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Chronic stress promotes tumor growth through increased BDNF production and neo-innervation

Julie K. Allen

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**Chronic stress promotes tumor growth through increased BDNF
production and neo-innervation**

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**Chronic stress promotes tumor growth through increased BDNF
production and neo-innervation**

A

DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M. D. Anderson Cancer Center
Graduate School of Biomedical Sciences

in Partial Fulfillment
of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

By

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Houston, Texas
May, 2012

Dedication

I dedicate this thesis first and foremost to my Aunt Sue, who is the reason I am where I am today. It was her battle with ovarian cancer that turned me from my lifelong ambition to become a veterinarian towards cancer research. In March 2005 she lost her battle with cancer, but hopefully someday someone will win their battle because of the research I have done.

Secondly, to my parents, without whom I wouldn't have made it half this far. To my mother, who has always been there for me and loved me despite the myriad of reasons I gave her not to, and to my father, who never let me settle with "good enough" but always encouraged me to go further.

I also dedicate this to my wonderful boyfriend, Achutha Bava, who put up with all the ranting and raving about graduate school for the last few years. You are the best thing in my life and I love you more than anything.

To my best friends who have been there along the way: Sarah May for putting up with my animals and loving them in my place when I wasn't around to do it. Maggie Atkins, for her brains and friendship for the last 11 years. Sarah Strunk, for her lifelong friendship, support, and fellow Nintendo fanaticism.

To the Texas Medical Center Orchestra for allowing me the privilege of playing my French horn with them for the last 4 years. Frank, Danielle, Pat...I will miss you guys more than you will ever know.

To my wonderful pets/kids, Lance, Violet, and Dexter: Your cold noses and warm hearts fill my life with unspeakable joy. Not a day goes by that you don't make me laugh and smile. Nothing cures a bad day like coming home to wagging tails, face licks, and purring. You're the best furry family I could ask for.

Finally to all the rest of my friends and family, without whose love, friendship, and support I never would have made it to where I am today.

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I'd like to thank all the members of the Sood lab from the last 5 years. Their friendship and support has made all the difference in my graduate school experience. We've had lots of good times and too many laughs to count. I'd like to particularly thank Dr. Guillermo Armaiz-Peña, who has been an invaluable source and mentor to me.

I'd also like to acknowledge the past and present member of my advisory and supervisory committees for their guidance and assistance in helping guide this project to what it has become. These members include Dr. Rosemarie Schmandt, Dr. Joseph McCarty, Dr. Lopez Berestein, Dr. Gary Gallick, Dr. Janet Price, Dr. Andrew Bean, Dr. Eric Wagner, and Dr. Menashe Bar-Eli. My particular thanks go to Dr. Gary Gallick whose guidance, support, and words of wisdom have been so important to me during my graduate training.

I'd also like to thank several other collaborators who lent me their knowledge. Donna Reynolds, for her abundant help with IHC troubleshooting. Dr. Michael Deavers, for his help interpreting IHC. Archana Nagaraja, for helping whenever I needed her. Also to all the other employees of the Smith Research Building who have shared their expertise along the way.

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Abstract

Background: Activation of the sympathetic nervous system (SNS) in response to chronic biobehavioral stress results in high levels of catecholamines and persistent activation of adrenergic signaling, which promotes tumor growth and progression. However it is unknown how catecholamine levels within the tumor exceed systemic levels in circulation. I hypothesized that neo-innervation of tumors is required for stress-mediated effects on tumor growth.

Results: First, I examined whether sympathetic nerves are present in human ovarian cancer samples as well as orthotopic ovarian cancer models. Immunohistochemical (IHC) staining for neurofilament revealed that catecholaminergic neurons are present within tumor tissue. In order to determine whether chronic stress affects the density of nerves in the tumor, I utilized an orthotopic mouse model of ovarian cancer that was exposed to daily restraint stress. IHC analysis revealed that nerve density in tumors increased by more than three-fold in stressed animals *versus* non-stressed controls. IHC analysis suggested that this results from both recruitment of existing neurons (axonogenesis) as well as new neuron formation (neurogenesis) within the tumor. To determine how tumors are recruiting nerve growth, I utilized a PCR array analysis of 84 nerve growth related genes and their receptors, which showed that stimulation of the SKOV3 ovarian cancer cell line with norepinephrine (NE) leads to increased expression of several neurotrophins, including brain-derived neurotrophic factor (BDNF). Neurite extension assays showed that media conditioned by ovarian cancer cell lines is capable of inducing neurite outgrowth in differentiated neuron-like PC12 cells, and NE treatment of cancer cells potentiates this effect. Norepinephrine-induced neurite extension was abolished after BDNF silencing by siRNA, suggesting that BDNF is critical to tumor cell-induced nerve growth. *in vivo* BDNF

inhibition resulted in complete abrogation of stress-induced increases in tumor weight and nerve density, as well as downstream markers of stress.

Discussion: These studies indicate that adrenergic signalling induced by chronic stress promotes neo-innervation in the tumor microenvironment. This results in a mutually beneficial relationship between the tumor cells and neurons. This work is crucial for providing a link between chronic stress and its effects on the tumor and its microenvironment. The data shown here aims to open new venues that can be used in development of therapies designed to block the stress effects on tumor growth.

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List of abbreviations

ADRB – β Adrenergic Receptor

ANS – Autonomic nervous system

BDNF – Brain Derived Neurotrophic Factor

CNS – Central nervous system

CRH – Corticotropin-releasing hormone

DCX – Doublecortin

DOPC - 1,2-dioleoyl-sn-glycero-3 phosphatidylcholine

ECM – extracellular matrix

FAK – Focal Adhesion Kinase

GDNF – Glial cell-derived neurotrophic factor

hBDNF – human BDNF

HPA – Hypothalamic Pituitary Adrenal Axis

HpF – High Powered field

IHC – Immunohistochemistry

IL-6 – Interleukin 6

mBDNF – murine BDNF

MMP – Matrix Metalloproteinase

NE – norepinephrine

NGF – Nerve Growth Factor

PBS – phosphate buffered saline

PCR – Polymerase Chain Reaction

PNS – peripheral nervous system

PSNS – parasympathetic nervous system

siRNA – Small interfering RNA

TrkB – Tropomyosin-related Kinase B

Introduction

1. **Rationale and Significance**

Modern science has demonstrated that biobehavioral factors can affect disease states. This interaction was coined the “mind-body” model of illness by psychiatrist George Engel (1). Since then, experimental and clinical evidence has shown that psychological factors can affect several types of diseases, including cardiovascular disease and cancer. Altered mental and physical states, such as depression or stress, activate the sympathetic nervous system and hypothalamic-pituitary-adrenal axis to cause release of hormones, including catecholamines. Recent evidence has shown that catecholamines are capable of promoting tumor growth and progression (2) through increases in tumoral catecholamine levels (3). We sought to find a link between chronic stress and the high levels of catecholamines seen within tumor tissue. Catecholamines may be released into circulation by the adrenal gland or locally by sympathetic nerve endings. I hypothesized that sympathetic neurons within the tumor would be responsible and that chronic stress would be capable of inducing growth of these neurons into the tumor. My work demonstrates 1) nerve density in tumors from chronically stressed animals is increased compared to non-stressed controls; 2) Norepinephrine signals through the β_3 -adrenergic receptor to stimulate BDNF production in tumor cells which is then responsible for promoting nerve growth into the tumor; 3) high nerve density is associated with high BDNF expression and poor outcome in ovarian cancer patients. Together these data represent the first demonstration that tumors are capable of promoting neo-innervation. It also demonstrates that neo-innervation of tumors is required for chronic stress-

mediated tumor progression. This work provides evidence that BDNF may be a viable therapeutic target in chronically stressed patients in order to block the deleterious effects of chronic stress on patient outcome.

2. Historical views of biobehavioral impact on disease

In 1977 Engel published a seminal article in *Science* proposing what he called the biopsychosocial model of human disease. In this model, he theorized that human disease is affected not only by biological factors, but also by psychological and social factors. Though his study is seen as a turning point in the field, the idea that psychosocial factors might influence disease states had been around for centuries. In Roman times the philosopher Galen noted that “melancholy” women were more likely to develop cancer (4). Alexandre Dumas’ physician wrote that he thought the author’s stomach cancer was due to the agitations of a public lifestyle (5). Dogen, a Buddhist monk, discussed in his *Shobogenzo* that people who tend towards a less complicated lifestyle are less likely to be sick.

In more modern research, scientists have begun to elucidate the mechanisms behind these observations. These studies have given us a thorough understanding of the systems our bodies use to cope with stress, yet have only begun to elucidate how and why these mechanisms can benefit, or sometimes be detrimental to, our health. More studies are necessary to further illuminate the mechanism of how stress affects disease.

3. The Stress Response

In a healthy, non-stressed individual, the body's homeostasis is maintained through a balance between virtually every system in the body. Each system runs at counterbalance to the others at normal basal levels to keep the body healthy and functional. As shown in Figure 1A, the sympathetic and parasympathetic nervous systems (SNS and PSNS, respectively) act as opposites to regulate many body functions. Stress, however, causes a disruption in this balance. The catecholamines of the sympathetic nervous system overbalance and cause hyperactivation of critical systems, vasoconstriction, and overall heighten the body's capability of dealing with a perceived stressor. In the case of chronic stress, the body remains in this constant state of hyper-activation and awareness, and eventually results in a multitude of health issues

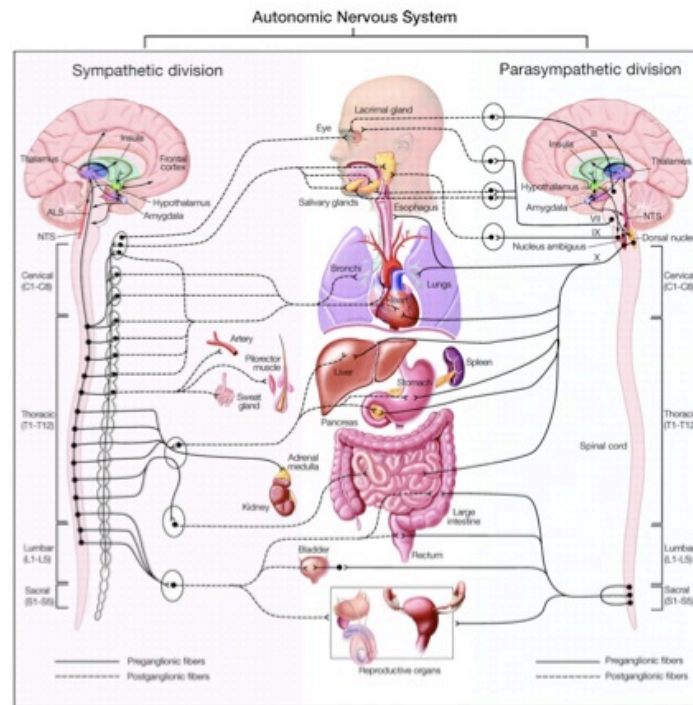
Stress is an extremely complex process that has widespread and diverse effects throughout the body, ranging from cardiovascular effects to changes in gastrointestinal function and immunity. Once the body detects a stressor, the central nervous system (CNS) activates both the autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenal axis (HPA). Stimulation of the ANS results in activation of the sympathetic nervous system (SNS) and subsequent release of catecholamines. Among other functions, Norepinephrine (NE) and epinephrine control blood pressure and heart rate. They act as vaso-constrictors, increasing blood pressure, and increasing blood flow to organs critical to the fight or flight

stress response. The third catecholamine, dopamine, is involved in learning, sociability, and promoting reward-seeking behavior (6). Its release is used to control levels of prolactin, which has numerous functions in the body, from reproduction to neurological function and immunity (7).

Activation of the HPA axis causes release of corticotrophin releasing hormone (CRH) from the hypothalamus, setting off a cascade that results in release of glucocorticoids from the adrenal gland (Figure 1B). Receptors for these hormones are found on virtually every cell type. In normal physiology, glucocorticoids are responsible for regulating immunity, cardiovascular function, and metabolism, among others (8, 9). Under stress conditions these hormones are responsible for the defeat/withdrawal response. Under chronic stress conditions they cause prolonged inhibition of CRH and subsequent inhibition of immunity.

Stress responses are highly adaptive systems that affect not only physiological changes, but also behavioral and neurological systems. Combined, these changes make up the collective “stress syndrome.” First, the body prepares for its best chance of survival by shutting down physiological functions that are not immediately deemed essential, and reroutes those resources to more critical functions. For example, following detection of a stressor the body slows digestion and growth as well as inhibits immune function and stalls reproductive urges. Conversely, it increases cardiac output, respiratory rate, and energy production.

A.



Lane R D et al. Psychosom Med 2009;71:117-134

B.

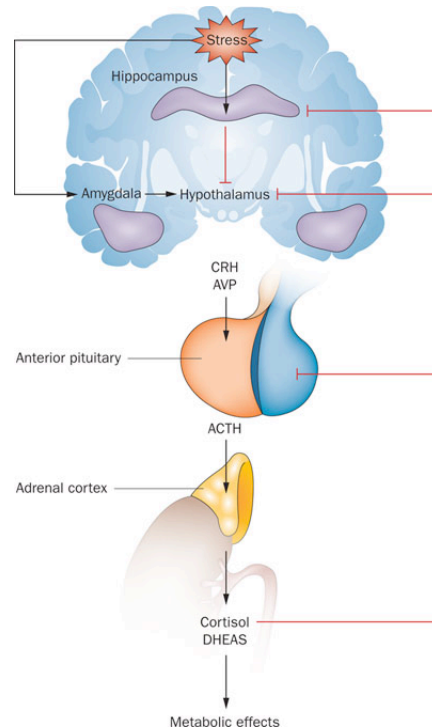


Figure 1. **Systems involved in the stress response.** A. Counterbalancing effects of the Parasympathetic and Sympathetic Nervous Systems. B. Activation of the HPA axis leading to cortisol secretion.

(Figures used with permission from 158-159)

Behaviorally, the individual becomes more alert, focused, and has increased mental faculties for processing his/her surroundings and circumstances (10).

4. Acute versus chronic stress

It is important to distinguish between the types of stress the body can experience. Although stressors can be physical, mental, emotional, social, or biological, a critical distinction is whether the stress is acute or chronic. Acute stressors are short-lived, infrequent, and beneficial to the body. A good example would be an individual giving a public speech or taking a test. Following an acute stressor the body prepares by releasing catecholamines and glucocorticoids to increase blood flow and prepare the body to cope. Once the stressor is removed, the stress response systems return to baseline and normal function resumes.

On the other hand, chronic stressors recur frequently or are sustained over long periods of time, typically from months to years. Good examples of chronic stressors are depression, chronic fatigue, a feeling of isolation or poor social support, or even the daily strain of a stressful job. While chronic stressors activate the same pathways as acute stressors, their continual or persistent nature does not allow these systems time to shut off, leaving the body in a constant state of overdrive. This continual response causes a prolonged disruption of the body's homeostasis as it attempts to cope. Over time this increases risk of several diseases, most particularly cardiovascular disease, and has been associated with

disruptions in metabolism, reproduction, immune activity, and even cancer initiation (11-13).

5. Chronic stress impacts on disease

A. Chronic stress and Cardiovascular disease

Effects of chronic stress on the cardiovascular system are perhaps the most well-studied and characterized. This link has been investigated for more than six decades, and has shown from virtually every angle that psychological and biobehavioral factors play a major role in promoting cardiovascular disease. These studies show that psychological factors can cause myocardial infarctions, coronary heart disease, hypertension, atherosclerosis, and other cardiovascular health issues (14). For example, studies have demonstrated that even short periods of chronic stress can increase risk of cardiovascular problems decades later (15). Large epidemiological studies have correlated feelings of irritability or anxiety with increased incidence of myocardial infarctions (16).

Effects of chronic stress on risk of cardiovascular disease are primarily a result of increased levels of cortisol and catecholamines. Catecholamines signal through adrenergic receptors (ARs), primarily the β -family of adrenergic receptors (ADRB) (see section 7B below). As a result, many patients with cardiovascular disease are treated with β -blockers, a class of drugs that inhibits catecholaminergic activation of β -ARs, resulting in lower blood pressure and heart rate.

B. Chronic stress and immunity

Chronic stress effects on the immune system are mediated primarily through activation of the HPA axis and release of glucocorticoids, as well as some control through the ANS and catecholamines. Several studies have demonstrated interactions between the CNS, endocrine, and immune systems, and suggest that disruption to one system will have subsequent effects on all.

The dual nature of glucocorticoids in acute versus chronic stress is highly pertinent to immune function. Normal physiologic levels of glucocorticoids are immunoregulatory, maintaining normal immune surveillance. Chronic stress significantly increases levels of glucocorticoids, at which point they become immunosuppressive. Within the immune system T and B cell, neutrophils, monocytes, and macrophages all carry glucocorticoid receptors. This allows for chronic stress to disrupt both cellular and humoral immune responses, such as inhibiting inflammation, causing a shift in the balance between Th1 and Th2 cytokines, and causing antigen-presenting cells to cease production of IL-12, all of which are important for immune adaptation (17,18). High levels of glucocorticoids can also cause apoptosis in monocytes, macrophages, and T lymphocytes (19).

C. Chronic stress and the central nervous system

Chronic stress can also have significant effects on neurological function. Specific brain regions (notably the hippocampus) express high densities of glucocorticoid receptors. Chronic stress levels of glucocorticoids can therefore

have very prominent effects on memory, both through interfering with new memory formation (20,21), as well as in preventing access to existing memories. For example, hippocampal function is significantly reduced following chronic stress, and several studies support that this is due to dendritic retraction of CA3 neurons, ultimately resulting in impaired spatial memory (22). Additionally, chronic stress is known to impair neurogenesis in the dentate gyrus region of the hippocampus, thereby impairing new memory formation and increasing anxiety-like responses (23).

6. Chronic stress and cancer (Figure 2)

More recent studies have demonstrated that chronic stress has a pronounced impact on virtually every facet of cancer, from playing a role in cancer initiation to promoting tumor growth and metastasis through several mechanisms. These studies have looked at both animal models of chronic stress as well as human clinical data.

A. Animal models of chronic stress

Several different methods have been used to mimic the effects of psychosocial stress in laboratory animals. Methods have included swim stress, hypothermia, rotation, restraint, social isolation, and social domination. Our group routinely uses the restraint stress model. Studies have demonstrated that physical restraint stress causes modulations in levels of both catecholamines and

glucocorticoids (2, 24). We have found that chronic restraint stress increased tumor growth, and exhibited higher tumor levels of catecholamines and glucocorticoids, suggesting that this model activates both the HPA axis and sympathetic nervous system, and therefore functions as a good model for mimicking chronic psychological stress in humans (2, 24).

B. Chronic stress and cancer initiation

Human tumorigenesis is a complex, multistep process. Hanahan and Weinberg postulated that six steps are required for transformation of cells: self-sufficiency in growth signals, insensitivity to antigrowth (growth inhibitory) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (25). Evidence that chronic stress may play a role in promoting this transformation is, however, difficult to reconcile due to often contradictory results. Two separate clinical studies have been performed on women following identification of a suspicious lesion, and prior to diagnosis by biopsy. The first of these studies found a significant correlation between malignant lesions and the occurrence of at least one major stressful life event in the previous 5 years (26). Conversely the second study concluded that day-to-day stress actually decreased risk of breast cancer by as much as 40% (27). The confounding results demonstrated by these studies as well as others may be the result of variations in statistical analysis, differences in methods of determining stress, and the inability to differentiate whether stress is actually causing cancer or whether its role is more important for progression of a

tumor to a malignant lesion. Additional difficulties arise in that these studies are all epidemiological, retrospective studies. A long-term longitudinal epidemiological study may be needed to more effectively elucidate the involvement that chronic stress has on tumor initiation; however no such study has been done, most likely due to restraints such as feasibility and cost. It is also difficult due to the fact that there are very few ways of mimicking this process in pre-clinical models, and similar restraints exist to prevent the feasibility of any such model. To date, very few studies have demonstrated solid biological mechanisms that may suggest chronic stress can initiate tumor formation, though those that exist seem to indicate it is through enhancement of DNA damage. Glaser et al demonstrated that stress can impair DNA repair through downregulation of the critical DNA repair enzyme Methyltransferase (28). Hara et al also demonstrated that stress enhances DNA damage through activation of β -arrestin-1 (29).

7. Stress and cancer progression

In the last few decades, there have been significant data supporting chronic stress effects on cancer progression from both preclinical and clinical settings. Clinically, chronic stress has been associated with poorer outcome in cancer patients (30-34). Preclinical evidence has demonstrated that chronic stress can promote cancer progression through increased angiogenesis, invasion, and migration leading to metastasis, cell survival, and immune escape.

A. Stress, Immunity, and cancer progression

There are a significant amount of data supporting the role of chronic stress in suppressing the immune system to allow tumors to progress. As previously mentioned, chronic stress causes a shift in immune function. Stress hormones inhibit cellular, or Th1, immunity, allowing tumor cells to evade surveillance. Stress inhibits release of pro-inflammatory cytokines, impairing maturation and tumor eliminating capacity of NK cells, T cells, and macrophages (35). Epinephrine and prostaglandin, two hormones that increase following chronic stress, were found to promote progression of leukemia by suppressing NK cell activity (36). Clinically, patients with poorer social support had lower levels of anti-tumorigenic cytokines and increased IL-4, which indicates poorer immune-mediated clearance of tumor cells (37). Additionally, tumor-infiltrating leukocytes (TILs) demonstrate increased type-2 immune response, which is generally considered pro-tumorigenic (38-41). Poor social support has also been seen to reduce NK cell activity (42-43). Catecholamines have been shown to have a significant effect directly on immune activity. Inhibition of SNS activity promotes adaptive immunity through increases in Th1 immunity (44). Cunnick et al demonstrated that adrenalectomy recovered T-cell responses previously inhibited by stress (45).

B. Stress and adrenergic signaling in cancer

Effects of catecholamines are mediated through adrenergic receptors (ARs). This family of 7-transmembrane G-coupled receptors consists of a total of 8

receptors divided into α and β sub-families (46). Several studies have found these receptors to be expressed on a variety of tumor types, with increased expression found in several cancers such as oral, liver, and colon (47-50). G-coupled receptors act as molecular switches to control a diverse array of downstream signaling pathways, and have been found to contribute to several pathways of tumor initiation and progression. These receptors have a dichotic function, acting something like on/off switches for their downstream signaling. Binding of the G_s subunit results in activation of downstream pathways, while binding of the G_i subunit inhibits signaling.

When members of the ADRB family are activated, they initiate downstream signaling through the cAMP/PKA and Epac signaling pathways. Downstream of PKA signaling can activate the cAMP Response Element Binding (CREB) protein which can activate a wide array of human genes, many of which are crucial to tumor growth and migration (51-52). Signaling through Epac (Exchange Protein activated by Adenylyl Cyclase) activates the MAP Kinase signaling pathway, which is well known to have potent effects promoting tumor proliferation.

C. Neuroendocrine effects on tumor cell proliferation

Cell growth, proliferation, and survival are controlled through a balance between positive and negative signals. Stress hormones can interfere with this balance (53). Studies have shown conflicting evidence for neuroendocrine

impact on tumor cell proliferation. For instance, several studies have found that adrenergic activation inhibits tumor cell proliferation, while others suggest it may enhance cell proliferation. One group found that ADRB2 signaling was required for pancreatic cell survival, and that blockade of ADRB2 induced cell apoptosis through the NFkB pathway (54). Conversely, another group found that NE treatment of breast cancer cells stimulated proliferation through an α_2 -AR specific pathway, and that use of an ADRB agonist countered this effect and decreased proliferation (55). Yet another group found that NE treatment inhibited proliferation of mouse chondrocytes through the ADRB2 receptor (56). Perhaps one could argue that response is simply cell-type specific, and depends largely on which adrenergic receptor family members are expressed on the cell.

Although the impact of adrenergic activation on proliferation is conflicting, the vast majority of studies all agree that chronic stress promotes tumor growth. This begs the question, if adrenergic activation is not promoting growth through cell proliferation, then which mechanisms are being modulated? Studies from our group and others show that it is in fact through promoting cell survival and inhibition of apoptosis.

D. Adrenergic influences on tumor cell survival

Tumor cells must adhere to extracellular matrix (ECM) compounds in order to metastasize and to avoid anoikis (Greek for “homelessness”). As previously

mentioned, activation of adrenergic receptors causes downstream activation of cAMP signaling, which controls cellular adhesion through PKA-mediated activation of RhoA and Rac (57). Additionally, activation of Epac downstream of β -ARs has been found to increase ovarian cancer cell adhesion (58). Focal Adhesion Kinase (FAK) aids cell adhesion to the ECM. Non-transformed cells will undergo apoptosis when their connection to the ECM is broken, due to a process called anoikis. Norepinephrine increases FAK activation to increase tumor cell adhesion and survival. Blocking FAK *in vivo* inhibits chronic stress effects on tumor growth by failing to protect cells from anoikis (59).

Sastry et al also demonstrated that NE acts through the β_2 -AR to protect cancer cells from apoptosis (60). Their studies demonstrated that activation of PKA resulted in downstream phosphorylation of BAD at Ser¹¹², the site thought to have primary control over antiapoptotic signaling downstream of BAD. Adrenergic activation is also thought to convey chemoresistance in some types of cancers (61-62). Studies in both breast and colon cancer have demonstrated that α -adrenergic receptor activation increased expression of MDR1, a gene long known to convey chemoresistance to tumor cells.

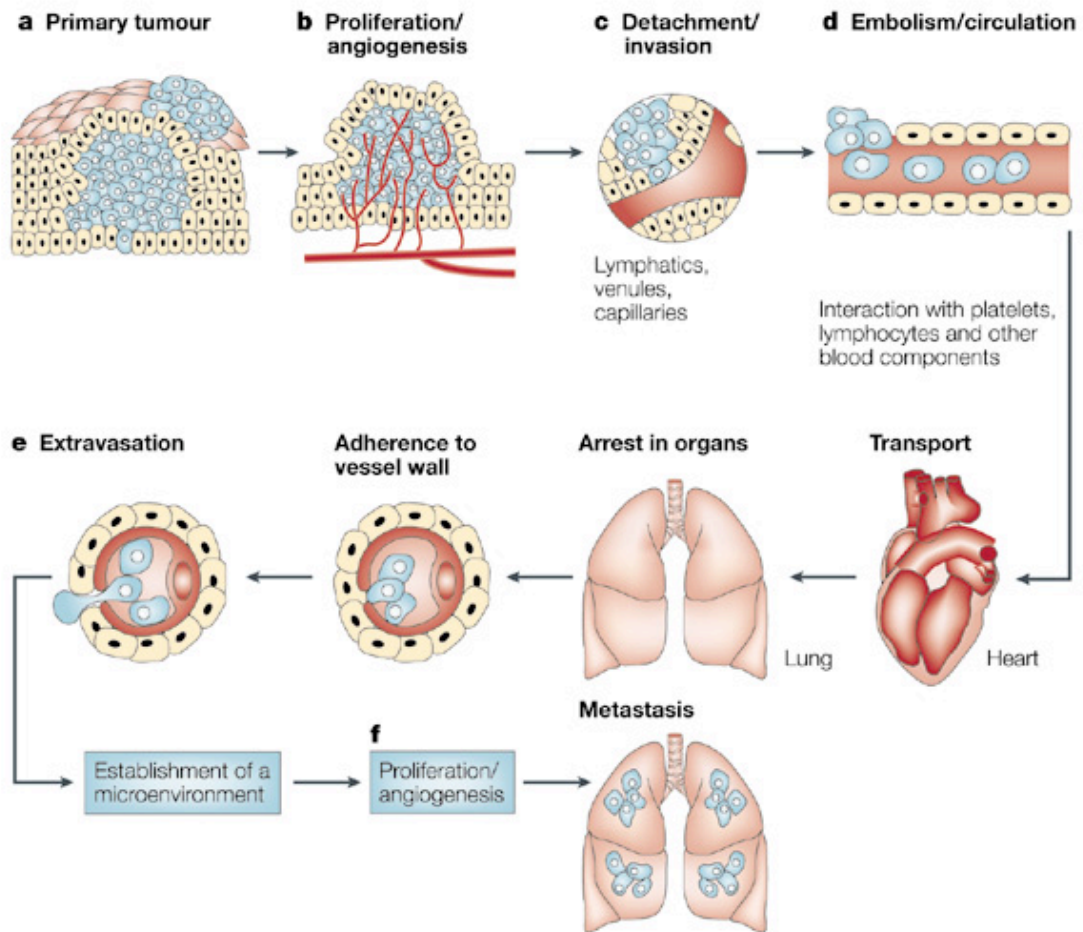
E. Stress and metastasis

In the vast majority of cases, patients suffering from cancer do not die as a result of their primary tumor, but much more commonly as a result of metastatic

lesions. It is therefore important to understand how a tumor metastasizes, and how chronic stress can promote this process. Metastasis is a complex process in which the cells must sequentially gain several characteristics or the cascade fails (63) (Figure 3). First, a developing tumor must develop a blood supply, as nutrients and oxygen from the bloodstream are only capable of diffusing <1 mm through tissues. This also provides a direct route for metastasis of cancer cells to other locations in the body. Secondly, tumor cells must be capable of detaching from the main tumor, secreting enzymes which break down the basement membrane and allow embolization into the blood stream. Once the cell becomes entrapped in a capillary bed, it must then be capable of extravasation from the blood stream and begin to grow in the new tissue bed, at which point the cycle begins again, and the new tumor must establish its own blood supply. During every step of this cycle the tumor cells must also be capable of avoiding surveillance of the immune system (64). Increasing evidence shows the stress response can affect many of the processes in this cascade.

F. Stress and angiogenesis

When a tumor begins to grow it must establish a vascular source to provide oxygen and nutrients to the cells. Nutrients and oxygen can only diffuse a matter of millimeters through tissue, and as such, formation of an adequate blood supply is a critical early step in tumor growth. This process has been termed angiogenesis and it has been extensively studied in human cancers.



Nature Reviews | Cancer

Figure 3. Steps of tumor metastasis

(Figure used with permission from (157))

Several factors may be responsible for promoting growth of blood vessels. There is a great deal of literature describing the effects of stress on two potent angiogenic factors, Vascular Endothelial Growth Factor (VEGF) and Interleukin-6 (IL-6).

Norepinephrine has been shown to increase production of VEGF through activation of β -AR/cAMP/PKA signaling (65), which can be mimicked by isoproterenol (a β -agonist) and blocked with β -blockers (66). In animal models norepinephrine increased CD31 positive staining, representing an increase in vessel density and therefore angiogenesis (66). In patients, high social support was associated with lower levels of VEGF (67).

IL-6 is a pro-inflammatory cytokine. Although its primary function is in immunity, IL-6 also plays a role in promoting angiogenesis. Under chronic stress conditions, IL-6 expression is increased in tumor cells through adrenergic activation of Src signaling (68). Clinical evidence has shown that patients with poor social support have higher levels of IL-6 when compared to patients with good social support (69) and these high levels are associated with poorer outcome in patients (70).

G. Adrenergic impact on migration and invasion of tumor cells

Tumor cells must gain the ability to migrate and invade to become metastatic. Chronic stress has been shown to promote tumor cell migration in breast and ovarian cancer cells through activation of β -adrenergic receptors

(66,71). Chronic stress also promotes tumor cell invasion through activation of several promalignant pathways. This invasion is primarily mediated through activation of STAT3 signaling, which promotes invasion through increasing expression of MMP-2 and -9, both of which play a critical role in degrading the basement membrane and allowing tumor cells to invade the vasculature and thereby travel to metastatic sites (66,72-73).

8. Clinical impact of chronic stress

Several studies have now been done to investigate the possible patient benefit of psychological interventions. However, there is a great deal of controversy over the studies that have been done with psychological/behavioral interventions. For instance, many studies have looked only at so-called “soft” factors, such as quality of life and feelings of well-being, as opposed to “hard” factors, such as survival and cortisol levels, which a concrete value can be placed upon. Perhaps the most convincing of these studies was done at Ohio State University. The group performing these studies found that placing patients in small support groups resulted in decreased anxiety, improved social support, improved immune response, and overall improved health. Follow-up of 11 years on these patients also revealed that patients who participated in the groups had reduced chance of both recurrence and death (74-76). Other studies have found that psychological interventions reduce cortisol levels, improve immune response (through measures of lymphocyte proliferation), and provide patients with an

overall improved feeling of well-being (77-80). These studies suggest that behavioral therapies may be a viable option for improving patient quality of life as well as outcome, and merit further investigation.

Most of the preclinical studies of chronic stress effects on cancer have shown these effects to be mediated primarily through the β -family of adrenergic receptors. As such, β -blockers have been of interest for clinical use. To this point, most of the studies have been either retrospective or have contained confounding factors. For example, a retrospective study looked at the risk of developing prostate cancer in patients taking β -blockers, and found that these patients had significantly lower risk (81). Another retrospective study looked at overall risk of cancer in patients taking β -blockers for cardiovascular disease, and found that these patients also had reduced overall risk of developing cancer (82). Further observational studies have demonstrated improved relapse-free survival, secondary tumor formation, and overall mortality in breast cancer (83-84) as well as improved outcome in melanoma (85-86). No clinical trials have yet been performed to determine the efficacy of these drugs in treating a patient's cancer, however.

9. Neurogenesis

A. Embryonic neurogenesis

Development of peripheral neurons during embryogenesis is a fairly well-understood process. Neurons originate in the neural crest from

sympathoadrenal progenitor cells (87). These cells quickly begin migrating ventrally and begin differentiating into catecholaminergic neurons. There is then a period of cell death that results, as neurons are initially produced in excessive numbers. This period is controlled by neurotrophic factors, primarily Nerve Growth Factor (NGF) and its family members, Neurotrophin-3 (NT-3) and Brain derived neurotrophic factor (BDNF) (88-89). NGF and NT3 signaling through Tyrosine receptor kinase A (TrkA) maintain survival and direction in developing neurons (90-91). Conversely, signaling through the p75^{NTR} receptor can lead to apoptosis of neurons (92) in the absence of Trk receptor activation (93) or when co-activation of Trk and p75 receptors shifts in favor of p75. Once activated by neurotrophins, the internal domain of p75 activates ceramide production leading to cell death (93-94). Most neuronal circuits track along blood vessels. Some studies suggest that this may be a result of the high amounts of NT-3 and moderate levels of NGF secreted by vessels (95-97), which may provide growth advantages and direction as these circuits are developing.

B. Adult Neurogenesis and nerve growth

In human adults, neurogenesis has previously been thought to occur in only two specific brain regions: the subventricular zone of the forebrain (98) and the dentate gyrus region of the hippocampus (99) (Figure 4). More recent studies are beginning to demonstrate that neurogenesis can also take place in other regions of the brain, suggesting that this process may be more widespread than previously thought (100-102). In adult CNS neurogenesis, maturing neurons are able to

integrate into existing neuronal circuits (99). These maturing neurons migrate along existing pathways (the rostral migratory stream or the mossy fiber pathway) as they begin to integrate into signaling circuitry by extension of dendritic extensions. This formation of new neurons within the CNS appears to play many major roles in memory formation, learning, and normal emotional function. To my knowledge, the only study previously describing adult neurogenesis outside of the CNS showed that the carotid body contains glia-like stem cells capable of forming new glomus cells following hypoxia (103).

Much of what is known about adult peripheral neural growth and repair has come from studies of wound healing. The first studies demonstrating that nerves are capable of regenerating following damage appeared in the mid-19th century. Since then, many of the mechanisms and factors involved in this process have been elucidated. Many of the mechanisms are similar to those involved in neural development during embryogenesis, with cells of the extracellular matrix producing growth factors and guidance factors that first break down damaged axons, and then induce axonogenesis for replacement (104). Most of these processes appear to be mediated primarily through release of NGF and GDNF family members as well as guidance provided by Schwann cells.

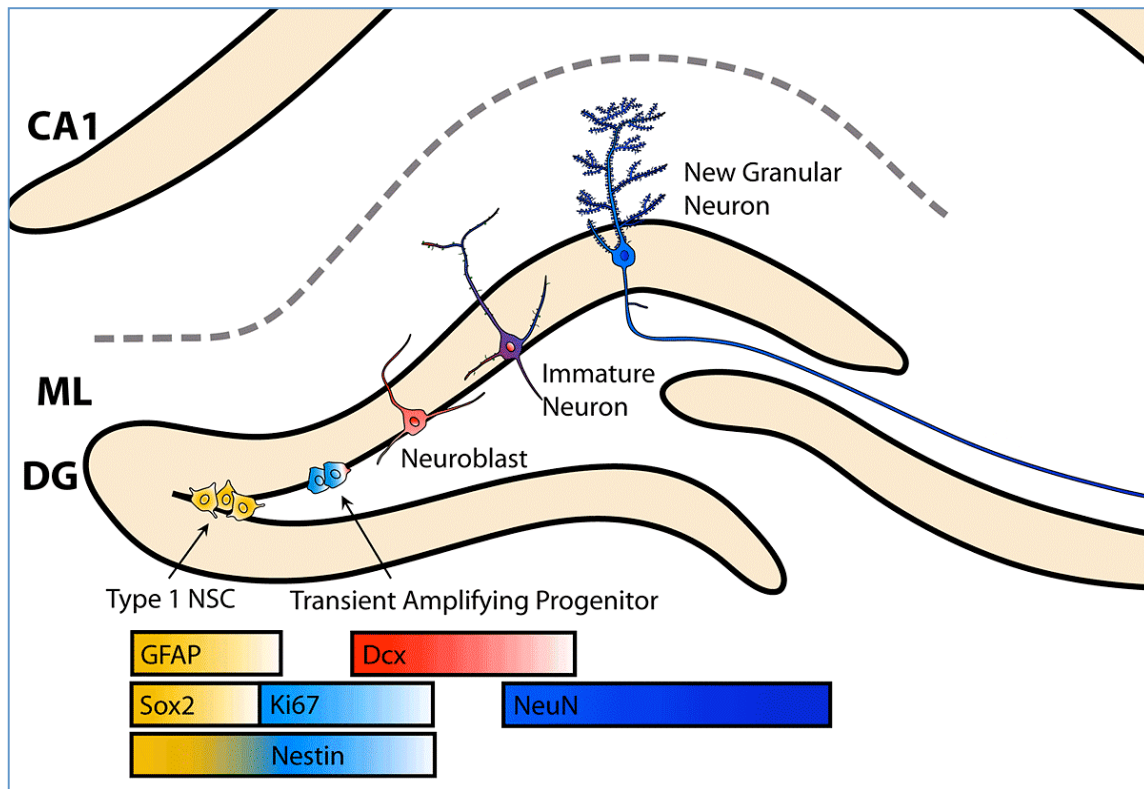


Figure 4. Adult neurogenesis in the hippocampus

(Figure courtesy of Richard Smart, University of Arizona)

C. Doublecortin

During neuronal maturation, precursors express several different markers, some of which fade as the neurons mature, others of which increase throughout maturation. One of these markers is doublecortin (DCX) protein. Found in all neural progenitor cells and neuroblasts, doublecortin is frequently used as a marker of adult neurogenesis. As neurons mature and differentiate, DCX expression decreases rapidly, as expression of mature neuronal markers increase. Expression is usually completely absent after about 14 days (Figure 4). Brown et al. first demonstrated use of DCX as a marker for adult neurogenesis due to its transient expression (105). The high expression levels in new neurons, and quick fading as more mature neuronal markers increase, makes this an ideal marker for newborn neurons (105)

D. Brain Derived Neurotrophic Factor

Brain Derived Neurotrophic Factor (BDNF) plays a significant role in adult neurogenesis as well as embryonic development of neural circuits. In the adult brain, high expression of BDNF supports neurogenesis and neuroplasticity. It plays critical roles in learning and memory, as BDNF deficient animals demonstrate severe impairment of these functions (106). Overexpression of BDNF causes both anxiogenic and anti-depressant behavior (107). Interestingly, BDNF expression is significantly downregulated under chronic stress conditions in neurogenic brain regions (108-109).

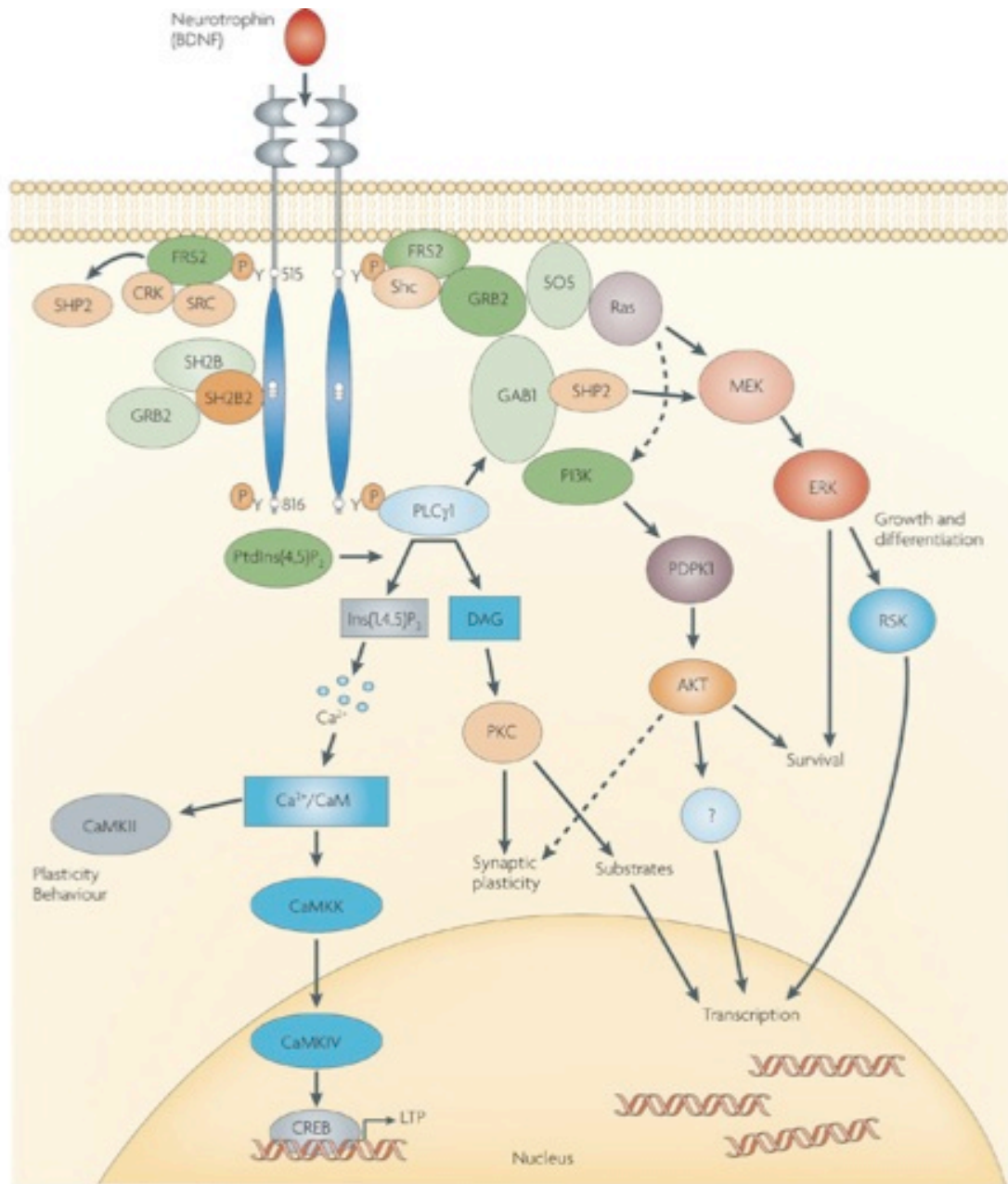
E. Tropomyosin-related Kinase B (Figure 5)

TrkB (Tropomyosin-related kinase B) is the membrane-bound receptor for BDNF. This member of the Trk family of receptors is critical for BDNF effects on neuronal growth and survival. During development, BDNF signaling through TrkB activates MAPK/ERK, PLC γ , and PI3K pathways (100). Activation of PLC γ promotes neurite outgrowth and both initiation and maintenance of long-term potentiation in target cells (110). Trk signaling through MAPK is essential for differentiation of neuronal precursors (111). Neuronal survival is mediated through activated PI3K signaling (112). Activation of TrkB signaling in neurons is critical not only for development and survival at the neuron/cellular level, but also at the organismal level, as TrkB(-/-) knockout is neonatally lethal, with animals dying by 3 weeks of age due to neuronal death (113). Interestingly, TrkB expression is limited almost exclusively to nervous tissue, but its expression has been found in ovarian cancer (114).

10. Neurogenesis and chronic stress

A. Association of neurogenesis with disease

Several diseases are thought to have roots in neurogenesis. Major depression, for instance, is thought to arise from defects in neurogenesis (115-117). Many studies demonstrate that neurogenesis is inhibited during periods of chronic stress, and many antidepressant drugs work by promoting neurogenesis (118-120).



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Figure 5. BDNF/TrkB signaling in neurons

(Figure used with permission from 160)

Interestingly, several studies have implicated BDNF expression as an important factor in stress-induced impairment of neurogenesis in the CNS. Under conditions of chronic stress, BDNF expression is downregulated in neurogenic brain regions (108-109) by histone methylation (121) and corticosterones (122). In contrast, very recent studies have demonstrated that chronic immobilization (restraint) stress induces dendritic growth in the amygdala (123). Perhaps most interesting was a study by Lakshminarasimhan and Chattarji that demonstrated that chronic restraint stress not only caused dendritic atrophy in the hippocampus and dendritic growth in the amygdala, but that this also correlated with significant decreases in BDNF in the hippocampus and increases in the amygdala (124). These exciting data demonstrates that stress may play contrasting roles on neuron growth through modulations in BDNF.

B. Nervous system impact on tumor progression

The connection linking the nervous system and tumor growth has been relatively little studied. While many groups have demonstrated the presence of nerves in tumors, hypotheses on their function and origin have, to this point, been weak and highly correlative. Work done by several different groups has demonstrated that various tumor types secrete neurotrophic factors, and that media conditioned by these cells is capable of inducing neurite outgrowth (125). However, no direct mechanism has been demonstrated. Additionally, many studies have demonstrated that neurotransmitters are capable of influencing tumor

progression in both stress and non-stress conditions. A limited number of studies exist showing innervation of tumors (126-128). Some recent data have suggested that tumors are capable of recruiting nerves into the tumor (129). These studies also demonstrate that there is a correlation between positive staining for nerve markers and poorer outcome in cancer patients (129-130). However, these studies are very limited in scope and inconclusive about the role the nerves are playing and how the tumors are recruiting them. Also, to date, no study has revealed the mechanism by which nerves are recruited into tumor tissue.

11. Study Impact

To date, no study has yet provided a mechanism for why tumoral catecholamine levels are higher than systemic levels following chronic stress. Nor has any study yet provided a mechanistic understanding of how nerves are recruited into tumors, and what role they play there. The work presented in this thesis seeks to fill these gaps by demonstrating that catecholamines are delivered by neurons directly into tumor tissue under chronic stress conditions, and that chronic stress contributes to neuronal recruitment through increasing expression of brain derived neurotrophic factor in ovarian tumor cells.

Methods

1. Reagents

Table 1.

Reagent	Provider	Working Concentration
norepinephrine	Sigma	10 μ M
epinephrine	Sigma	10 μ M
isoproterenol	Sigma	10 μ M
dibutyryl cAMP	Sigma	100 μ M
8cpt-2Me-cAMP	Tocris	100 μ M
cortisol	Sigma	10 μ M
dexamethasone	Sigma	10 μ M
forskolin	Sigma	10 μ M
tanshinone IIA	Sigma	100 μ M
KT5720	Calbiochem	1 μ M
BRL37344	Sigma	10 μ M
SP600125	Tocris	25 μ M
prazosine	Sigma	10 μ M
yohimbine	Sigma	10 μ M
atenolol	Tocris	10 μ M
butoxamine	Sigma	10 μ M
propranolol	Tocris	10 μ M
U0126	Sigma	10 μ M
U73122	Sigma	10 μ M
LY294002	Sigma	1 μ M
SB203580	Tocris	5 μ M
Brefeldin A	Tocris	100 μ M
GGTI-298	Sigma	1 μ M
API-2	Tocris	10 μ M
hexamethonium Bromide	Sigma	1 mg/kg/day
Recombinant NGF- β	Sigma	100 ng/mL

Methods Table 1. Reagents Used, provider, and working concentration.

Table 2.

Antibody	Provider	Catalog Number
Neurofilament (anti mouse)	millipore	MAB1615
Neurofilament (anti human)	abcan	ab9035
BDNF	Abcam	ab72438
doublecortin	abcam	ab18723
tyrosine hydroxylase	millipore	mab318
CD31	PharMingen	
GFP	abcam	ab13970
RFP	abcam	ab62341
anti mouse HRP IgG1	Jackson ImmunoResearch	
anti mouse HRP IgG	Jackson ImmunoResearch	
anti rabbit secondary HRP	Jackson ImmunoResearch	
anti-Rabbit Alexa 488	jackson ImmunoResearch	
anti-Rabbit Alexa 594	Invitrogen	
anti-mouse Alexa 488	Invitrogen	
anti-mouse Alexa 594	Invitrogen	
AMCA-conjugated anti-Rabbit	Jackson ImmunoResearch	code 111-156-047
Hoechst	invitrogen	

Methods Table 2. Antibodies used, provider, and catalog number (where available).

Lipfectamine 2000 transfection reagent was purchased from Invitrogen (Carlsbad, CA). Unless otherwise noted, all media for tissue culture were obtained from Invitrogen.

2. Cell lines

The source and derivation of HeyA8, SKOV3, and A2780 ovarian cancer cells has previously been reported (2). Cells were grown in RPMI medium supplemented with 15% fetal bovine serum and 0.1% gentamicin sulfate. PC12 cells were a kind gift from Dr. Yixin Yao and its derivation and source has been

described previously (131). These cells were maintained in RPMI-1640 supplemented with 10% horse serum and 5% fetal bovine serum. Colon cancer cell lines were a kind gift from Dr. Lee Ellis and were maintained in MEM supplemented with NAA (Mediatech), sodium pyruvate (Gibco), Glutamine (Gibco), and 10% FBS. Their source and derivation have previously been described (132). Cells were screened for mycoplasma, and all experiments were carried out while cells were ~70% confluent unless otherwise noted.

3. siRNA

SiRNA was used for gene expression knockdown both *in vitro* and *in vivo*. All siRNAs were obtained from Sigma-Aldrich (St. Louis, MO). Cells were transfected according to manufacturer's protocol. Briefly, for *in vitro* transfection cells were plated at 15-20% confluency. 8 µg (10 cm) or 2 µg (6-well) of siRNA was diluted in 100 µL serum-free media and allowed to incubate for 5 minutes. 30 µL (10 cm) or 5 µL (6-well) of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was also diluted in 100 µL serum-free media and allowed to incubate 5 minutes. Following incubation the diluted siRNA and transfection reagent were combined and allowed to incubate for 25 minutes before being added to cells. Total volume was then brought up to 15 mL (10 cm) or 2 mL (6-well) using serum-free media. The reactions were allowed to continue for 5 hours, at which point 15% fetal bovine serum was added to halt transfection. Knockdown efficiency was tested by qPCR at 24, 48, and 72 hours. For all siRNAs tested 48 hours was sufficient, therefore all experiments were carried out at 48 hours post-transfection.

For *in vivo* studies, siRNAs were incorporated into 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC), a neutral liposome delivery molecule extensively used by our lab and previously described by (133). Incorporations were done in the laboratory of Dr. Gabriel Lopez-Berestein as follows: DOPC and siRNA were mixed in the presence of excess tertiary butanol at a ratio of 1:10 siRNA:DOPC. Tween 20 was added to the mixture in a ratio of 1:19 Tween 20:siRNA/DOPC. The mixture was vortexed, frozen in an acetone/dry ice bath, and lyophilized. Prior to *in vivo* administration, the lyophilized mixture was hydrated with 0.9% saline to 20 µg/mL concentration. Animals were treated at a 5 µg/mouse/treatment dose, therefore 100 µL of the hydrated mixture was injected intra-peritoneally into each mouse.

4. Neurite extension assay (FIGURE 6)

Neurite extension assays were used to assess neurite outgrowth in neuron-like PC12 cells. 24-well inserts with 1 µM pore size were purchased from Millipore and uniformly coated with type 1 rat-tail collagen. PC12 cells were plated at 30% confluency and maintained for 72 hours prior to plating for the assay in DMEM containing 1% fetal bovine serum and 1 ng/mL recombinant NGF (nerve growth factor) to induce differentiation. After 72 hours of treatment the cells were lifted and plated in the coated inserts. Culture media containing rNGF was placed in the top of the inserts and in the bottom of control wells. Media conditioned by ovarian cancer cell lines was placed in the bottom of wells (see section 5 below). Plates were placed in the incubator for 48 hours to allow neurite extension. After 48

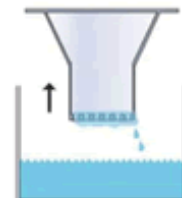
1. Type 1 rat tail collagen solution is placed in well



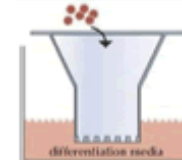
2. 1 μ M pore inserts are incubated in solution for 2 hours to allow coating of membrane



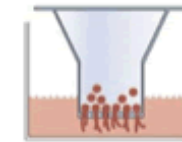
3. Insert is removed and the membrane allowed to dry



4. Insert is placed in conditioned media and PC12 cells plated on membrane



5. Inserts are incubated at 37° C for 48 hrs to allow neurite extension



6. Membranes are fixed and stained. Cell bodies are removed from the membrane and axonal extensions counted

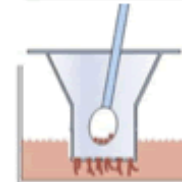


Figure 6. Neurite Assay Protocol

hours the wells were removed. Media was removed using a vacuum manifold. Cells were fixed in ice-cold methanol for 20 minutes then washed in PBS. They were then stained in neurite stain solution (Millipore) and again rinsed in PBS. Once thoroughly rinsed, cell bodies remaining on the upper surface of the membranes were removed using cotton swabs and PBS. The membranes were then allowed to dry overnight before being removed from inserts, placed on a microscope slide and coverslipped. Five 40x objective fields were counted per membrane, and all treatments were done in duplicate, for a total of 10 fields per treatment group. Each branch was counted as a separate neurite extension, with no bias given to length or size of the extension.

5. Conditioned media

For experiments requiring media conditioned by ovarian cancer cell lines, serum-free media was used and all media remained on cells for 24 hours prior to being collected. For cells that were treated with norepinephrine, the cells were serum starved overnight, and new media was added at the time of treatment. The conditioned media was then collected 24 hours post-treatment. For media conditioned by cells treated with siRNA, the cells were transfected at time 0, media replaced with serum-free media at 48 hours post-transfection, and collected at 72 hours post-transfection. For cells treated with both siRNA and norepinephrine (NE), cells were serum-starved from 36-48 hours post-transfection, new media containing NE given at 48 hours, and collected at 72 hours post-transfection.

Conditioned media from each treatment group was used for subsequent neurite extension assays.

6. Migration and Invasion assays

For all migration and invasion assays, 24-well inserts containing a polycarbonate membrane with 8 μ M pore size was used. For migration assays the inserts were uniformly coated with 0.1% gelatin at room temperature for 2 hours. After allowing the matrix to dry, a single cell suspension of 5×10^4 cells was plated per well with media containing 1% fetal bovine serum in the upper well and media containing 5% fetal bovine serum in the lower chamber to encourage migration. After an 8 hour incubation in a humidified incubator at 37C with 5% CO₂, the membranes were fixed and cells stained using a kit by Fisher Scientific. Cells remaining on the upper surface of the membrane were removed following fixation. Once dry the membranes were placed on microscope slides and coverslipped. Counting was performed using light microscopy on a total of 5 high power fields per insert. When required, cells were treated with siRNA 48 hours prior to migration to ensure complete gene knockdown.

For invasion assays, inserts were coated with a defined basement membrane matrix consisting of human laminin, type IV collagen, and gelatin. This coating is in place to mimic the basement membrane that tumor cells must degrade in order to invade into the surrounding tissues. Cells were plated as described for migration assays and incubated for 24 hours to allow time for matrix breakdown.

7. In vivo Models

All experiments using human cell lines were done in 8- to 12- week old female athymic nude mice received from Taconic Farms (Hudson, NY). Experiments using mouse cell lines were performed in C57/B6 lineage mice. All experiments were approved by the M. D. Anderson Institutional Animal Care and Use Committee (IACUC)

8. Chronic stress model

In order to mimic chronic adrenergic activation caused by chronic stress in humans we utilize a daily restraint stress system developed by our lab (2). In our system, boxes have been created that allow 10 mice to be placed in individual slots, each containing a moveable wall that allows us to restrain the animals in a movement-restricting space for the desired length of time. Our group has previously tested various lengths of time for daily restraint and found that 2 hours daily produces significant increases in tumor growth, while longer lengths of time did not seem to add any additional effect (2). I therefore utilized 2 hours of restraint stress daily for 1 week prior to tumor cell injection, and continuing for 2 weeks post injection. Unless otherwise noted, animals are necropsied at approximately 28 days following tumor cell inoculation.

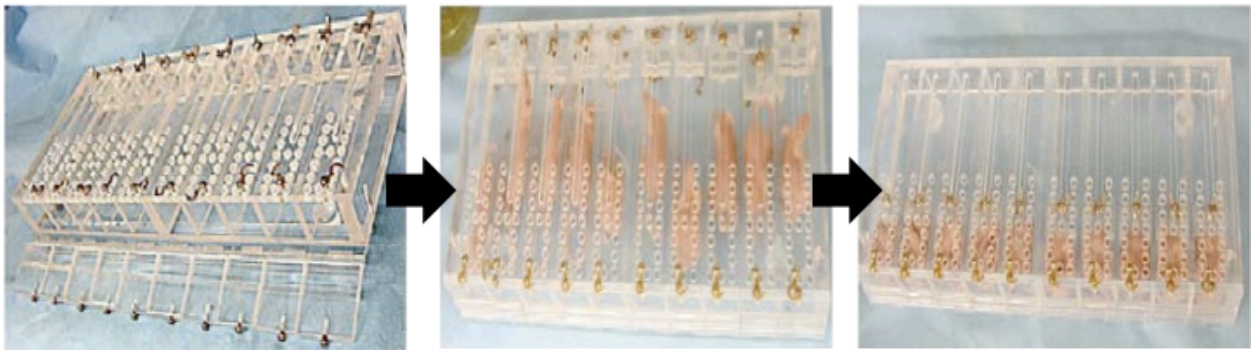


Figure 7. Restraint stress boxes. Animals are placed in individual compartments and moveable walls are put in place to confine the animals to a small, movement-restricting space.

9. Tumor cell inoculation

To prepare tumor cells for inoculation, cells were plated and allowed to grow until 60-70% confluent to ensure they were still in the exponential growth phase. Cells were then washed and lifted using 0.25% trypsin/EDTA. After lifting, trypsin was inactivated using media supplemented with FBS. Cells were spun down for 5 minutes at 1300 rpm, washed and resuspended in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hanks Buffered Saline Solution (HBSS). Cells viability was tested using trypan blue and then counted. The volume containing the desired number of cells was then removed and spun down again, and re-suspended in HBSS to the desired concentration. Injections were done in a volume of 200 μL per mouse intraperitoneally. Cell numbers used for injection were as follows:

Cell line	Cell number injected
SKOV3-ip1	1×10^6
HeyA8-ip1	2.5×10^5
RKO-ip1	3×10^6
ID8-VEGF	1×10^6

10. In Vivo siRNA treatments

SiRNA treatments began on day 5 following tumor cell inoculation. This time lapse was allowed to ensure that knockdown of the target gene would not affect tumor cell adhesion within the peritoneal cavity. Previous *in vitro* and *in vivo* studies in our laboratory have shown effective knockdown for up to 72 hours following a single siRNA treatment (134). We therefore treated our animals

biweekly with 5 µg siRNA per mouse. As described above, siRNA was incorporated into the DOPC liposome and delivered in a 0.9% saline solution intraperitoneally. Expression knockdown was confirmed using quantitative PCR.

Our laboratory has shown that Chitosan nanoparticles have more effective delivery to peripheral nerve endings. Therefore, in order to achieve optimal delivery to the neurons, the mTrkB siRNA was incorporated in Chitosan nanoparticles rather than DOPC. The particles were injected intravenously through the tail vein twice weekly.

11. Surgical models

Animals receiving hexamethonium bromide received surgical implantation of Alzet mini-osmotic pumps. These pumps ensure continual delivery of a drug and are approved for up to 42 days of continual delivery. Animals were anesthetized using Isoflurane inhalant anesthesia. A 1 cm incision was made dorsally between the shoulder blades through only the skin, not penetrating the muscle layers. Blunt ended scissors were used to separate connective tissue beneath the skin. Pumps were inserted into the gap made and the incisions were closed using surgical wound clips, which were removed 7 days post-surgery. Osmotic pumps were dry when loaded with drug. This allowed a delay of up to 72 hours from the initial time of implantation before drug was secreted. Tumor cells were injected at the time of surgery, and this time was to allow tumor cells time for implantation.

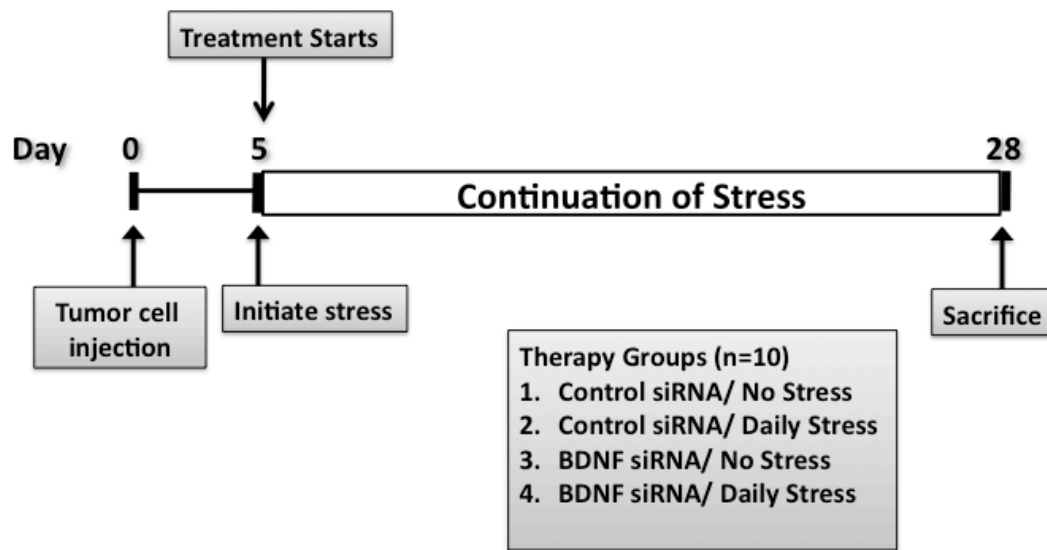


Figure 8. Schema for *in vivo* experiments utilizing BDNF siRNA.

Adrenalectomized animals were received from Taconic Farms. Animals were shipped 3 days following surgery and allowed 10 additional days for recovery prior to onset of stress. Sham surgeries were performed on age-matched C57/B6 mice provided by M.D. Anderson department of Experimental Radiation Oncology. Animals were anesthetized using a ketamine-based anesthesia (100 mg/kg Ketamine, 2.5 mg/kg Xylazine, 2.5 mg/kg Acepromazine), and a dorsal incision measuring approximately 1 cm was made on each mouse through the skin. A flank incision was then made through the muscle layer on the right side of each animal. Sterile cotton swabs moistened with sterile PBS were inserted into the incision and manipulated within the animal to mimic adrenalectomy surgery. Muscle incisions were sutured using Lycril. External skin incisions were closed using wound clips. Post-surgery all animals were maintained on 0.89% saline water.

For intraovarian injections animals were anesthetized with ketamine-based anesthesia. Flank incisions were made on the right side of the animal and the ovary located with the aid of PBS-moistened cotton swabs. 1×10^6 SKOV3-ip1 ovarian cancer cells were injected into the ovary as a single cell suspension using a 30-gauge hypodermic needle. Tumors were allowed 5 weeks to grow and metastasize before necropsy.

12. Patient Sample Immunohistochemistry

Human ovarian tumor clinical specimens were received from the M.D. Anderson gynecology oncology tumor bank following approval by the Institutional

Review Board. Formalin-fixed, paraffin-embedded tumors (both primary and metastatic sites) from 67 patients were stained for Neurofilament, Tyrosine hydroxylase, and BDNF. Slides were placed in an oven overnight at 65°C and passed through xylene to remove the paraffin and were rehydrated in PBS. Antigen retrieval was performed in a 10 mM citrate solution in the microwave at 98°C for 10 minutes and allowed to cool to room temperature over approximately 1.5-2 hours. Endogenous peroxidase activity was blocked by incubation in a 3% hydrogen peroxide solution in methanol for 12 minutes. Tissues were incubated at room temperature in a solution of 4% cold-water fish gelatin in PBS for protein blocking. Tissues were incubated with primary antibodies diluted in protein block temperature in a humidified chamber overnight at 4°C. Secondary antibodies were diluted in protein block and incubated on tissues for 1 hour at room temperature in a humidified chamber overnight at 4°C. DAB was applied and development was monitored under a light microscope to prevent over-exposure (approximately 4-6 minutes). Gill's hematoxylin was used for 10 seconds on samples for nuclear counterstain. Samples were dried and coverslipped. Light microscopy was used for visualization of nerves using the 40x objective. The 20x objective was used for BDNF visualization. Samples were scored according to both percentage and intensity of staining.

13. Statistical Analysis

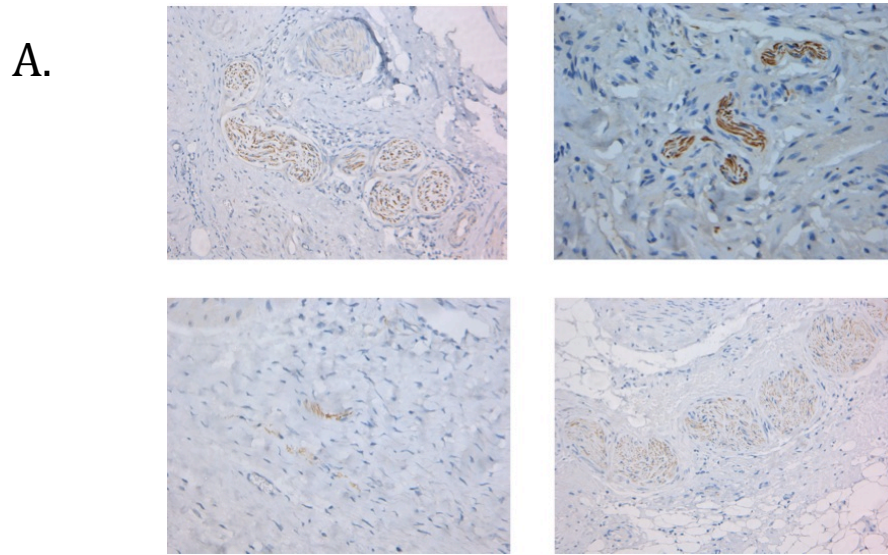
Continuous variables were compared using either a student's t-test or analysis of variance (ANOVA). We used the non-parametric Mann-Whitney test to compare differences. Two-way ANOVA was used to determine sample size. We determined a sample size of 10 animals per group would provide an effect size (ration of fixed effect and residual standard deviation) of 1.3 with 80% power at a significance level of $p=0.05$, which we considered to be significant.

Results

1. Nerve density in human ovarian cancer

My first goal was to determine whether nerves are present in human ovarian cancers. I therefore examined 160 high-grade epithelial ovarian cancers from both primary and metastatic sites from 67 patients. It is postulated that these tumors develop from the surface epithelium of the ovary, and, while the cortex of the ovary has significant nervous supply, this layer does not. Additionally, ovarian cancer tends to metastasize within the peritoneal cavity, frequently developing in the omentum and mesentery, neither of which is highly innervated. My analysis revealed that neurofilament, a marker for mature and developing neurons, was present in > 60 percent of samples analyzed, indicating that ovarian cancers are innervated (Figure 9A). Nerves were found in both primary tumors from the ovary and metastatic tumors found in the omentum, peritoneum, and a variety of other sites. There was no evidence that innervation of tumors was site specific.

Samples were divided into those with high versus low nerve density and survival analysis performed. This analysis showed that those patients whose tumors had high nerve density had an average survival time of 39 months, versus 65 months in patients with low nerve density (Figure 9B). This statistically different result suggests that tumor neo-innervation may be an important marker for patient outcome.



Representative photos of neurofilament in human ovarian cancer specimens

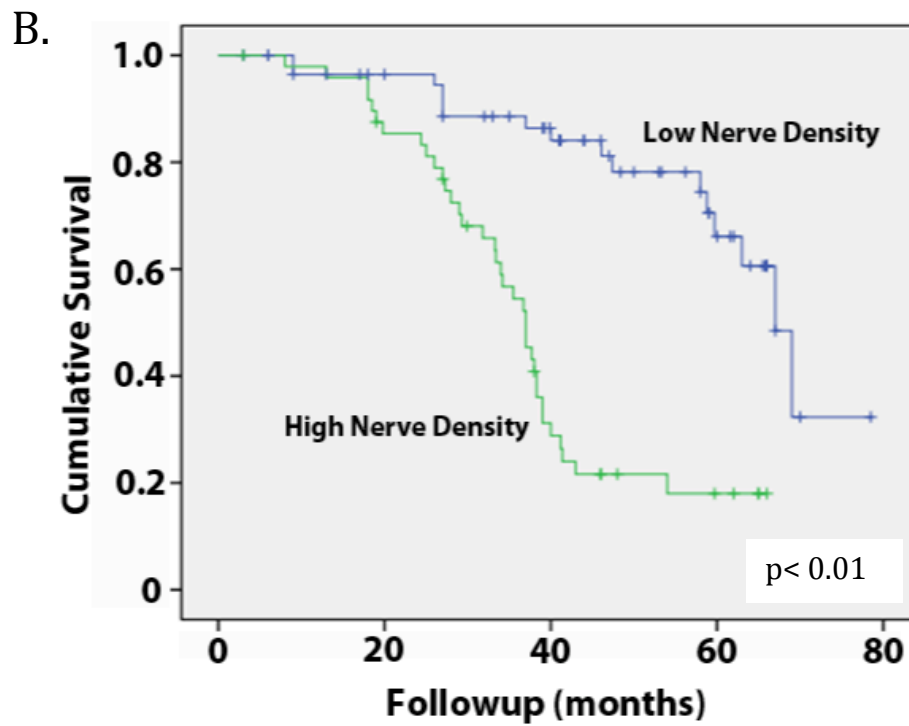


Figure 9. Nerve Density in human ovarian cancers. A. Representative sections of neurofilament staining in human ovarian tumor samples. B. Overall patient survival in patients with high versus low nerve density.

2. Norepinephrine stimulates neurotrophin expression in ovarian cancer cells

Recent work in our lab and others has demonstrated that chronic stress can significantly promote tumor progression through high levels of catecholamines present within the tumor. Catecholamines are primarily produced by sympathetic nerve endings. I therefore hypothesized that neurons present within the tumor may be responsible for the high levels of catecholamines found in tumor tissue. This led me to question what signals promoted nerve growth into the tumor.

Neurotrophic factors are responsible for promoting survival, growth, and proliferation of neurons, and are ubiquitously expressed throughout the body. I therefore chose to screen a panel of human ovarian cancer cell lines to determine whether tumor cells also express these factors. Using quantitative reverse transcription PCR I found that 7 different ovarian cancer cell lines expressed members of the nerve growth factor family of neurotrophins, and that their expression was increased over non-transformed ovarian surface epithelium cells (Figure 10A). Previous work from our lab has demonstrated that chronic restraint stress results in increased tumor growth and progression through activation of β adrenergic receptors (ADRB) and glucocorticoid receptors on tumor cells.

Adrenergic stimulation has been shown to activate signaling pathways known to regulate neurotrophic factors. I therefore hypothesized that stress

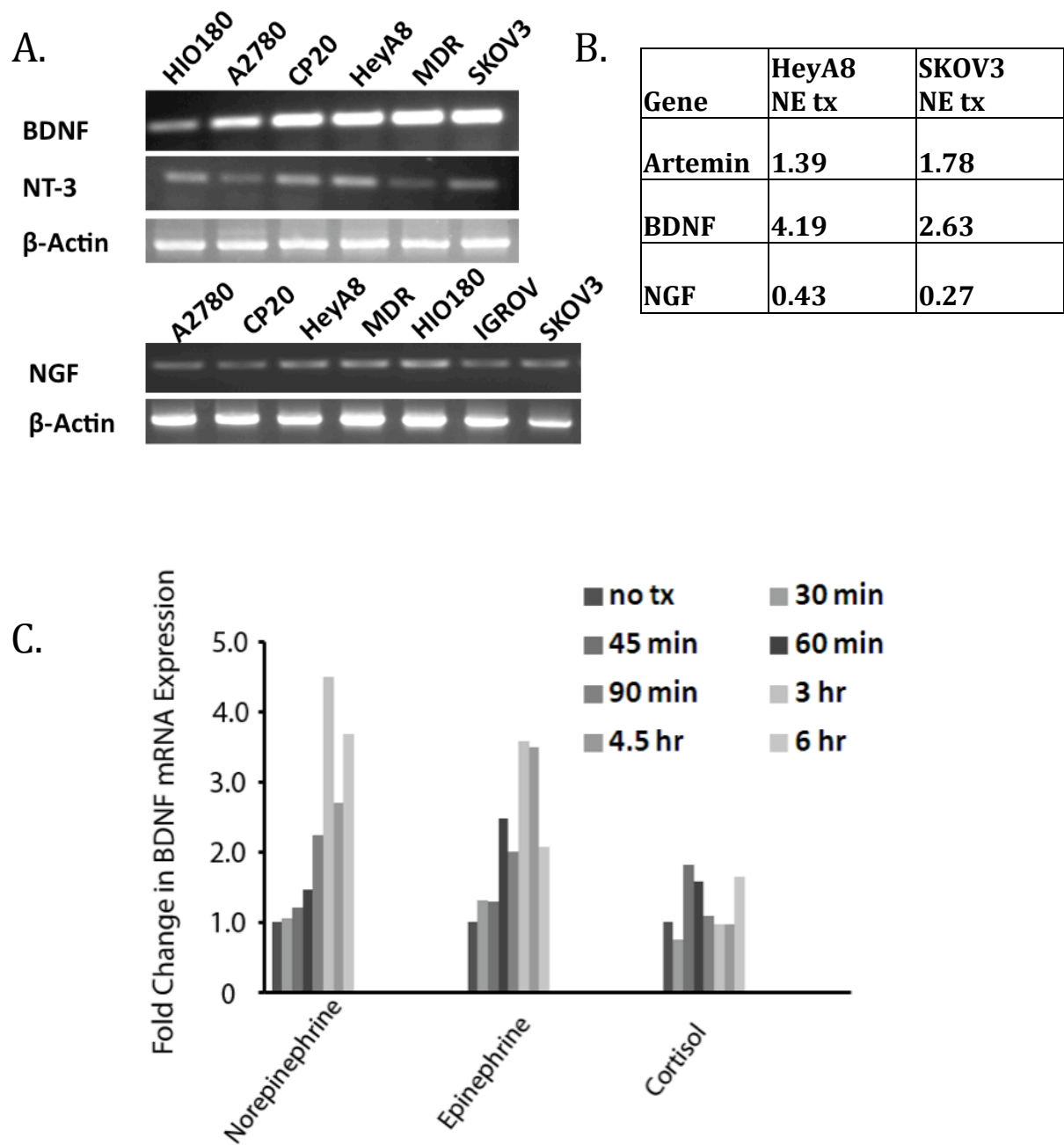


Figure 10. Neurotrophin expression in ovarian cancer cells. A. Expression of neurotrophins in ovarian cancer cells. B. Neurotrophic factors significantly increased in both HeyA8 and SKOV3 cells following norepinephrine stimulation. C. Timecourse of BDNF expression in SKOV3 cells following stimulation with norepinephrine, epinephrine, or cortisol.

hormones would increase expression of neurotrophic factors. I first treated several ovarian cancer cell lines with norepinephrine (NE), epinephrine (Epi), isoproterenol (a nonspecific ADRB agonist), and cortisol. Quantitative PCR analysis showed increased expression of Nerve Growth Factor (NGF) and Glial cell-derived neurotrophic factor (GDNF) family members such as BDNF and artemin following stimulation with NE, isoproterenol, and, to some extent, epinephrine. Cortisol had little to no effect on the neurotrophic genes examined, suggesting that these increases are in fact mediated through the ADRB family of receptors rather than glucocorticoid receptors. NE treatment caused increased expression in neurotrophins within 30 minutes, with peak increases seen between 3 and 6 hours post-treatment (Figure 10C). To expand my analysis to a broader spectrum of neurotrophins, I used a quantitative PCR array to examine members of the NGF and GDNF families as well as other known neurotrophic factors and related receptors. After exposing cells to NE for 6 hours, my analysis showed upregulation of two genes of particular interest, BDNF and artemin (Fig 10B).

3. Ovarian cancer cells promote neurite extension

To determine whether ovarian cancer cells are capable of promoting neurite extension, I utilized a neurite extension assay (Figure 6). Due to the difficulty in isolating and culturing neurons of the peripheral nervous system, I used the PC12 pheochromocytoma cell line. When stimulated with Nerve growth factor (NGF),

these cells differentiate and closely resemble neurons of the peripheral nervous system (132). Media conditioned by ovarian cancer cells was capable of promoting neurite extension (Figure 11A), and addition of NE to ovarian cancer cells potentiated this effect (Figure 11B).

Taking into consideration the increase in BDNF and artemin seen following NE stimulation of ovarian cancer cells, I next chose to silence expression of these neurotrophic factors using siRNA to determine whether they might be playing a role in promoting neurite extension. Exposure to media conditioned by BDNF siRNA treated cells abrogated NE-induced increases in neurite extension (p value = 0.15 of treatment vs. no treatment) (Figure 11E) compared to exposure to media conditioned by control siRNA treated cells (Figure 11C). Silencing artemin had no significant effect (p value < 0.01 in treatment vs. no treatment) (Figure 11D). These results suggested that BDNF might be responsible for promoting nerve growth following adrenergic activation. Based on these results I next decided to test the role of BDNF in recruitment of nerves into tumors in an orthotopic mouse model of ovarian cancer.

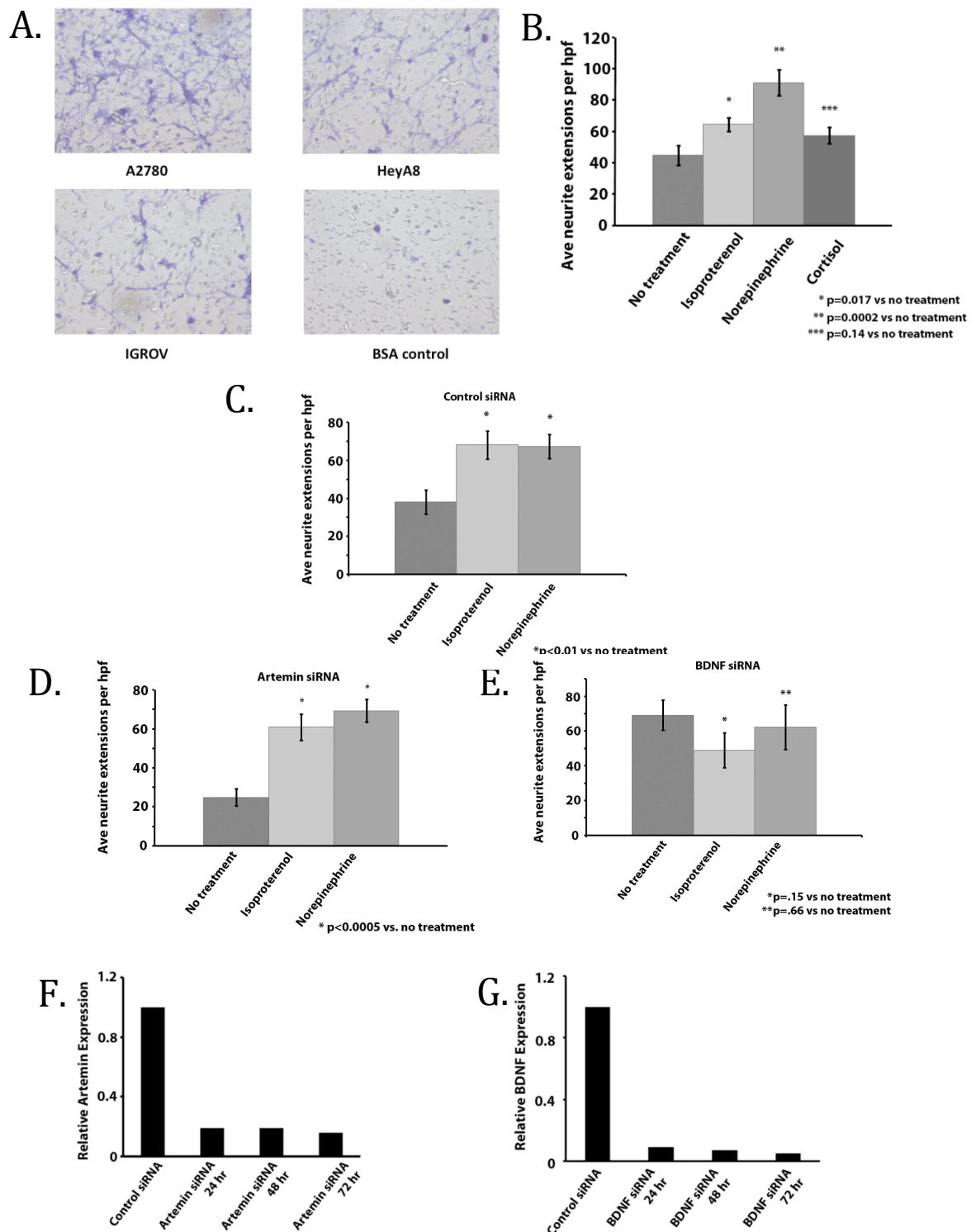


Figure 11. Neurite Extension Assays. A. Representative photos of neurite extension assays with tumor cell conditioned media versus BSA control media chemoattractant. B. Average neurite extensions per hpf of PC12 cells in response to media conditioned by SKOV3 ovarian cancer cells stimulated with isoproterenol, norepinephrine, or cortisol. C. Average neurite extensions per hpf of PC12 cells in response to media conditioned by SKOV3 ovarian cancer cells treated with control/non-targeting siRNA and subsequently stimulated with isoproterenol or norepinephrine. D. Average neurite extensions per hpf of PC12 cells in response to media conditioned by SKOV3 ovarian cancer cells treated with Artemin siRNA and subsequently stimulated with isoproterenol or norepinephrine. E. Average neurite extensions per hpf of PC12 cells in response to media conditioned by SKOV3 ovarian cancer cells treated with BDNF siRNA and subsequently stimulated with isoproterenol or norepinephrine. F. Knockdown efficacy of artemin siRNA. G. Knockdown efficacy of BDNF siRNA.

4. Chronic stress promotes neo-innervation of tumors in a BDNF-dependent manner

A. Tumors taken from chronically stressed mice have higher nerve density than tumors taken from non-stressed mice

To study the biological consequences of the increased neurite extension seen *in vitro*, I used a chronic restraint stress system to induce adrenergic activation *in vivo*. Daily restraint stress has been shown to activate the SNS and stimulate production of catecholamines, resulting in increased tumor growth. Semi-quantitative analysis of immunohistochemical staining of neurofilament in tumor samples taken from animals that underwent daily restraint stress exhibit a 3-fold increase in nerve density when compared to tumors obtained from non-stressed control animals (Figure 12).

B. Chronic stress impact on nerve density in normal tissue

The above results then led me to consider whether chronic stress increases native nerve density of normal tissues in non tumor-bearing animals. To test this I removed liver, spleen, omentum, ovary, and peritoneum from both chronically stressed and non-stressed non tumor-bearing mice. Analysis of nerve density in these tissues revealed no significant changes in nerve density between stressed and non-stressed animals (Figure 13B), suggesting stress-induced nerve growth may be tumor-specific.

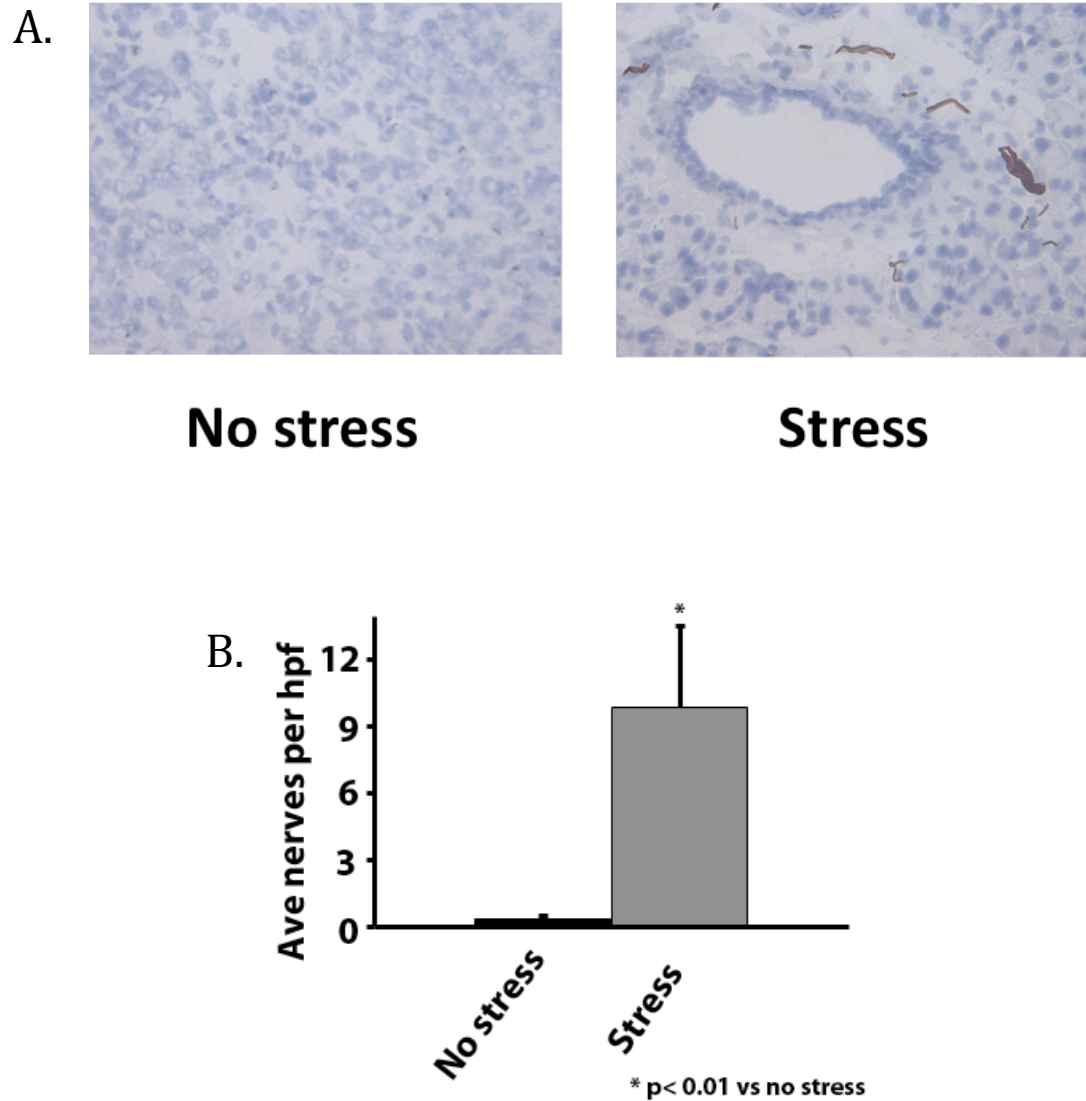


Figure 12. Nerve density in tumors of chronically stressed mice A. Representative photos of neurofilament immunohistochemical staining in SKOV3 tumors taken from stressed or non-stressed animals. B. Average nerve density per hpf in SKOV3 tumors taken from stressed or non-stressed animals.

C. The role of nerve density in metastasis

Evaluation of patient samples suggested that tumor innervation is not site specific. To confirm this observation, I utilized an intraovarian model of ovarian cancer metastasis with or without chronic stress. In this model, tumor cells are injected directly into the ovary and allowed to metastasize. Tumors were then collected and analyzed for nerve density at the metastatic site. Nerves were seen in tumors taken from spleen, ovary, peritoneum, omentum, and liver. As shown in figure 13B, nerve density in these tissues varies greatly, suggesting that native nerve density does not play a significant role in predilection of tumors to metastasize to certain sites.

In the intraovarian model of ovarian cancer metastasis, tumors most frequently metastasized to the peritoneum, surgical wound site (resulting from injections), and the mesentery. Analysis of tumors taken from each of these sites revealed that chronic stress increased nerve density in tumors from all three sites (Figure 13A), suggesting that chronic stress increases in nerve density are not site specific.

D. siRNA silencing of BDNF in tumor cells inhibits stress-induced neo-innervation of tumors

I next wished to investigate whether inhibition of BDNF expression in tumor cells would affect neo-innervation of tumors *in vivo*. To silence expression of BDNF

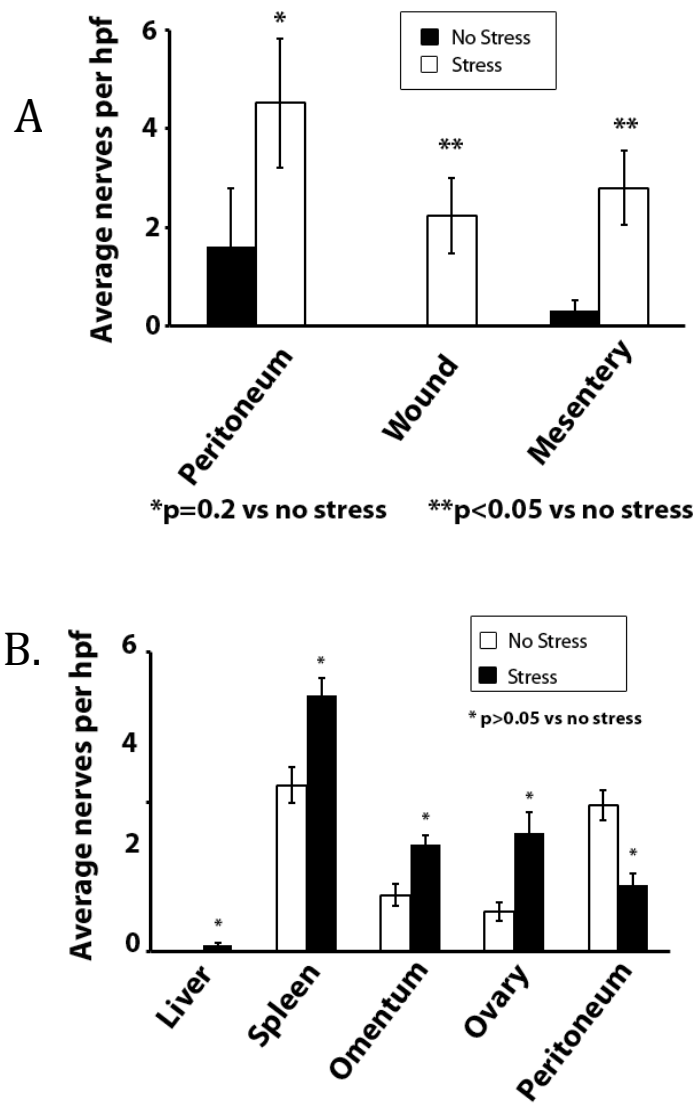


Figure 13. Nerve density of tumors taken from metastatic sites. A. Nerve density from metastatic ovarian tumors from various sites. **B.** Nerve density of various tissues from non tumor-bearing animals

I used siRNA incorporated into a neutral DOPC liposomal delivery system. Our lab has done extensive testing of siRNA in *in vivo* models, and has found that use of this liposome for high degree of delivery into tumor tissues (134). Additional testing has demonstrated that we can achieve significant inhibition lasting over 72 hours from one treatment (134). Animals were therefore treated biweekly to ensure continuous knockdown of BDNF expression. Use of an orthotopic mouse model of ovarian cancer allowed me to use a human-BDNF-specific (hBDNF) siRNA to inhibit BDNF production in the tumor without affecting BDNF production in other tissues. Tumors were collected from stressed and non-stressed animals treated with either scrambled, nontargeting control or hBDNF targeting siRNAs. Tumors were analyzed for BDNF expression using both qPCR and IHC. Additional IHC analysis was also used to count nerve density. Analyses revealed that BDNF expression increased over 9-fold in tumors from stressed animals versus non-stressed (Figure 14A and B), demonstrating that the increases in BDNF seen following NE stimulation *in vitro* are significantly amplified *in vivo*. As anticipated, tumor weight was doubled in stressed versus non-stressed control siRNA treated animals. More interestingly, this effect was entirely abrogated in animals treated with hBDNF siRNA. While hBDNF inhibition had no significant effect in non-stressed animals, it entirely abolished stress-induced increases in tumor growth in animals receiving daily restraint stress (Figure 14C). Further analysis revealed that hBDNF inhibition also abrogated stress-induced increases in tumor neo-innervation (Figure 14E). While tumors from control siRNA treated chronically stressed mice had an average of 6 nerves per high powered field,

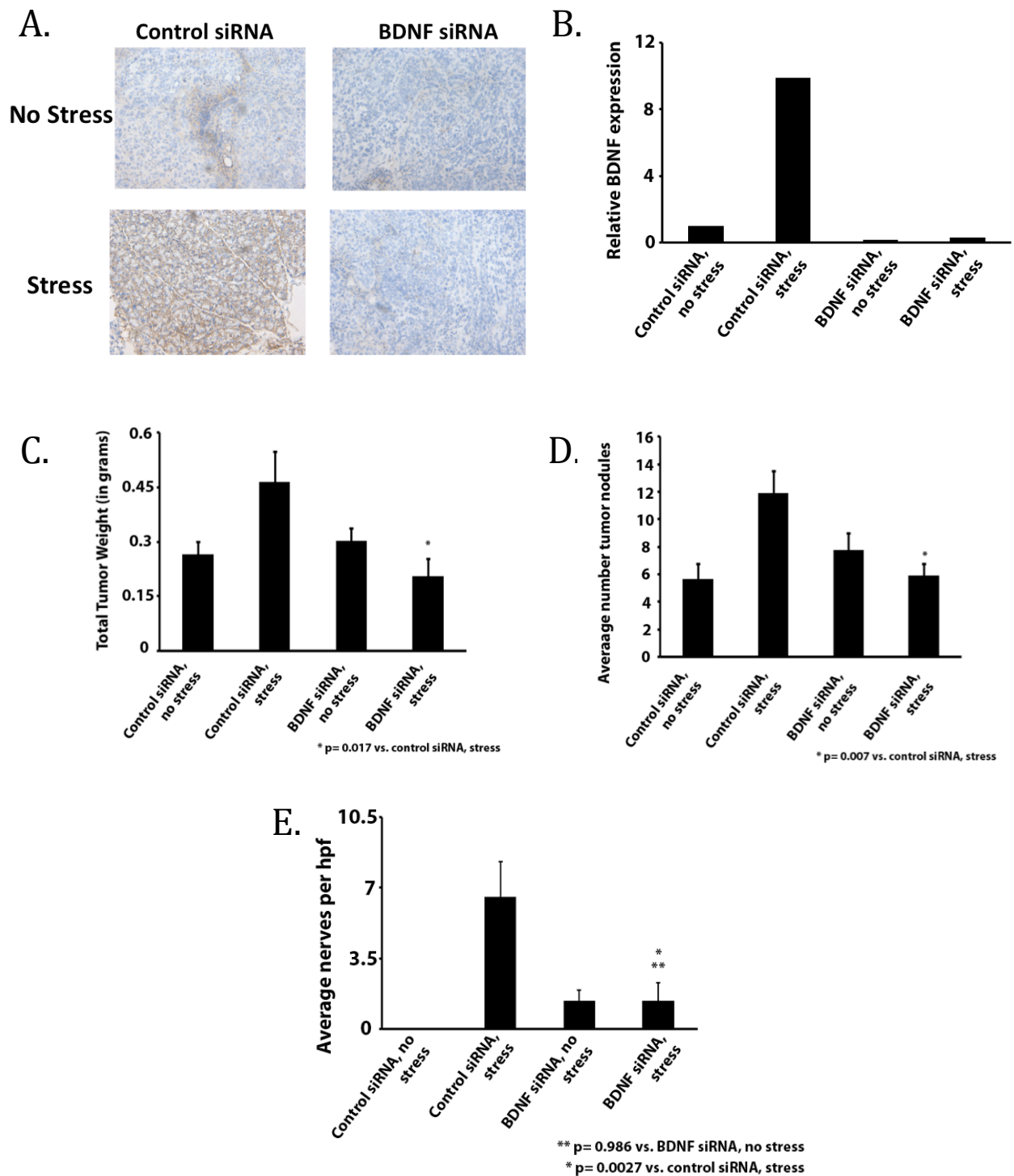


Figure 14. BDNF siRNA in SKOV3 tumors. Relative BDNF expression by A. IHC and B. qPCR in SKOV3 tumor samples C. Average total SKOV3 tumor weight, D. tumor nodules, and E. Average nerve density per hpf in stressed versus non stressed animals that underwent either adrenalectomy or sham surgery.

hBDNF siRNA treated stressed mice had no nerves present. Similar results were also seen in tumors from the HeyA8 ovarian cancer cell line (Figure 15).

To demonstrate that stress-induced tumor neo-innervation is not an ovarian cancer-specific phenomenon, I repeated these experiments using the RKO human colon cancer cell line. Upon NE stimulation *in vitro*, BDNF expression increases in these cells. *In vivo* chronic stress increased tumor weight and nerve density in these tumors by 2-fold (Figure 16), and as in the ovarian cancer models, BDNF inhibition using hBDNF-targeted siRNA abrogated stress effects on both tumor growth and nerve density. Taken together, these data suggest not only that chronic stress promotes neo-innervation of tumors, but also that tumor neo-innervation is critical for chronic stress-induced effects on tumor growth, and that BDNF production from the tumor is a crucial factor in this process.

5. BDNF expression correlates with nerve density and patient outcome in human ovarian cancer patients

I next wished to determine whether there was a correlation between high nerve density and BDNF expression in human ovarian cancers. I therefore measured BDNF levels semi-quantitatively by IHC analysis in patient samples previously stained for nerve density. Patients were divided into high and low BDNF expression (Figure 17A). This analysis revealed a positive correlation between

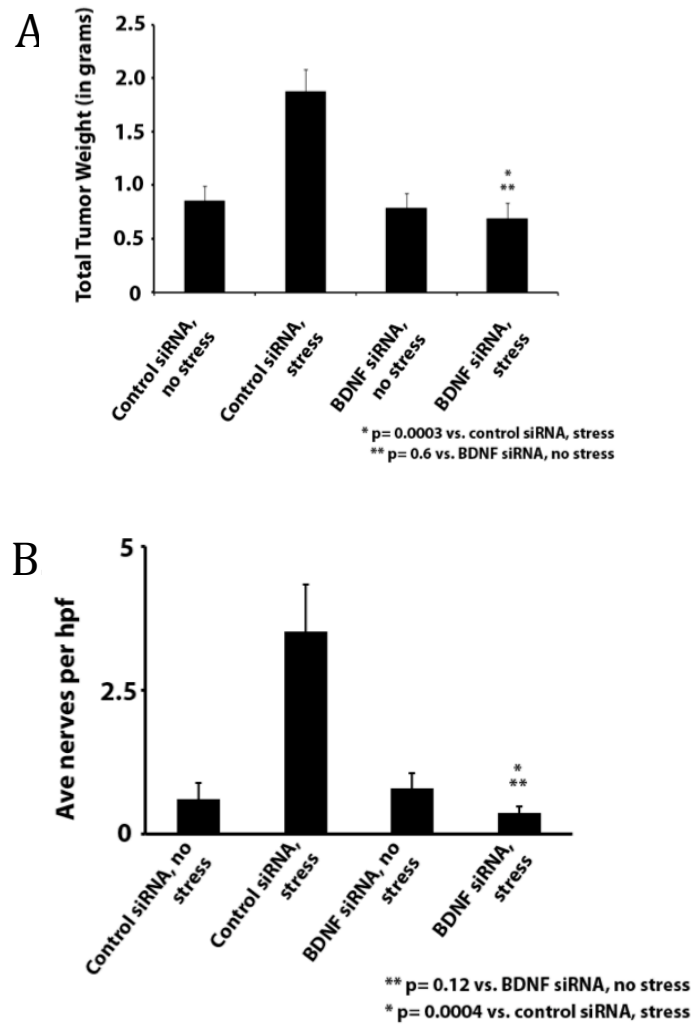


Figure 15. BDNF siRNA in HeyA8 tumors. A. Average total tumor weight of HeyA8 tumors and B. average nerve density in stressed versus non stressed animals treated with either control or BDNF siRNA.

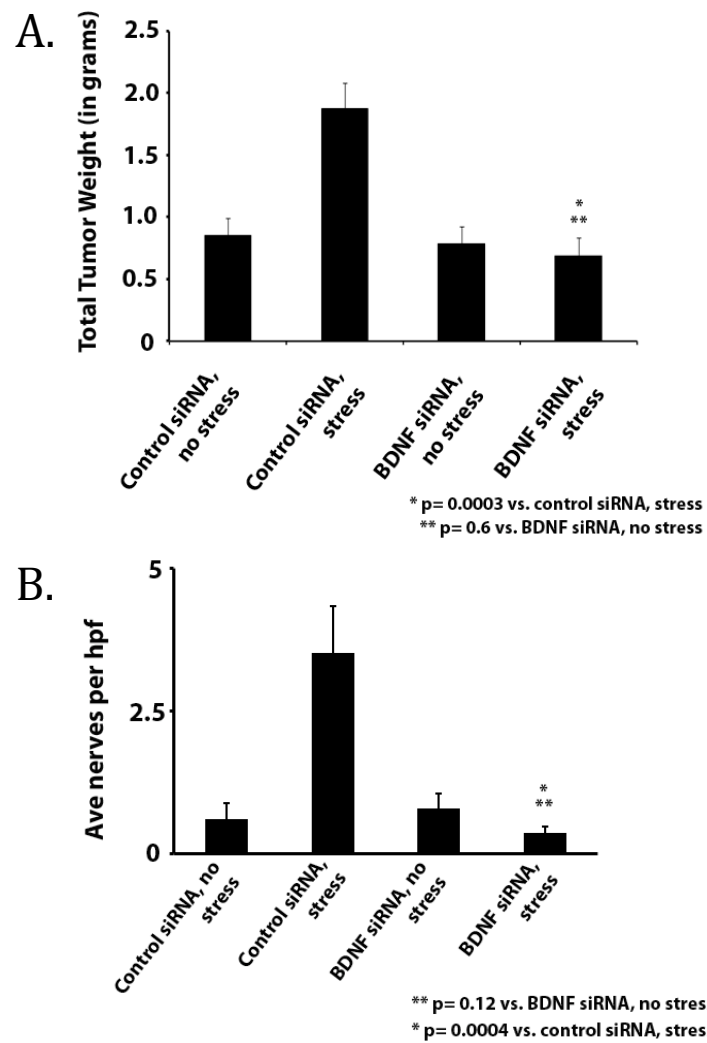


Figure 16. BDNF siRNA in RKO colon cancer. A. Average total tumor weight and B. Average nerve density per hpf of RKO tumors taken from stressed or non-stressed animals treated with control or BDNF siRNA

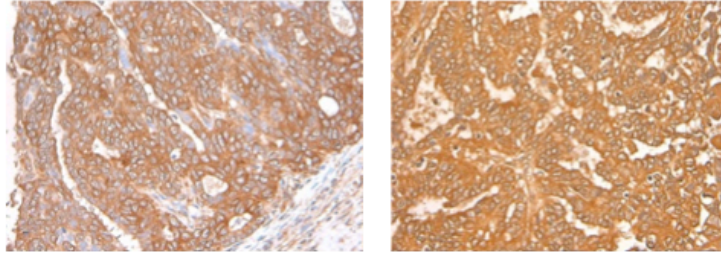
increased BDNF expression and increased nerve density. There was also a significant correlation between high BDNF expression and poor outcome ($p < 0.001$) (Figure 17B). Patients with high levels of BDNF expression survived an average of 39 months, compared with 65 months in patients expressing low levels of BDNF.

6. Neural precursors are present in tumors

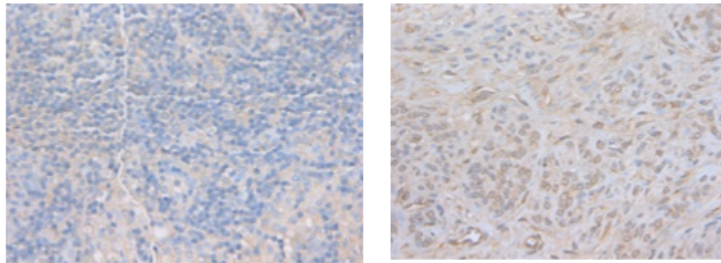
We next wondered about possible origins for the nerves noted in the tumors. Increased nerve density may be a result of branching of existing neurons, or occurs by formation of new neurons from precursor cells. Doublecortin is a protein expressed exclusively in neuronal precursors and immature neurons. Its expression decreases and ultimately disappears after the first 10-14 days of a neuron's existence (Figure 4). It is therefore used as a marker for new neuron formation. IHC analysis of tumors taken from chronically stressed mice revealed the presence of doublecortin positive cells within the tumors (Figure 18). This evidence demonstrates that signals activated by chronic stress, and most likely coming from the tumor itself, are initiating new neuron formation through the recruitment of neuronal precursors.

A

High intensity
BDNF expression



Low intensity
BDNF expression



B.

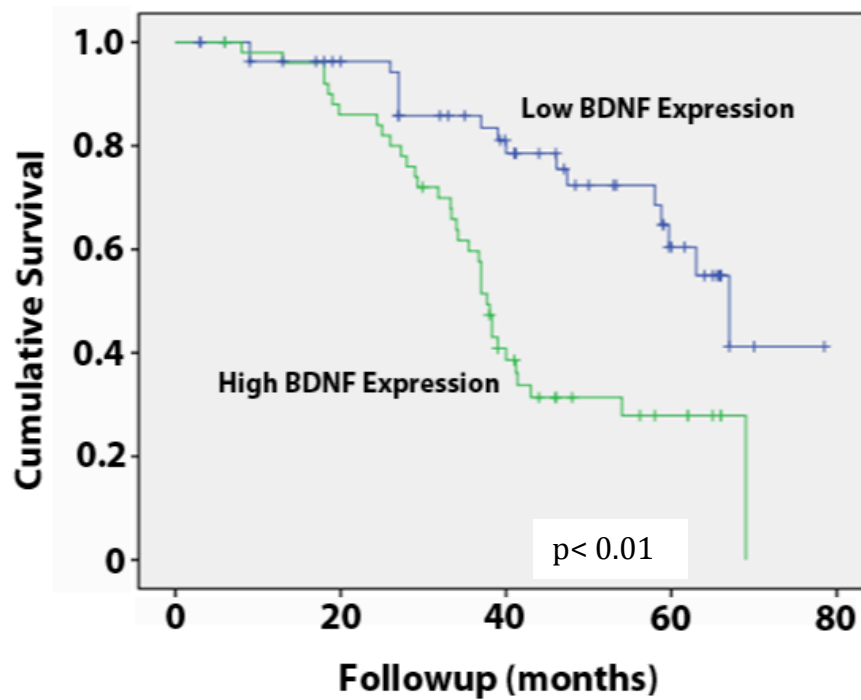


Figure 17. BDNF expression in human ovarian cancer. A. BDNF expression in human ovarian cancer samples. B. Overall survival of ovarian cancer patients with high versus low BDNF expression.

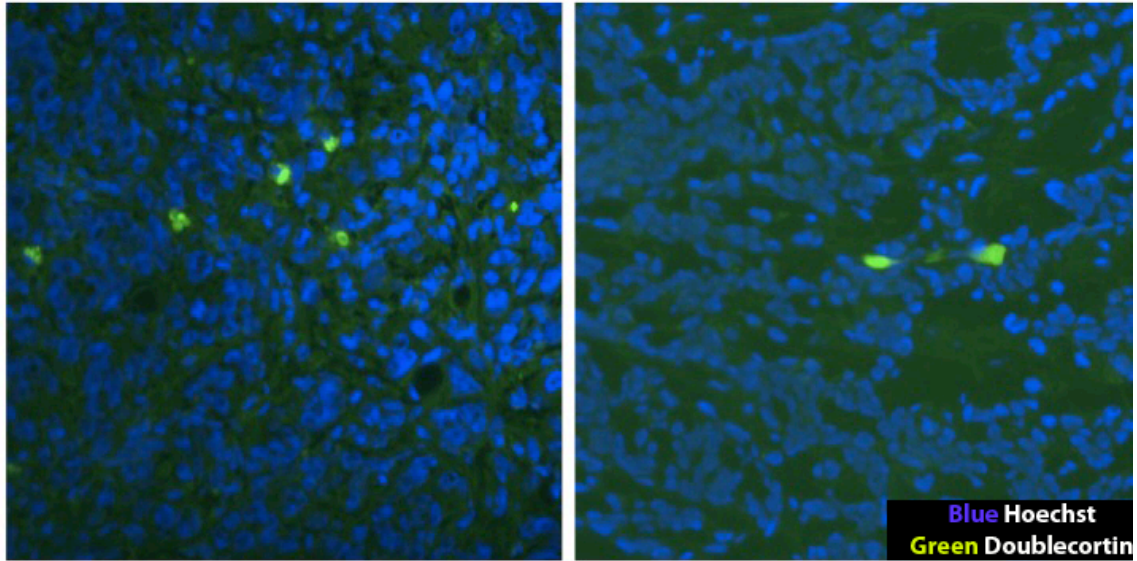


Figure 18. A. Doublecortin positive cells in SKOV3 ovarian tumors

7. Inhibition of neo-innervation of tumors abrogates downstream stress effects

Previous studies from our lab have shown that chronic stress activates several processes critical for tumor growth and progression. For a malignancy to progress it must gain an adequate blood supply to provide nutrients and growth factors to the tumor cells. IL-6 is a prominent factor in this process. Our previous studies have shown a several fold increase in IL-6 following chronic stress both in animal models and clinically (2, 68). Additionally, in order to metastasize tumors must be able to invade the surrounding basement membrane through the function of matrix metalloproteinases (MMPs). Our studies have also shown two MMPs, MMP-2 and -9, are significantly increased following chronic stress. I therefore wished to determine whether inhibition of tumor neo-innervation affected other markers of chronic stress in tumors. Quantitative-PCR analysis demonstrated that IL-6 expression in tumors of chronically stressed mice was increased over 7-fold compared to non-stressed controls, and this increase was entirely abrogated in hBDNF siRNA treated animals (Figure 19A). Additionally, MMP-2 and -9 production increased 19- and 10-fold respectively in tumors from chronically stressed animals, but no significant increase was seen in tumors taken from hBDNF siRNA treated animals (Figure 19B and C). These data suggest that by inhibiting neo-innervation of tumors, we are also inhibiting downstream effects of chronic stress.

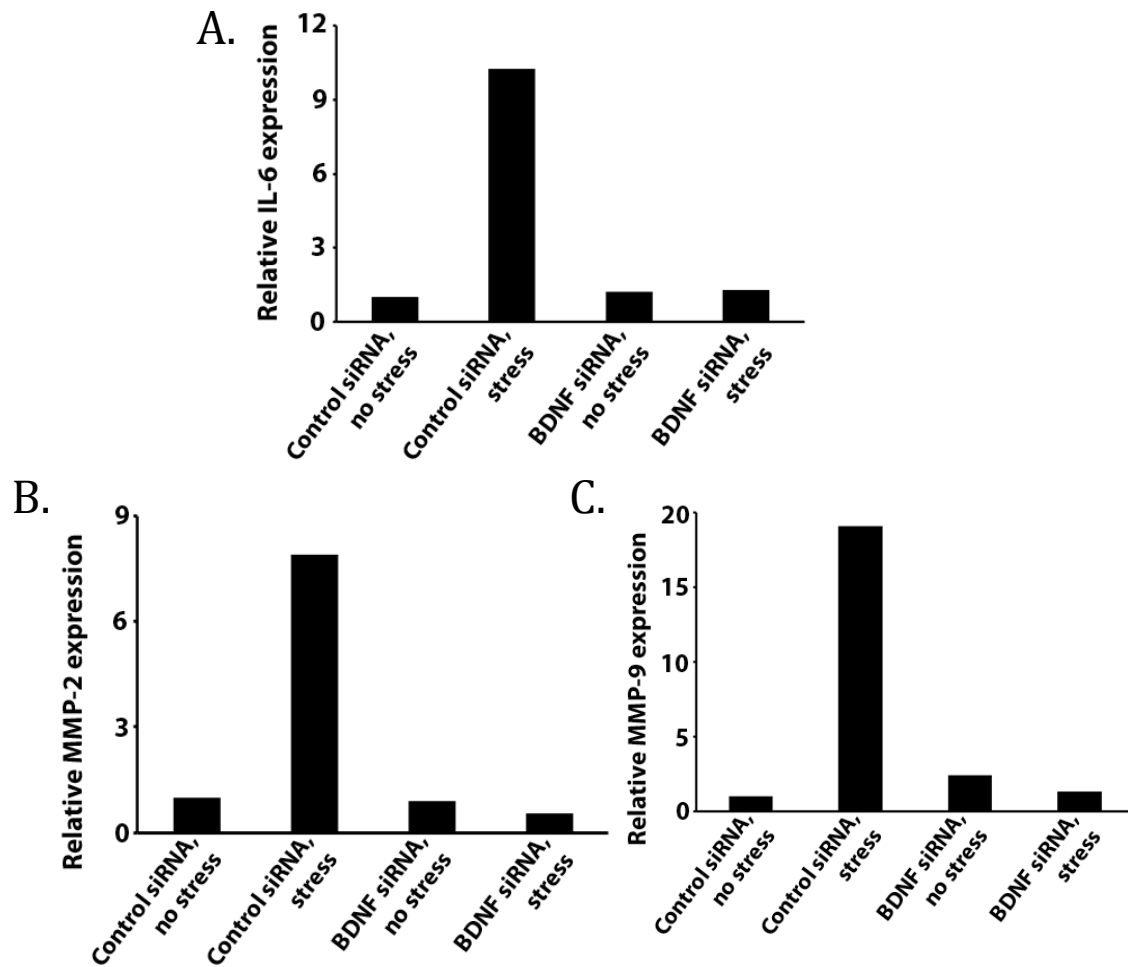


Figure 19. Downstream markers of chronic stress following BDNF inhibition *in vivo*. Relative expression of A. MMP-2, B. MMP-9, C. IL-6 in SKOV3 tumors taken from stressed or non-stressed mice treated with either control or BDNF siRNA

8. TrkB receptors on tumor cells play no significant role in stress-induced tumor growth

Members of the nerve growth factor (NGF) family bind to the Trk family of tyrosine kinase receptors. These receptors are known to be overexpressed in ovarian cancers. BDNF binds with high affinity to TrkB, and activation of TrkB by BDNF in ovarian cancer cells has been shown to slightly increase tumor cell migration, while inhibition of TrkB impaired migration and invasion (135). To verify that effects seen on tumor growth are an indirect effect mediated through decreased tumor neo-innervation as opposed to a direct effect on the tumor cells themselves, I treated nude mice bearing SKOV3 human ovarian cancer tumors with human TrkB targeted siRNA. hTrkB inhibition had no significant effect on total tumor weight in either stressed or non-stressed animals (Figure 20C). Conversely, inhibition of mTrkB was capable of entirely abrogating stress-induced tumor growth (Figure 21), suggesting that autocrine signaling of BDNF on TrkB receptors found on the tumor cells themselves are not a significant mechanism used by chronic stress to induce tumor growth., while paracrine signaling through other cells expressing TrkB receptor, mainly nervous tissue, does play a significant role.

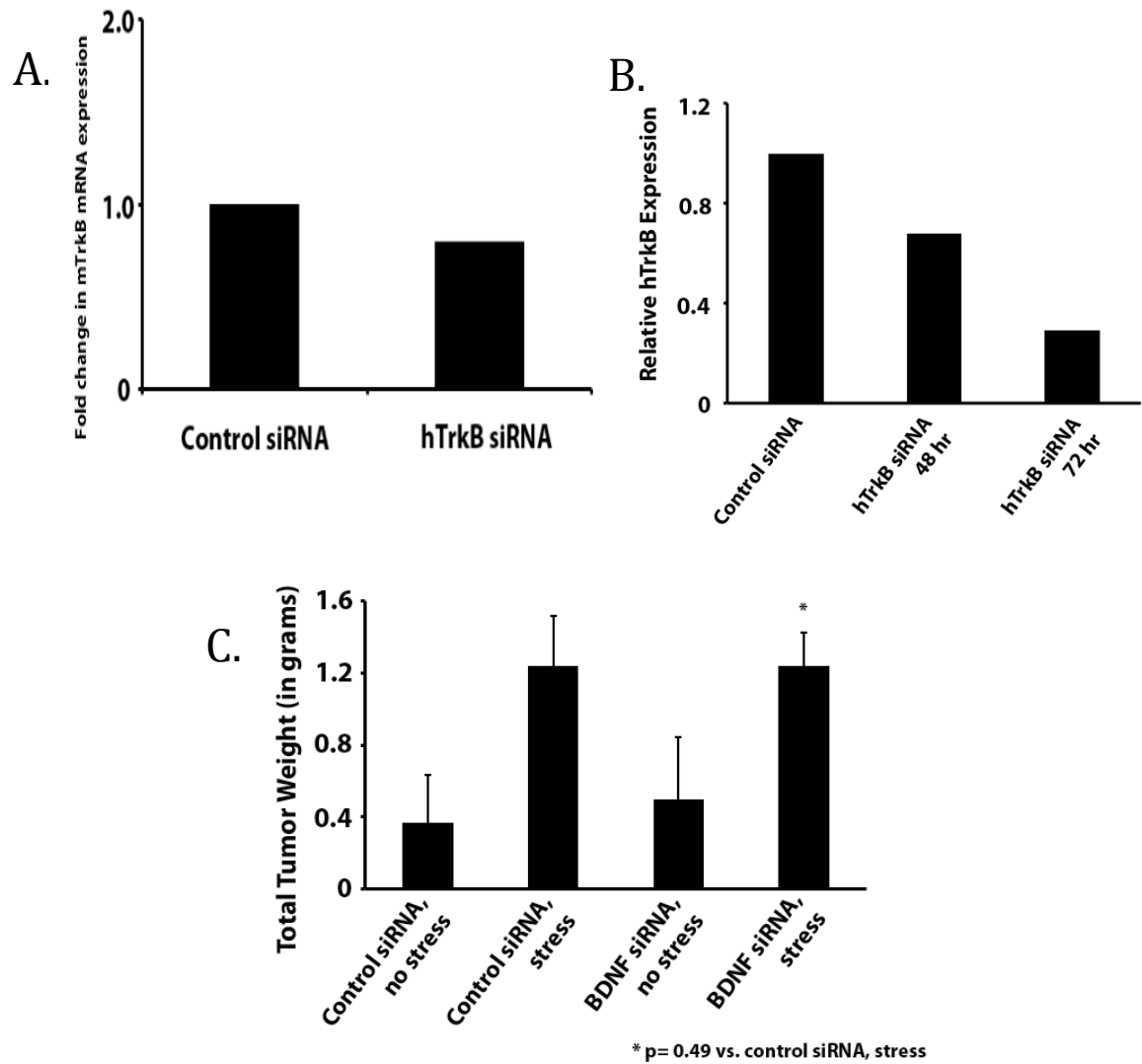


Figure 20. hTrkB siRNA *in vivo*. A. Relative mTrkB expression in ID-8 mouse ovarian tumor cells treated with hTrkB siRNA. B. Knockdown efficacy of hTrkB siRNA. C. Average total SKOV3 tumor weight from either stressed or non-stressed mice treated with either control or hTrkB siRNA.

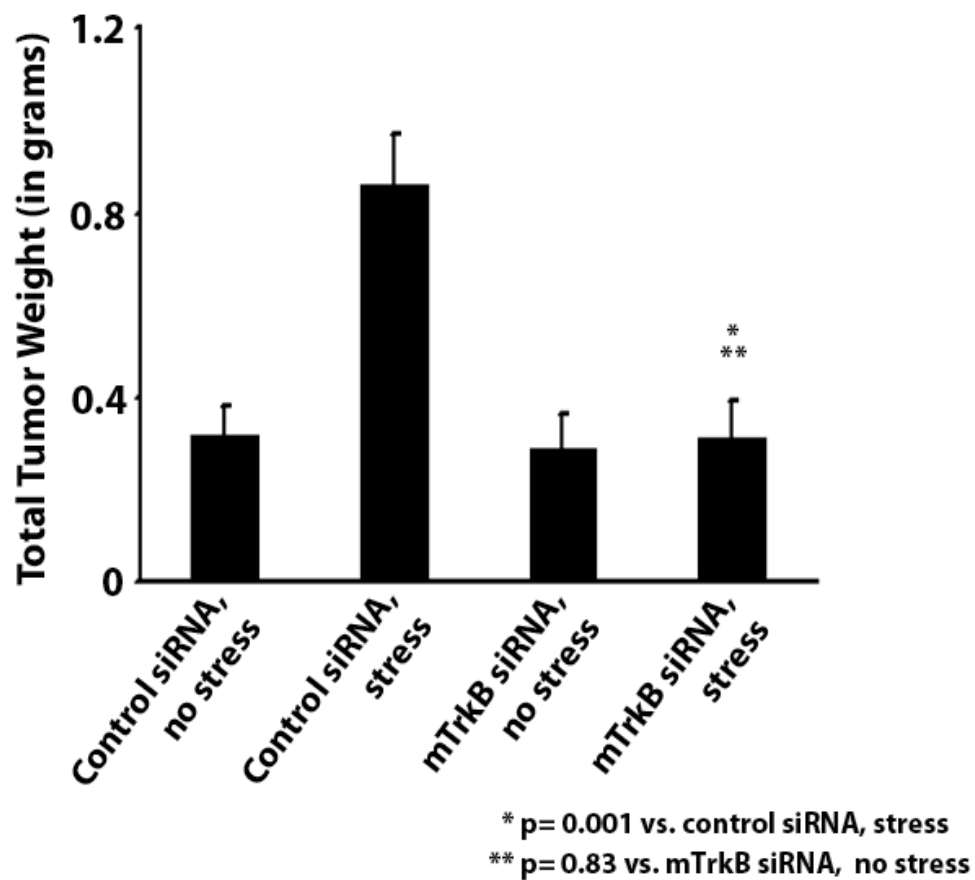


Figure 21. mTrkB siRNA *in vivo*. Average total SKOV3 tumor weight from either stressed or non-stressed mice treated with either control or mTrkB siRNA.

9. Inhibition of peripheral nervous system function blocks stress-mediated tumor growth and innervation

Hexamethonium Bromide is a ganglionic blocker that blocks nicotinic receptors of postganglionic neurons. Interestingly, hexamethonium is only capable of crossing the blood-brain-barrier at very high doses. Therefore, given at low doses it can serve as a peripheral nervous system-specific antagonist. As a result, this drug has been used for many years to study peripheral nervous system function. As shown in Figures 1 and 2, the central nervous system initiates the stress response systems through the hypothalamic-pituitary-adrenal axis and autonomic nervous system. In my studies, I sought to demonstrate that the effects of chronic stress that we see on tumor growth are mediated through the peripheral nervous system function rather than direct CNS control. I compared tumors taken from stressed and non-stressed animals that were given either vehicle (PBS) or 1 mg/kg/day hexamethonium bromide, a dose significant enough to inhibit PNS function, without crossing into the CNS. Average tumor weight in chronically stressed animals treated with hexamethonium was similar to the baseline tumor weight found in non-stressed vehicle treated animals, and significantly reduced when compared to tumor taken from chronically stressed vehicle treated animals (Figure 22A). Interestingly, I found that nerve density in these animals was also decreased (Figure 22B). These results suggest that peripheral nervous system function is a critical mediator of chronic stress effects on tumor growth, and

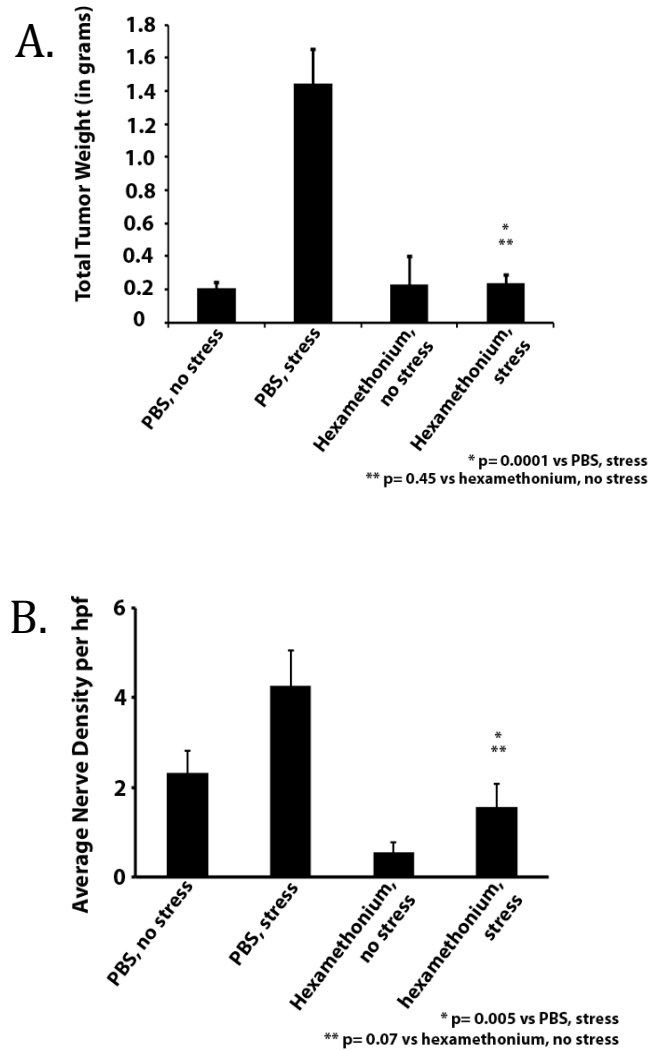


Figure 22. Hexamethonium Bromide treatment *in vivo*. A. Average total SKOV3 tumor weight and B. Average nerve density per hpf in stressed versus non stressed animals treated with either PBS control or hexamethonium Bromide.

supports my hypothesis that neurons of the peripheral nervous system found in tumors may be contributing to promoting these effects.

10. Circulating catecholamines do not significantly affect tumor progression in mouse syngeneic model of ovarian cancer

There are two main pathways downstream of the central nervous system that become activated as our bodies cope with a stressor. One pathway is the sympathetic nervous system, which signals through catecholamines released primarily by sympathetic nerve endings. The other pathway is the hypothalamic-pituitary-adrenal axis (Figure 1B). This pathway results in secretion of glucocorticoids and catecholamines from the adrenal glands to circulate systemically in the blood stream. Though this is not the primary method of secretion for catecholamines, I wished to verify that the hormones seen within the tumor tissue are in fact coming from sympathetic nerve endings, rather than being deposited in the tumor through circulation from the adrenal gland. To test this hypothesis I utilized an adrenalectomized mouse model. Due to low survival rates in nude mice when performing adrenalectomy surgeries, I used adrenalectomized C57/B6 mice. Our lab has previously used a line of ID8 mouse ovarian tumor cells that express high levels of VEGF for syngeneic models of chronic stress in ovarian cancer. Expression of neurotrophins and ability to induce neurite extension were similar in this cell line to those seen with the SKOV3 human ovarian cancer cell line,

so I chose to utilize this line for this model. Following either adrenalectomy surgery or sham surgery, adrenalectomized mice were given 10 days to recover prior to the initiation of daily stress. These animals were then treated according to our previously established model of chronic stress *in vivo* (Figure 8). Total tumor weight of tumors taken from chronically stress, adrenalectomized animals was very similar to total tumor taken from chronically stress, sham surgery animals (Figure 23A). This result suggests that stress hormones being delivered into the tumor from the adrenal gland play no significant role in promoting stress-induced tumor progression. Further analysis revealed that nerve density in tumors from both groups was also similar (Figure 23B), suggesting that hormones from the adrenal gland also do not play a role in initiating neo-innervation of tumors.

11. Norepinephrine stimulates BDNF production in ovarian tumor cells through the ADRB3-cAMP-Epac signaling pathway

Previous data from our lab have revealed that stress effects on tumor growth are mediated through the β_2 -Adrenergic receptor (ADRB2) (2). All members of the ADRB family are expressed in ovarian cancer cell lines. I therefore sought to discover which receptor and downstream signaling pathways are involved in adrenergic-induced increases in BDNF expression. Using receptor-specific inhibitors I found that NE-induced BDNF expression occurs through the β_3 -adrenergic receptor (ADRB3). Inhibition of α -adrenergic receptors (Figure 24A) as well as the β_1 - and β_2 - (Figure 24 B; atenolol, butoxamine, and propranolol)

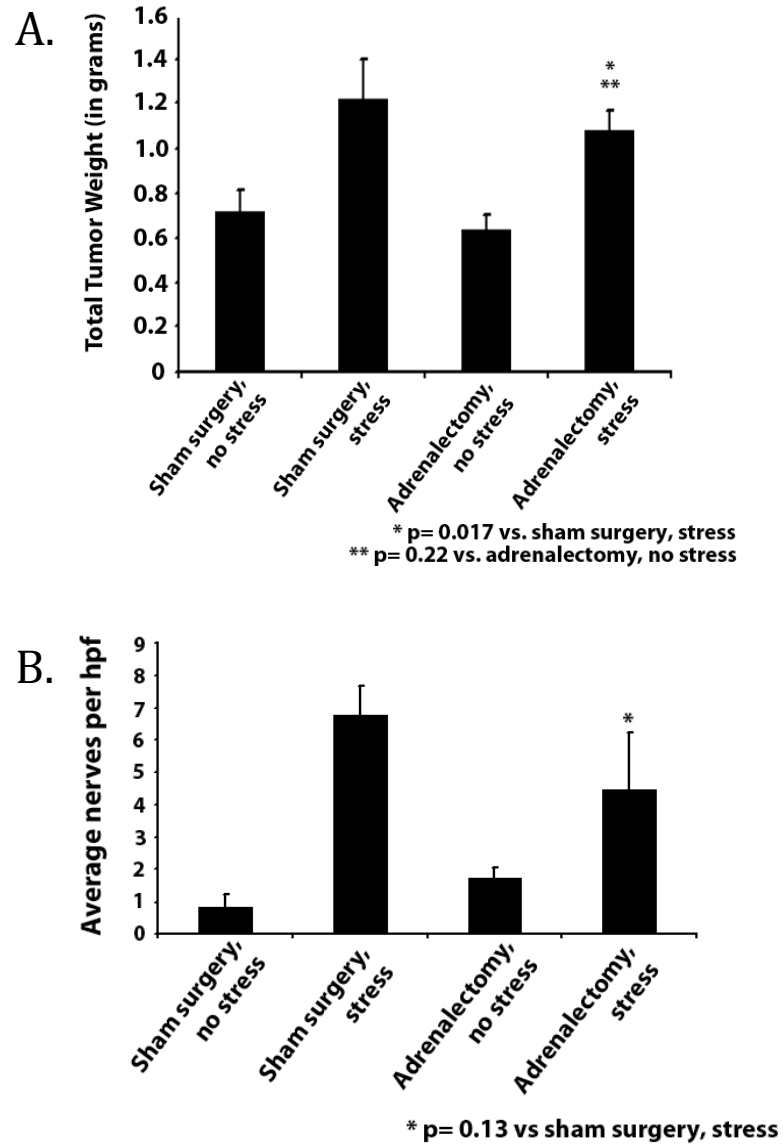


Figure 23. Tumor weight and nerve density in tumors of adrenalectomized mice. A. Average total SKOV3 tumor weight and B. Average nerve density per hpf in stressed versus non stressed animals that underwent either adrenalectomy or sham surgery.

adrenergic receptors had little or no impact on NE-induced BDNF expression, while inhibition of the β_3 family member entirely abrogated NE-induced BDNF expression in ovarian cancer cells (Figure 24B, SR59230). Conversely, stimulating with the ADRB3-specific agonist BRL37344 increased BDNF expression (Figure 24C). ADRB3 is a G-protein coupled receptor that functions primarily through the cAMP/PKA signaling pathway, though it can also signal through the Ras and PI3K pathways. I verified cAMP involvement by using forskolin, a cAMP agonist, which I found increased BDNF gene expression (Figure 25A). Activation or inhibition of PKA signaling had no effect on NE-induced BDNF expression (Figure 26F). I therefore chose to look into the less common Epac signaling pathway. Inhibition of Epac using Brefeldin A markedly decreased NE-induced increases in BDNF gene expression (Figure 25B). Conversely, activation of Epac using 8cPT-2Me-cAMP, a cAMP mimic that is specific to Epac family members, induced BDNF gene expression (Figure 25B). Further inhibition of factors downstream of Epac pointed to involvement of JNK activation (Figure 25C). Luciferase studies have confirmed that NE induces BDNF expression through ADRB3, and this signaling is mediated through Epac.

To further validate this pathway, I inhibited several other pathways known to be downstream of ADRBs, cAMP, or Epac. Inhibition of PLC, Akt, and MEK had no significant effect on NE-induced BDNF expression (Figure 26A). Additionally, inhibition of H- or K-Ras using siRNA no significant effect on NE-induced BDNF expression. Nor did inhibition of PKB or Rap signaling. These studies, summarized

in Figure 27, demonstrate that norepinephrine activates ADRB3 which then signals through cAMP to activate Epac and initiate downstream JNK signaling to promote BDNF gene expression.

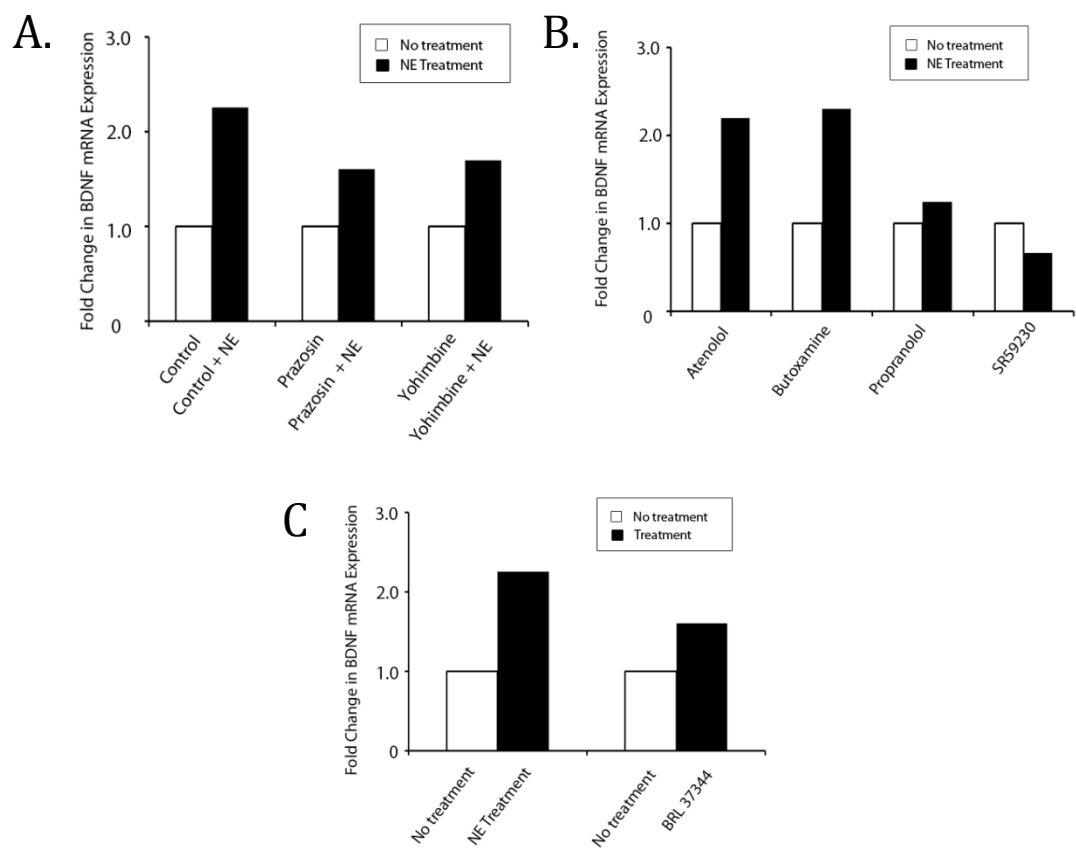


Figure 24. Norepinephrine signals through ADRB3 to increase BDNF expression. A. Relative BDNF expression in norepinephrine stimulated SKOV3 cells treated with Prazosin or Yohimbine. B. Relative BDNF expression in norepinephrine stimulated SKOV3 cells treated with atenolol, butoxamine, propranolol, or SR59230. C. Relative BDNF expression in SKOV3 cells treated with BRL 37344.

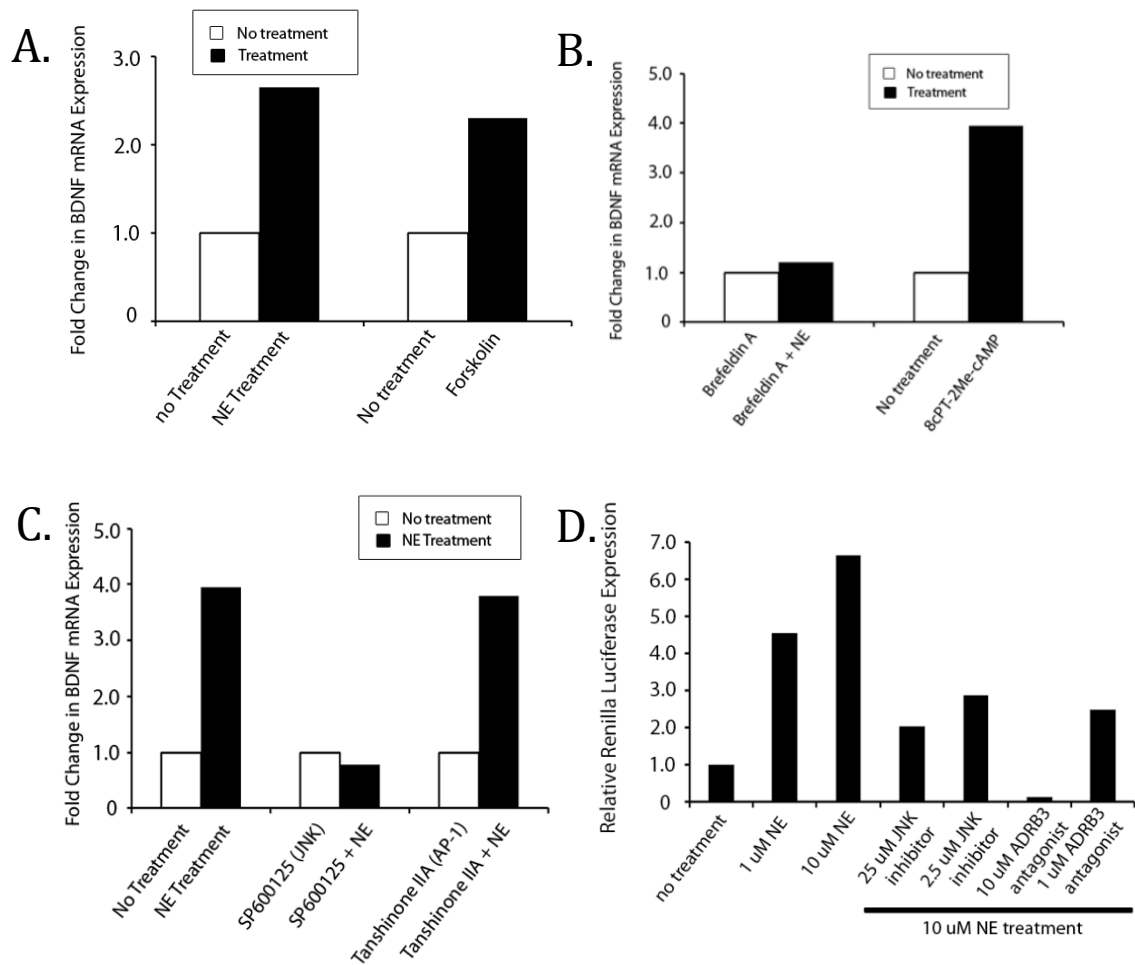


Figure 25. Norepinephrine-induced increases in BDNF expression are mediated through cAMP, Epac, and JNK. A. Relative BDNF expression in SKOV3 cells treated with norepinephrine or Forskolin. B. Relative BDNF expression in norepinephrin stimulated SKOV3 cells treated with Brefeldin A and non-stimulated SKOV3 cells treated with 8cPT-2Me-cAMP. C. Relative BDNF expression in norepinephrine stimulated SKOV3 cells treated with SP600125 or Tanshinone IIA. D. Relative BDNF promoter activity in norepinephrine stimulated SKOV3 cells treated with either SP600125 (JNK inhibitor) or SR59230 (ADRB3 inhibitor).

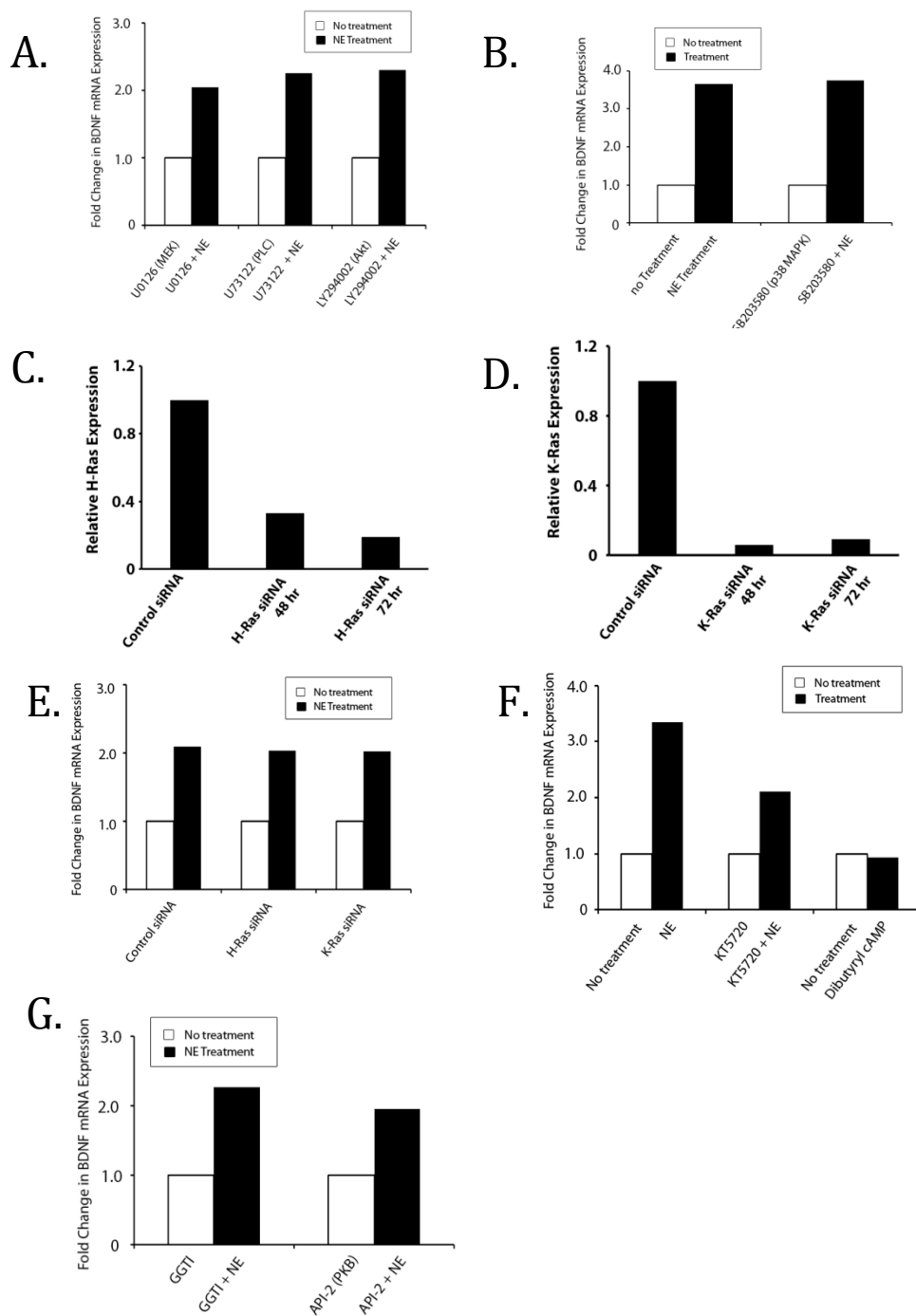


Figure 26. Downstream signaling of ADRB3. A. Relative BDNF expression in norepinephrine stimulated SKOV3 cells treated with U0126, U73122, LY294002 B. Relative BDNF expression in norepinephrine stimulated SKOV3 cells treated with SB203580. C. H-Ras siRNA knockdown efficiency. D. K-Ras siRNA knockdown efficiency. E Relative BDNF expression in norepinephrine stimulated SKOV3 cells treated with H-Ras siRNA. F. Relative BDNF expression in norepinephrine stimulated SKOV3 cells treated with K-Ras siRNA. G. Relative BDNF expression in norepinephrine stimulated SKOV3 cells treated with GGTI or API-2.

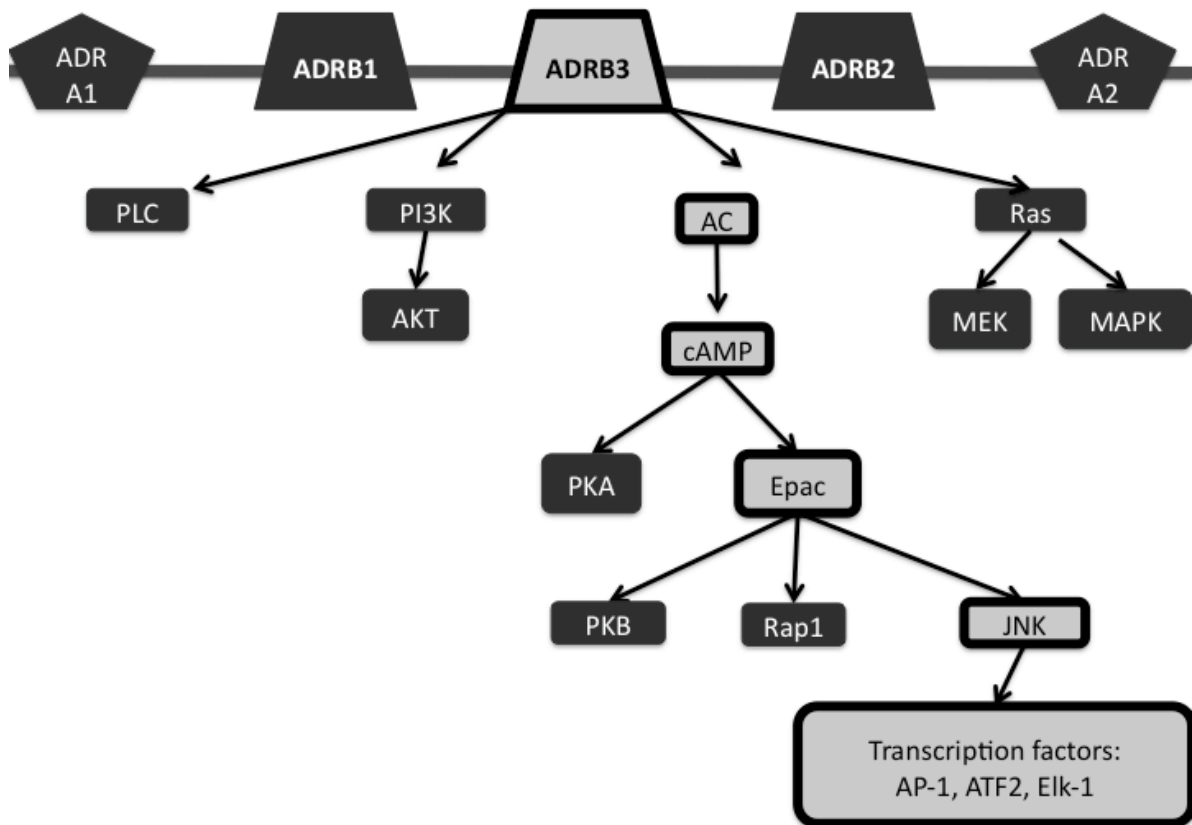


Figure 27. Mechanism of norepinephrine stimulated BDNF expression

Discussion

1. Summary (Figure 28)

In this dissertation, I have considered the impact that chronic stress may have on promoting neo-innervation of tumors and the effects of increased tumor innervations on tumor growth, as well as the mechanisms by which tumor cells stimulate nerve growth. Using both human and animal models I have demonstrated that chronic stress is capable of promoting neo-innervation of tumors through upregulation of BDNF gene expression activated by $\beta 3$ adrenergic signaling and that this increased nerve density is responsible for promoting tumor growth by releasing high levels of catecholamines into the tumor tissue. Specifically, after demonstrating that increased nerve density was associated with poorer outcome in ovarian cancer patients, I verified that ovarian cancer cells secrete neurotrophic factors and are capable of inducing neurite outgrowth. Stimulation of cells increased production of BDNF as well as promotion of neurite extension. Inhibition of BDNF using siRNA abrogated this effect. I also found that NE-induced BDNF gene expression is mediated through ADRB3 activation of cAMP/PKA signaling through Epac.

Based on *in vitro* evidence that BDNF is critical to stress-induced increases in neurite extension, I next examined human clinical samples for BDNF and found a positive correlation between BDNF expression, increased nerve density, and overall poorer outcome. I next utilized a mouse orthotopic model of ovarian cancer and found that chronic stress increased nerve density in tumors taken from chronically stressed animals, and that this increase was a result of both neurogenesis and

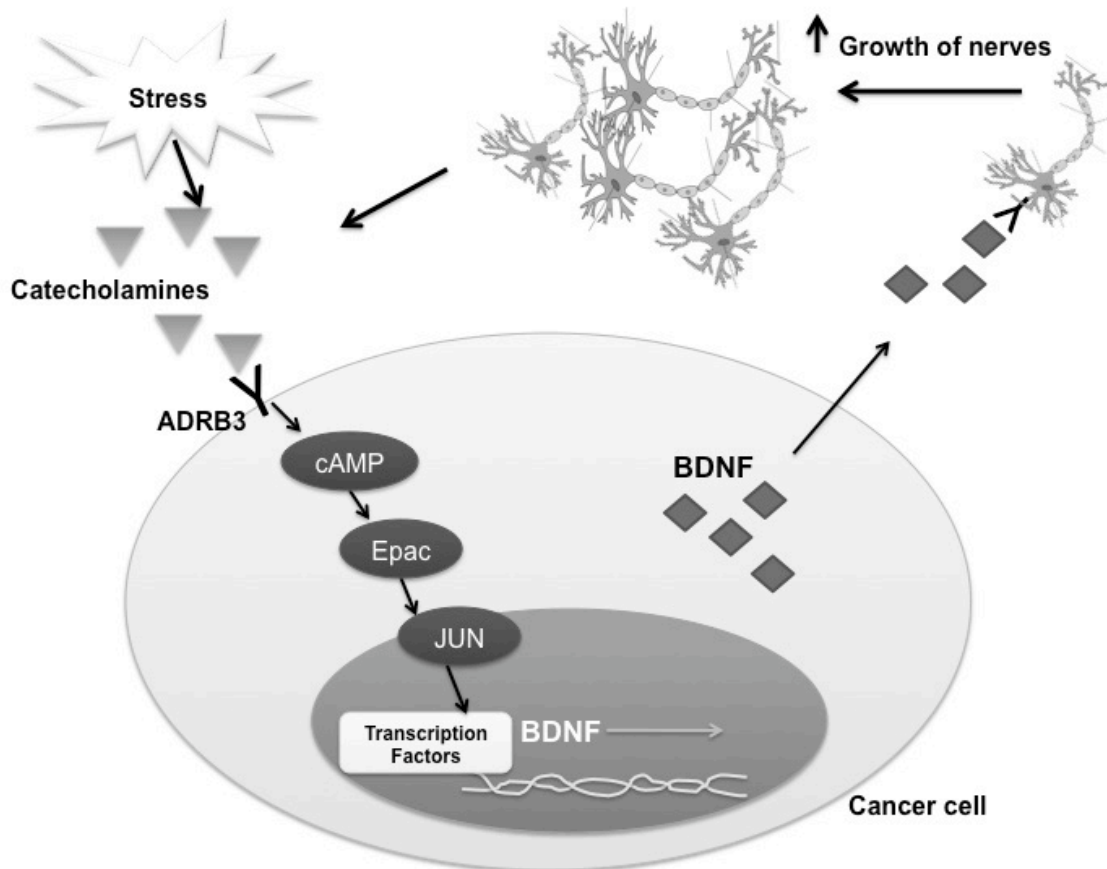


Figure 28. Overview model. Following a stressor, catecholamines are released which then bind to the β_3 adrenergic receptor on the tumor cell surface to initiate downstream signaling through cAMP and Epac. Activated Jun crosses the nuclear membrane to initiate transcription of BDNF gene expression. BDNF protein is produced and secreted from the cell where it binds TrkB receptors on sympathetic nerve endings. This activates growth and proliferation of neurons, increasing nerve density within the tumor. Increased nerve density results in higher catecholamine levels, resulting in a positive feedback loop in which nerves promote growth of the tumor through catecholamines, and tumor cells produce BDNF to support growth and survival of neurons.

axonogenesis. *In vivo* inhibition of BDNF abrogated chronic stress effects on tumor growth and nerve density. Using adrenalectomized models and *in vivo* administration of hexamethonium I demonstrated that chronic stress effects on tumor growth are mediated through sympathetic nerve endings, as opposed to systemic release of catecholamines from the adrenal gland, or by central nervous system control. hTrkB and mTrkB siRNA demonstrated that BDNF acts in a paracrine manner, stimulating nerve growth into tumors which, in turn, exert effects on tumor progression, rather than in an autocrine manner of self-activation of TrkB signaling on the tumor cells themselves. Taken together this work is the first to demonstrate stress-induced neurogenesis in tumors as well as the mechanism by which tumors may stimulate tumor neo-innervation.

2. Future Directions

A. TrkB Switch Kinase mouse model

In this work I have demonstrated a link between increased BDNF production by tumor cells and increased neo-innervation. Other studies have suggested that BDNF signaling may promote tumor growth in an autocrine fashion by activating TrkB receptors on the tumor cells. I utilized both human and mouse TrkB targeting siRNA to demonstrate that the growth advantage provided by this autocrine signaling is insignificant when compared to paracrine effects through increased

nerve density. However, further studies may be necessary to draw definitive conclusions on this effect. Due to the post-natal lethality of complete TrkB knockout, one model that may provide further insight into this mechanism is the TrkB SwitchKinase mouse model available from Taconic Farms (Hudson, NY). This model provides pure TrkB inhibition by inhibiting kinase function with a dietary additive that binds to genetically modified TrkB receptor. This is due to point mutations in the ATP-binding pocket of the kinase. Interestingly, this system is inducible and reversible, providing effective knockout of TrkB function without mortality of the animal (136). This model would extend our findings and provide support for our findings that, under conditions of chronic stress, BDNF exerts its effects through recruitment of nerves rather than signaling on tumor cells.

B. Other neurotrophic factors

In this study my primary focus was on the role of BDNF in promoting neo-innervation of tumors. However, other neurotrophic factors could also be playing a role. My preliminary studies that led us to choose BDNF were based on the critical role that NGF family members play in promoting growth and survival of neurons as well as the increase in gene expression following NE stimulation. I therefore did not consider the role that other neurotrophins may be playing in promoting neo-innervation of tumors at a basal level. Other groups and I have demonstrated that various types of tumor cells express numerous neurotrophins (130), however the role they play is, as yet, undetermined.

C. Other Neurotransmitters

Additional studies should also examine additional factors that nerves may be secreting that influence tumor progression. The work done in our lab has thus far focused primarily on the role of Norepinephrine in promoting tumor growth. Other groups have demonstrated an anti-tumorigenic role of dopamine (137). Neurons are capable of secreting several other factors which may influence tumor growth. For example, Substance P is well-known to be involved in anxiety and depression, and is responsible for controlling many aspects of inflammation and immunity (138-140). Additionally, several types of tumors express NK receptors, which bind Substance P (141-143). Binding of Substance P to NK receptors on tumor cells promotes cellular proliferation and metastasis (144). Acetylcholine, another neurotransmitter secreted by nerve endings, is also known to promote tumor growth (145). Its receptors are also found on many types of tumor cells, and may promote angiogenesis and epithelial to mesenchymal transition of tumor cells, a step associated with increased metastatic potential (146). Conversely, Dopamine, oxytocin, and GABA (gamma-amino butyric Acid) all have anti-tumorigenic effects. These are just a few of the factors nerves secrete that are known to affect tumor biology. In this study these factors were not considered, and future work should be done to examine their role in tumor neo-innervation.

D. Immune impact

In my experiments I used an athymic nude mouse model, with the exception of the adrenalectomized mouse model. These animals are immuno-deficient, and allowed me to eliminate the effects that the immune system may have in promoting or blocking tumor neo-innervation. While this allowed me to effectively study the role of stress and increased BDNF without the confounding addition of the various aspects of immunity, it also begs the question of what role these cells may be playing. For example, cells of the immune system produce high levels of pro-inflammatory cytokines such as IL-6, which can also act as a neurotrophic factor, promoting nerve growth and directional guidance (147-149).

E. Other models of chronic stress

Our lab and many other groups study the effects of chronic stress use the well-accepted restraint stress model of chronic stress. This model has been shown to affect behavior, immunity, endocrine function, neurological, and developmental processes. It causes activation of many pathways known to be involved in stress response systems, and is therefore a widely used and accepted model. There are, however, many other models of stress. Some groups utilize dozens of stressors, from cold, heat, and isolation, to swim, fear, and predator stress. Effects of these various stressors have yet to be studied in the context of tumor progression. Additionally, animals may adapt to repetitive administration of the same stressor, blunting the effects that may be seen if a different, randomized stressor were used

each day, and the time during which stress took place varied. Our studies have shown that stressing animals for a period greater than 3 weeks provides no further significant increase in tumor burden. Variations in stressors may in fact prove chronic stress can have much more substantial effects on tumor growth than current research has described.

F. Types of neurons found in tumors

In addition to the studies outlined here, several other aspects of this study may benefit from further investigation. For example, although the neurons identified were shown to be catecholaminergic, we have not yet investigated what type of neurons they are beyond this distinction. Neurons of the sympathetic nervous system can be afferent (sensory) or efferent (motor). Cancer patients frequently mention pain in the area of their tumor. While this could be due to the pressure put on surrounding tissues by a tumor, it may also indicate that some neurons within tumors may be sensory. This also begs the question of whether nerves in the tumors may be influencing CNS biology. Neuronal signals travel both to and from the CNS. Signals from the tumor may be capable of altering brain function and processes. By comparing brain regions of tumor- and non tumor-bearing animals we may be able to determine chemical or structure changes influenced by tumors.

G. Origin of neuronal precursors

My studies also demonstrated the presence of neuronal precursors in tumors. These exciting data suggest that neurogenesis may be taking place in developing tumors, in addition to axonogenesis of existing neurons. One question that remains to be answered, however, is from where these neuronal precursors are recruited. In adults, neural progenitor cells are thought to exist only within the CNS and bone marrow (150). As neural progenitors in the CNS are primarily located in the hippocampus and have only been shown to migrate to 5 mm through the Rostral migratory stream, it is unlikely that neurons in the tumor originate in the CNS. To provide an answer on whether these cells may be coming from the bone marrow, our group is using a fluorescent mouse model. These animals are C57/B6 mice which express RFP protein. The animals were irradiated and bone marrow transplants were done with marrow taken from GFP expressing donor mice. This model allows us to differentiate between native cells from the host mouse and cells derived from the bone marrow. Analysis will use IHC co-localization studies. Tumors will be analyzed for expression of neurofilament (nerve marker) or doublecortin (neuronal precursor marker) as well as GFP and RFP protein. Co-localization between either neurofilament or doublecortin and RFP would signify neurons originate from the host animal, most likely through axonogenesis of existing neurons. Conversely, co-localization between neurofilament or doublecortin and GFP would represent neurons developing from

bone marrow-derived progenitor cells, and would be, to our knowledge, the first evidence of adult neurogenesis taking place outside of the CNS.

3. Study impact in Cancer

This work provides a mechanistic link between observation that catecholamines are significantly increased locally in tumor tissue following chronic stress and how tumors are responsible for promoting this increase. Over the last several years there has been increasing interest into clinical impacts of chronic stress and potential treatment options. As previously mentioned, several epidemiological studies have shown that β -blockers may be a viable treatment option to improve patient care. Other groups are investigating the impact of psychological intervention and have demonstrated that this may also improve patient outcome. This work demonstrates the critical role that BDNF plays in promoting chronic stress effects on tumor progression. It therefore may serve as a viable target for improving patient outcome.

4. Study impact in other diseases

Chronic stress has long been associated with increased risk of cardiovascular disease. Several studies exist showing a correlation between sympathetic innervation and heart disease. Long QT syndrome has long been seen as a cause of sudden cardiac death and cardiovascular abnormalities (151). In patients for whom β -blockers are insufficient treatment, left cardiac sympathetic denervation is used to improve patient performance (151). Increased nerve density in various regions of the heart has been associated with several types of

arrhythmias (152). Experiments in dogs have demonstrated that dogs with atrial fibrillations have increased nerve density and sprouting in cardiac tissue (153). Increased cardiac nerve density has also been associated with poor or lacking recovery following myocardial infarctions (154). These studies suggest that innervation may also play a critical role in stress-promoted cardiovascular disease.

In humans, bowel tissues are some of the most highly innervated tissues in the body. This is largely a result of the high amount of control the parasympathetic nervous system has in controlling gut motility. Chronic stress has also been known to cause gastric diseases such as irritable bowel syndrome (IBS) (155). One study demonstrated that patients with IBS have increased nerve density in their colonic mucosa layer. Additionally, BDNF production is increased in the bowel of IBS patients (156). Taken together, these data suggest that BDNF expression may be important in promoting innervation in diseases besides cancer.

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Vita

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