** protein levels after siRNA induced silencing. **(f)** Number of tumor nodules in SKOV3ip1 mice treated with control or mTrkB siRNA-chitosan. **(g)** Number of tumor nodules in ID8-VEGF tumor-bearing $^{+/+}TrkB^{F616A}$ mice treated with vehicle or 1NaPP1. Means and standard error of the mean are shown; n = 10 mice per group. **P* < 0.05.

Supplementary Table 1

Alteration in pathways associated with neuronal growth and function after NE treatment (HeyA8 and SKOV3ip1 cells)			
Annotation ID	**Functional Annotation**	**Hyper-geometric p-value**	**Genes**
GO:0051969	regulation of transmission of nerve impulse	9.41E-05	EDN1, IL6, BDNF, EIF2AK3
GO:0007406	negative regulation of neuroblast proliferation	1.24E-04	BDNF
GO:0031644	regulation of neurological system process	1.33E-04	EDN1, IL6, BDNF, EIF2AK3
GO:0023061	signal release	1.48E-04	EDN1, SLC38A2, IL6, BDNF, EIF2AK3
GO:0045666	positive regulation of neuron differentiation	2.46E-04	IL6, BDNF
GO:0043523	regulation of neuron apoptosis	3.66E-04	IL6, NR4A2, BDNF
GO:0051402	neuron apoptosis	5.15E-04	IL6, NR4A2, BDNF
GO:0070997	neuron death	5.78E-04	IL6, NR4A2, BDNF
GO:0043524	negative regulation of neuron apoptosis	0.0012	NR4A2, BDNF
GO:0051960	regulation of nervous system development	0.0016	IL6, BDNF, EIF2AK3, MEIS1
GO:2000177	regulation of neural precursor cell proliferation	0.0030	BDNF
GO:0045664	regulation of neuron differentiation	0.0033	IL6, BDNF, MEIS1
GO:0007405	neuroblast proliferation	0.0039	BDNF
GO:0050767	regulation of neurogenesis	0.0069	IL6, BDNF, MEIS1
GO:0021675	nerve development	0.0073	BDNF
GO:0050804	regulation of synaptic transmission	0.0092	EDN1, BDNF
GO:0048167	regulation of synaptic plasticity	0.0141	BDNF
GO:0007409	axonogenesis	0.0451	RHOB, NR4A2, BDNF

Supplementary Table 2. Association of Clinicopathologic variables with BDNF protein expression

	BDNF expression		
variable	**low**	**high**	**p value**
stage			
I-II	11	3	0.03
III-IV	46	48	
ascites			
no	31	18	0.047
yes	26	33	
cytoreduction			
optimal	11	20	0.02
suboptimal	46	31	

Supplementary Table 3. Association of Clinicopathologic variables with Nerve Density

	Nerve Density		
variable	**low**	**high**	**p value**
stage			
I-II	12	2	0.01
III-IV	47	47	
ascites			
no	37	12	<.01
yes	22	37	
cytoreduction			
optimal	14	17	0.21
suboptimal	45	32	