THE INTERACTION BETWEEN BRAIN-DERIVED NEUROTROPHIC FACTOR AND SYMPATHETIC DYSFUNCTION IN PERIVASCULAR INFLAMMATION

by

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ABSTRACT

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Nerve growth factor (NGF) plays a role in sympathetic neuron integrity and survival. Brain-derived neurotrophic factor (BDNF) also has trophic effects on sympathetic neurons. We report here the serendipitous finding that co-treatment of hippocampus with BDNF and the NGF antagonist TrkA-Fc leads to perivascular inflammation and marked vasoconstriction. This effect is not observed with either reagent alone or in combination with other control proteins. Because NGF supports sympathetic neuron health, we tested the hypothesis that BDNF combined with sympathetic compromise caused this effect. Superior cervical ganglia were removed bilaterally with concurrent BDNF infusion into hippocampus. Perivascular inflammation was observed at 3 days, but not 12 days post treatment, when sympathetic terminals had receded, suggesting that the presence of these terminals was necessary for inflammation. Since sympathetic dysfunction may lead to compensatory overactivity of norepinephrine (NE) signaling, we co-infused BDNF with NE in the hippocampus and observed perivascular inflammation. In humans, sympathetic overactivity has

been reported in a variety of vascular diseases. Some of these diseases, e.g. primary Raynaud's, are not accompanied by serious inflammatory disease whereas others, such as scleroderma and systemic lupus, are. In order to test the interaction between sympathetic dysfunction and BDNF in inflammatory disease, we induced autoimmunity against sympathetic nerve tissue in autoimmune-prone animals. We found that animals with sympathetic autoimmune damage showed perivascular inflammation which was further enhanced by the presence of BDNF. We speculate that BDNF may contribute to the transformation of sympathetic dysfunction to inflammatory disease.

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LIST OF ABBREVIATIONS

ANS	Autonomic Nervous System
BBB	Blood-Brain Barrier
BDNF	Brain-derived Neurotrophic Factor
CNS	Central Nervous System
EAE	Experimental Autoimmune Encephalomyelitis
EC	Endothelial Cell
eNOS	Endothelial Cell Nitric Oxide Synthase
IDDM	Insulin Dependent Diabetes Mellitus
IL	Interleukin
MS	Multiple Sclerosis
NE	Norepinephrine
NFKB	Nuclear Factor KB
NGF	Nerve Growth Factor
nNOS	Neuronal Cell Nitric Oxide Synthase
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NPY	Neuropeptide Y
NT	Neurotrophin
PNS	Peripheral Nervous System
PSNS	Parasympathetic Nervous System
RP	Raynaud's Phenomenon

SCG	Superior Cervical Ganglion
SLE	Systemic Lupus Erythematosus
SMC	Smooth Muscle Cell
SNS	Sympathetic Nervous System
SSc	Scleroderma / Systemic Sclerosis
TH	Tyrosine Hydroxylase
VEGF	Vascular Endothelial Cell Growth Factor
α2-NA	α2-Noradrenergic Receptor
β-NA	β-Noradrenergic Receptor

Chapter 1: INTRODUCTION

Autoimmune diseases are debilitating illnesses that preferentially effect women between the ages of 30 and 50. Symptoms of autoimmunity vary widely and are accompanied by a multitude of biological abnormalities including alterations in neurotrophin levels, sympathetic dysfunction, vascular abnormalities, and enhanced inflammatory responses. It is unclear what factors contribute to these changes seen in vastly different systems, but it is likely that genetics and the environment play an important role. While there are theories identifying the origins of certain autoimmune diseases, evidence to support these theories is not conclusive. Traditionally, research on autoimmune disease has focused primarily on their immune abnormalities and not on other abnormalities accompanying them including sympathetic and vascular dysfunction. While autoimmune diseases are undoubtedly linked with immune dysfunction, recent evidence supports the possibility of complex interactions between the immune system, sympathetic nervous system, and vascular system in the development of autoimmunity. This dissertation focuses on the interactions between sympathetic nervous system dysfunction and brain-derived neurotrophic factor in mediating perivascular inflammation.

1. Neurotrophins

The neurotrophins (NTs) are a family of protein factors involved in neuronal growth, differentiation and support. Nerve growth factor (NGF) was the first neurotrophin discovered when tumors transplanted into chick embryos secreted a protein factor that promoted sympathetic neuron survival and

differentiation (Levi-Montalcini et al., 1951). At first it was unclear if this factor, NGF, acted only locally or was able to act over larger distances. Subsequent research demonstrated that NGF was a diffusible factor and did not require cell-cell contact to exert its trophic effects (Cao and Shoichet, 2003; Politis et al., 1982). This characteristic long-distance effect of NGF led to the theory that this protein was a target-derived neuronal growth factor. In other words, NGF was involved in guiding neurons to the appropriate location through end-organ derived release (Staeker et al., 1996; Elkabes et al., 1994; Shelton and Reichardt, 1984).

Subsequent to the discovery of NGF, homologous proteins were identified which also had trophic effects on neurons including brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) (Barde et al., 1982; Hohn et al., 1990; Maisonpierre et al., 1990; Berkemeier et al., 1991; Ip et al., 1992). The neurotrophins all bind to the low affinity receptor $p75^{NTR}$ (Johnson et al., 1986; Radeke et al., 1987; Teng and Hempstead, 2004). In addition, they bind to their high-affinity tryosine kinase receptors (trks). Tyrosine kinase receptors are a family of similar proteins that contain a ligand binding site, an intracellular tyrosine kinase domain which phosphorylates proteins on tyrosine residues, and a C-terminal end containg multiple tyrosine phosphorylation sites (Kaplan et al., 1991 b; Klein et al., 1991). NGF preferentially binds to trkA but BDNF and NT-4 also serve rarely as non-preferred ligands (Kaplan et al., 1991; Ip et al., 1992); BDNF and NT-4 bind trkB (Soppet et al., 1991; Squinto et al., 1991; Klein et al., 1992; Ip et al., 1992); NT-3 preferentially binds trkC but can

also bind readily to trkA and trkB (Soppet et al., 1991; Lamballe et al., 1991; Glass et al., 1991; Barbacid, 1993; Fig. 1).

1.1 Neurotrophin Receptor Function

Signaling through trk neurotrophin receptors occurs by the dimerization of specific trk receptors and autophosphorylation of the intracytoplasmic domain (Jing et al., 1992). This phosphorylation leads to the activation of pathways including MAPK (Erk), PI3K, PLC, and PKB/AKT (Sometani et al., 2002; Chen et al., 2005; Kelly-Spratt et al., 2002; Barnabe-Heider and Miller, 2003; Vetter et al., 1991; for review see Lee et al., 2002) which have been shown to inactivate pro-apoptotic pathways such as Bcl-2 (Pincelli et al., 1997) and lead to differentiation, neurite outgrowth and survival (Belliveau et al., 1997; Muller et al., 1997; Rabin et al., 1993; Hempstead et al., 1992). In addition to signaling through receptor phosphorylation, recent studies have shown that trk receptors, when bound to neurotrophins, form a complex that is internalized and retrogradely or anterogradedly transported along axon terminals (Senger and Campenot, 1993; von Bartheld et al., 1996; Bhattacharyya et al., 1997; Butowt and von Bartheld, 2001). Anterograde transport is the movement of substances from the soma to nerve terminals. In contrast, retrograde transport is the transport of receptor-ligand complexes from the synaptic region to the soma, and is important in mediating genomic responses to singnalling events.

Signaling through the p75 receptor occurs via interaction of the cytoplasmic domain with cytoplasmic interactors. The cytoplasmic domain of p75 is similar to the death-domain found in related receptors (Chapman, 1995;

Feinstein et al., 1995). Although it was believed that p75 could not signal via autophosphorylation, studies have shown that this receptor is capable of autophosphorylation and this signaling mechanism leads to downstream activation of NF-κB (Dobrowsky et al., 1994; Carter et al., 1996; Rao et al., 1995). Evidence has suggested that the $p75^{NTR}$ can also dimerize with the trk receptors to form heterodimeric receptors (Hantzopoulos et al., 1995; Huber and Chao, 1995), which may contribute to different downstream signaling cascades than those seen with homodimeric trk or p75 receptor binding. In addition, recent studies have shown that p75 can also interact with Sortilin (He and Garcia, 2004), a member of the Vps10p family of cell sorting receptors (Mazella, 2001; Nielsen et al., 2001), thus leading to differential downstream effects. Sortilin has recently been shown to be a ligand for the precursor forms of the neurotophins (Teng et al., 2005; Hempstead et al., 2006).

While signaling through the different neurotrophin receptors has acute effects on neurons and glia, these cells can also exhibit long-term changes in cellular function and gene expression. Gene transcription activated by trk receptors has been shown to be mediated by STAT5 (Klein et al., 2005), binding of CREB to target genes (Gaiddon et al., 1995), and upregulation of fos and subsequent AP-1 activation (Vidal et al., 2001; Seaman et al., 1996; Gaiddon et al., 1995). Interestingly, the binding of CREB to DNA has been shown to be dependent on nitric oxide (NO) pathways, and in particular, neuronally expressed NOS (Riccio et al., 2006), an important mediator of vasodilation.

1.2 Neurotrophin Receptor Localization

Neurotrophin receptors are widely expressed in the CNS (for review see Barbacid, 1994; Lessman et al., 2003) and the periphery (Sheard et al., 2002; Mu et al., 1993; Goettl et al., 2004; Hikawa et al., 2002), predominantly on neurons and glia (Pitts and Miller, 2000; Wang et al., 1998) though they can also be found on non-neural cells such as immune cells (Noga et al., 2002; Zhang et al., 2003), vascular smooth muscle cells (Nemoto et al., 1998), and endothelial cells (Kim et al., 2004). TrkA mRNA is highly expressed in the dorsal root ganglia (DRG), sympathetic ganglia and spleen, while trkB and trkC mRNA levels are high in DRG somato-dendritic membrane and axon terminals (Salio et al., 2005) as well as the spinal cord (Yamamoto et al., 1996). TrkB and trkC expression is widely distributed throughout the brain. Areas expressing trkB or trkC include olfactory formations, neocortex, hippocampus, thalamic and hypothalamic nuclei, brainstem nuclei, and cerebellum (Merlio et al., 1992; Masana et al., 1993).

Hippocampal neuronal cells are dependent on the trkB ligand, BDNF, for survival, connectivity and higher functioning including learning & memory and LTP (Minichiello and Klein, 1996; Martinez et al., 1998; Dragunow et al., 1997). Expression of trkB in the cortex has been shown to co-localize with its ligand, BDNF, in specific cortical layers (Pitts and Miller, 2000). In the adult, trkB expression is not limited to the full-length receptor which contains the intracellular signaling domain. TrkB is also expressed as a truncated form of the receptor which lacks the intracellular signaling domain (Middlemas et al., 1991; Valenzuela et al., 1993). Several theories about the function of truncated trkB

receptors have been suggested. One theory includes playing a modulatory role in neuronal plasticity by regulating neurotrophin concentrations in the hippocampus (Beck et al., 1993). Another function proposed for truncated BDNF is that it acts as an inhibitory modulator of neurotrophin responsiveness by forming non-functional heterodimers with full-length trkB receptors (Eide et al., 1996). In the forebrain, full-length trkB is expressed during development and the truncated form is not expressed until postnatal maturation (Fryer et al., 1996).

Similar to trkB, full-length trkC protein is expressed at low levels during development in the prefrontal cortex (PFC) but in adulthood, it is truncated trkC protein which is expressed (Beltaifa et al., 2005). Though the form of trkC expressed changes in the PFC, trkC mRNA is expressed throughout the lifespan (Beltaifa et al., 2005). Both trkC protein and mRNA have been found in PFC pyramidal and non-pyramidal neurons, while in PFC glial cells only trkC protein has been identified (Beltaifa et al., 2005), indicating transport of trkC protein from afferent structures.

In contrast to the wide expression of trkB and trkC in the adult brain, trkA has a more limited distribution. In particular, trkA expression is specific to the basal forebrain and hippocampus (Kordower et al., 1994; Venero and Hefti, 1993; Holtzman et al., 1992; Cellerino, 1996), though trkA expression has been shown in thalamic and hypothalamic structures as well (Venero and Hefti, 1993; Kordower et al., 1994).

In the CNS, p75 is differentially expressed in the cortex and cerebellum across development and into adulthood (Meinecke and Rakic, 1993; Chen et al.,

1994). $p75^{NTR}$ mRNA is expressed extensively throughout PNS tissues and in the spleen (Yamamoto et al., 1996; Schatteman et al., 1993) and is frequently found co-expressed with trk receptors (Sobreviela et al., 1994).

1.3 Neurotrophin Production and Transport

Mature neurotrophins are derived from proteolytic precursor molecules, pro-neurotrophins (Edwards et al., 1988; Seidah et al., 1996). Pro-neurotrophin synthesis occurs in a variety of cell types including neurons and glia (Hasan et al., 2003; Marcinkiewicz et al., 1999) and processing to the mature form is mediated by pro-hormone convertases, e.g. furin (Marcinkiewicz et al., 1999; Farhadi et al., 2000). Both NGF and NT-3 are usually released as the fully mature form (Barth et al., 1984; Gotz et al., 1992). However, studies have shown that both NGF and BDNF can be released as pro-hormones which, like the mature neurotrophins, can activate receptors (Mowla et al., 2001; Srinivasan et al., 2004). Recent studies have shown that receptor activation by pro-neurotrophins leads to neuronal or glial apoptosis (Srinivasan et al., 2004; Pedraza et al., 2005). In a study looking at SCG survival in culture, it was found that application of pro-BDNF to culture media led to a significant increase in cell death. Apoptosis of these cultured SCGs was dependent upon the interaction of pro-BDNF, not mature BDNF, with a p75-sortilin complex (Teng et al., 2005).

Injections of exogenous neurotrophins into the brain have elucidated receptor localization and transport of these molecules in the CNS. When NGF is injected into the lateral ventricle of a rat, the neurotrophin quickly circulates throughout the CSF and is also quickly cleared from the system (Anderson et al.,

1995). Subsequent labeling of NGF has shown that structures close to the ventricles retain NGF and, after a delay of several hours, distant structures such as the basal forebrain are positive for NGF (Anderson et al., 1995). Ventricular injection of exogenous BDNF revealed that BDNF does not penetrate as deeply into the parenchyma as NGF, most likely due to the high abundance of truncated trkB receptors located on the ventricular walls (Anderson et al., 1995).

Although neurotrophins do not penetrate the parenchyma deeply, evidence of exogenous neurotrophins could be found in distant brain regions. This supported the idea that neurotrophins were either retrogradely or anterogradely transported, or both. Studies have shown that NGF, injected into the hippocampus, is transported to brain regions with afferents to that structure including the basal forebrain (DiStefano et al., 1992), indicating retrograde transport. Indeed, even NGF administered in the periphery reaches the nervous system through retrograde transport. NGF given systemically is detected in the autonomic nervous system 4 hours after injection, indicating retrograde transport (Stoeckel et al., 1976). Similar mechanisms have been shown for BDNF, where injection of BDNF into the hippocampus led to positive BDNF staining in hippocampal efferents from structures including the basal forebrain and entorhinal cortex (DiStefano et al., 1992). Additional evidence for retrograde transport has been shown in studies where exogenous BDNF is injected into the eye of a chick and subsequently found in brainstem nuclei (vonBartheld et al., 1996). Studies using the axonal transport blocker colchicine have shown increased levels of BDNF in cortical regions and decreased BDNF levels in

cortical afferent connections disrupted by the drug, including the striatum (Altar et al., 1997). The above studies demonstrate that disruption of axonal transport can lead to subsequent changes in neurotrophin levels distal to the site of neuronal injury.

1.4 Brain-derived Neurotrophic Factor (BDNF)

BDNF has a complex three dimensional shape and is comprised of non-covalently associated homodimers (Bothwell and Shooter, 1977; McDonald et al., 1991). Mature BDNF is 50% homologous to mature NGF with the following common characteristics: (1) a signal peptide following the initiation codon (Maisonpierre et al., 1990a, 1991; Ip et al., 1992) (2) a pro-region including a proteolytic cleavage site for prohormone convertases, e.g. furin, followed by the mature sequence (Bresnahan et al., 1990; Seidah et al., 1996a).

BDNF has been localized to chromosome 11 in humans and chromosome 2 in mice (Maisonpierre et al., 1991; Ozcelik et al., 1991). The mature form is identical and the distribution pattern is highly conserved between species (Maisonpierre et al., 1991).

PNS expression of BDNF has been shown in dorsal root ganglia (Heppenstal and Lewin, 2001) and spinal cord (Yamamoto et al., 1996), as well as in target organs of sensory neurons including epithelial tissue (Buchman and Davies, 1993). BDNF has trophic effects on sympathetic neurons, although not as pronounced as NGF (Glebova and Ginty, 2004). BDNF has also been implicated as a trophic factor in a number of non-neuronal systems. The expression of BDNF, and its receptor Trk B, has been seen in tissues such as

the heart, muscle, inflammatory cells, and vasculature (Scarisbrick et al., 1993, Timmusk et al., 1993, Donovan et al, 1995, Hiltunen et al., 1996). BDNF has also been shown to be an endothelial cell survival factor (Donovan et al., 2000) which is important for proper vessel stability.

BDNF is widely expressed in the CNS, in both neurons and glia (Riley et al., 2004) and has been localized to the hippocampus (CA1, CA2, CA3 and dentate gyrus; Furukawa et al., 1998; Conner et al., 1997), most cortical areas (Eagleson et al., 2001; Conner et al., 1997), the hypothalamus (Conner et al., 1997), basal forebrain (Conner et al., 1997), striatum and amygdala (Conner et al., 1997).

In the developing PNS, BDNF has been shown to effect survival and growth of a variety of neurons including dorsal root ganglion cells (Acheson et al., 1995) and vestibular neurons (Huang and Reichardt, 2001), as well as proper functioning of these neurons. Perinatal BDNF -/- mice show reduced C-fiber responses in DRG electrophysiological preparations without concurrent changes in fiber number or synaptic density. This finding suggests that BDNF is responsible for normal fiber functioning and not proper anatomical placement or neuronal morphology in this system (Heppenstal and Lewin, 2001).

In the developing CNS, BDNF plays a significant role in the development of neurons in the visual cortex, hippocampus and other cortical areas (Huang and Reichardt, 2001). For instance, monocular deprivation in rats has been shown to decrease the expression of BDNF mRNA in retinal cells (Mandolesi et al., 2005). The functional outcome of monocular deprivation is the dominance of

cortical neurons by the open eye. This outcome of monocular dominance has been reversed by supplying exogenous BDNF (Mandolesi et al., 2005). In addition, exogenous BDNF can rescue retinal ganglion cells from apoptosis during development (Ma et al., 1998).

1.5 Nerve Growth Factor (NGF)

NGF has been localized to chromosome 1 in humans and chromosome 3 in mice (Francke et al., 1983; Zabel et al., 1985) and is highly homologous between species (Ullrich et al., 1983). Mature NGF is made up of non-covalently associated homodimers (Radziejewski et al., 1992) and contains three subunits which interact to form a complex. Within this complex, a 118 amino-acid sequence is responsible for the trophic effects of NGF on neurons (Ullrich et al., 1983).

In the developing CNS, NGF and both its receptors, TrkA and p75, are expressed in the basal forebrain cholinergic (CBF) system and its associated afferents: the hippocampus and cerebral cortex (Mobley et al., 1986; Auburger et al., 1987; Buck et al., 1988; Rossi et al., 2002); this expression persists into adulthood (Vazquez and Ebendal, 1991). Alternatively, other CNS areas which express NGF or its receptors or both, including the retina and cerebellum (Schatteman et al., 1988), show expression of these proteins until just before or shortly after birth (Schatteman et al., 1988). The role of NGF in CNS development has been studied using antibodies directed against the protein, delivered either systemically (Levi-Montalcini and Angeletti, 1966; Gorin and Johnson 1979) or locally (Li et al., 1995). It has been difficult for scientists to

study the role of NGF in the developing CNS using genetic techniques since disruption of NGF leads to a lethal phenotype. Deletion of specific NGF exons in mice allow for a short period of survival after birth (Crowley et al., 1994). NGF heterozygous mice (NGF +/-) however, survive and show only a minimal reduction in CBF neurons (Chen et al., 1997). Recent advances in technology have allowed the development of phenotypic knockouts, using neuronally produced antibodies against a specific protein, which circumvent the problems associated with lethal knockouts (Picciolli et al., 1991). Phenotypic knock out of NGF reveals a marked decrease in the number of cholinergic neurons in the basal forebrain and the hippocampus (Ruberti et al., 2000) though this phenomenon only manifests in adulthood and is not present during the post-natal time period (Ruberti et al., 2000). These data indicate more of a role for NGF in the adult CNS versus the developing CNS and is supported by functional deficits seen in NGF-deficient animals. Animals lacking NGF, showing decreased cholinergic neurons in the basal forebrain and hippocampus, show deficits in working memory and retention of spatially learned tasks (Rubertini et al., 2000).

NGF is more widely expressed in peripheral nervous system (PNS) target organs than in the central nervous system (CNS) supporting the idea that NGF is important for peripheral sympathetic nerve growth and differentiation during development. Mice lacking NGF exon IV show decreased sensitivity to pain and temperature, a functional outcome of decreased sensory and sympathetic ganglia, especially small neurons with unmyelinated or lightly myelinated axons (Crowley et al., 1994). Depletion of NGF during development leads to significant

decreases in the size of superior cervical ganglia and dorsal root ganglia in mice (Ruberti et al., 2000). Low levels of both NGF receptors, p75 and trkA, are expressed in trigeminal neuron axons as they migrate towards their targets (Davies et al., 1987). As the axons reach their targets, an increase in p75 and trkA mRNA is seen as well as an increase in the synthesis of NGF from target tissue (Davies et al., 1987). Survival of a subset of the trigeminal ganglia is dependent upon the presence of NGF (Buj-Bello et al., 1994). NGF, p75, and trkA are expressed in many target organs of the sympathetic nervous system (SNS) during development including the testis (Robinson et al., 2003), kidneys (Durbeej et al., 1993), and heart (Scarisbrick et al., 1993).

SNS development and survival is guided, in part, by neurotrophic factors. Nerve growth factor appears to be vital for sympathetic integrity even in the adult (Aloe et al., 2000). For example, experimentally-induced decreases in circulating NGF levels in adult animals lead to atrophy of sympathetic ganglia, reduced number of sympathetic neurons, decreased length of sympathetic dendrites, and decreased levels of norepinephrine in certain organs (Gorin and Johnson, 1980; Ruit et al., 1990). Unlike developing sympathetic neurons, adult sympathetic neurons are able to survive *in vitro* in the absence of NGF (Orike et al., 2001). Though no longer dependent on NGF for survival, adult sympathetic neurons show increases in neurite extensions and branching in the presence of NGF, mediated through the high-affinity trk receptor (Orike et al., 2001).

2. The Autonomic Nervous System

The autonomic nervous system (ANS) is involved in regulating the body's responses with respect to internal and external environmental factors, or maintaining homeostasis. There are three branches of the ANS including the sympathetic nervous system (SNS), the parasympathetic nervous system (PSNS) and the enteric nervous system. The former two branches, sympathetic and parasympathetic, are commonly known for their roles in "fight or flight" and "rest and digest" functions respectively.

The way in which the ANS maintains homeostasis is through a complex network of nerves which relay information about internal and external conditions from the periphery to the central nervous system (CNS). In turn, autonomic areas of the CNS coordinate proper responses of certain body systems to these local or global environmental factors. Autonomic regulation in the CNS is mediated by structures located in the brainstem, diencephalon, and telencephalon. Areas of importance within these larger central brain regions include the ventrolateral medulla (VLM), the nucleus tractus solitarius (NTS), the periaqueductal gray (PAG), the hypothalamus, amygdala, insular and prefrontal cortex. Signals from these central brain areas reach effector organs in the periphery, such as the pupils, lacrimal glands, sweat glands, salivary glands, heart, lungs, adrenal glands, intestines, bladder, and blood vessels, through peripheral nerves (Mosqueda-Garcia, 1996). Transmission of impulses through the ANS involves at minimum two neurons, or is disynaptically mediated. The first neuron is thinly myelinated and is called the pre-ganglionic neuron. The pre-

ganglionic neuron is located in the brainstem or spinal cord and synapses with one or more post-ganglionic neurons. Post-ganglionic cells are located outside the spinal column, are usually unmyelinated and synapse onto effector cells. The peripheral autonomic nerves are organized into three groups: the paravertebral ganglia, the prevertebral ganglia, and the terminal ganglia. Paravertebral ganglia are arranged in a segmented fashion along the vertebral column and comprise the sympathetic chain. Pre-ganglionic sympathetic nerves terminating in paravertebral ganglia synapse onto post-ganglionic fibers which mediate involuntary functions of structures above the diaphragm. Prevertebral ganglia are distal to the paravertebral ganglia and are not as organized as the latter. Pre-ganglionic fibers terminating in prevertebral ganglia synapse onto fibers which mediate involuntary functions of structures below the diaphragm. Terminal ganglia are located in close proximity to their effector organs and are parasympathetic in nature (Hamil, 1996; Fig. 2).

2.1 The Parasympathetic Nervous System

The parasympathetic nervous system (PSNS) is organized in such a way that central efferents innervate spinal ganglia predominantly with cholinergic input into the pre-ganglionic synapse. Post-ganglionic synapses on effector organs are also cholinergic in nature. While the major neurotransmitter in the PSNS is acetylcholine (ACh), other neuropeptides have been co-localized to parasympathetic cholinergic neurons including enkephalins in the pre-ganglionic neurons and vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY) in post-ganglionic neurons. The functional outcome of ACh release from post-

ganglionic neurons depends on the predominant cholinergic receptor in the target organ. The receptors typically found on effector organs are nicotinic (nAChR) and muscarinic (mAChR) cholinergic receptors, each with a variety of α and β subtypes, leading to distinct actions including pupil constriction, increased salivary secretion, bronchiole constriction, decreased heart rate, gastrointestinal stimulation, and vasodilation (Hamil, 1996).

2.2 The Sympathetic Nervous System

The sympathetic nervous system (SNS) has been classically regarded as the opposing system to the PSNS due to the fact that stimulation of the SNS leads to opposite effects of those discussed above including pupil dilation, reduced salivary secretion, bronchiole dilation, accelerated heart rate, inhibition of the gastrointestinal tract, and vasoconstriction. Though the effects of the SNS are opposite to those of the PSNS, there are some commonalities including the pre-ganglionic neurotransmitter, ACh, and the disynaptic connection between the CNS and effector organs. However, unlike the PSNS, the post-ganglionic neurotransmitter in the SNS is predominantly norepinephrine (NE), although other neuropeptides associated with the SNS include neuropeptide Y (NPY), dynorphin (DYN), enkephalins, and others depending on the target organ innervated (Hamil, 1996). Norepinephrine (NE), the major post-ganglionic neurotransmitter in the SNS, is released onto target organs which contain post-synaptic NE receptors (Zschauer et al., 1997), including lymphoid organs and blood vessels, by sympathetic nerve fibers (Elenkov et al., 2000; Flavahan et al., 2000).

2.3 Norepinephrine in the Sympathetic Nervous System

NE is a catecholamine which is synthesized in the cytoplasm of sympathetic neurons from the amino acid tyrosine. Tyrosine hydroxylase (TH) catalyzes the conversion of tyrosine into dihydroxyphenylalanine (DOPA), and is the rate-limiting step in catecholamine synthesis (Merrillis and Offerman, 1966). L-aromatic amino acid decarboxylase (dopa-decarboxylase, DDC) in the cytoplasm then converts DOPA into dopamine (DA), and dopamine-β-hydroxylase (DBH), located in vesicles, catalyzes the conversion of DA into NE (Musacchio and Goldstein, 1963; Goldstein and Musacchio, 1963). NE is stored in small dense-core vesicles located in varicosities of sympathetic neurons (Neuman et al., 1984). Vesicles produced near the Golgi apparatus of neurons reach sympathetic nerve terminals via anterograde axonal transport (Campenot et al., 2003). Release of NE can occur through both calcium dependent and independent mechanisms, resulting in exocytosis of NE containing vesicles and non-exocytotic release respectively (Noon et al., 1975; Sweadner, 1985). As with PSNS fibers, the effects of SNS fibers are dependent upon the receptor subtype in target organs, typically the distribution of α and β noradrenergic receptors (Chaudhry and Granneman, 1999; Townsend et al., 2004).

2.4 Sympathetic Neurons and Neurotrophins

The development and maintenance of sympathetic neurons is dependent, in part, on the neurotrophin family of growth factors including NGF, BDNF, NT-3, and NT4/5 (Lewin and Barde, 1996; Fritzch et al., 1997). Expression of neurotrophins in target organs ensures the proper interactions between the

sympathetic neuron and the specific organ. For example, the cardiovascular system expresses BDNF and NGF during development to guide noradrenergic neurons to their appropriate target organs (Scarisbrick et al., 1993; Hassankhani et al., 1995). Additionally, endothelial and vascular smooth muscle cells express both NTs and NT receptors (Nakahashi et al., 2000; Clegg et al., 1989; Angus et al., 1986; Aikawa and Akatsuka, 1990) leading to proper innervation and vasoconstrictive functioning of vasculature.

It is clear that the interaction between the sympathetic nervous system and neurotrophins plays an important role in maintaining normal vascular tone. Antagonists to neurotrophins lead to decreased investment of sympathetic terminals on target organs such as vasculature (Rush et al., 1997) while the addition of sympathetic agonists, such as NE, to certain cell types leads to decreases in neurotrophin levels including NGF (Peeraully et al., 2004). Additionally, alterations in sympathetic tone have been shown to result in increased blood pressure (Rossoni et al., 2003).

3. Neurogenic Control of Vasculature

One function of the ANS which is relevant to the work discussed in this dissertation is its role in regulating vascular tone. The ANS regulates vascular tone in both the periphery and the central nervous system, although autonomic input is not the only way in which vascular homeostasis is maintained. Other influences on vascular constriction and dilation include chemical, hormonal, and cellular mechanisms. Vasodilation and vasoconstriction are important physiological responses to such factors as changes in metabolic demand,

changes in oxygen levels, temperature fluctuations, postural adjustments, and stress (Crowley, Jr. and Franchini, 1996).

3.1 General Properties of Blood Vessels

Peripheral arteries, arterioles and veins are comprised of endothelial cells (ECs), basement membrane, and smooth muscle cells (SMCs). The smooth muscle investment is denser in arteries than veins (Fig. 3). Capillaries consist of a single layer of endothelial cells and lack smooth muscle cells associated primarily with arteries and arterioles. Normal arterial vascular tone is determined by intrinsic properties of vascular smooth muscle cells as well as additional neural and hormonal inputs. Blood vessels, arteries in particular, are heavily innervated by autonomic fibers. As autonomic fibers approach blood vessels, they display a beaded morphology, known as varicosities, and in general do not form a typical synapse. In fact, autonomic neurons are relatively far from their effector cells, smooth muscle or endothelial cells (20-2000 nm) in comparison to other neuro-effector cell distances (Crowley, Jr. and Franchini, 1996).

In the periphery, sensory organs such as arterial baroreceptors, detect changes in blood pressure (e.g. decreases in blood pressure occurring when changing from a lying down position to an upright position) and send signals to central autonomic nuclei. The brainstem autonomic nuclei can then coordinate vasoconstriction in order to prevent postural hypotension and syncope upon standing (Dampney, 1994; Singewald and Philippu, 1996; Aicher et al., 2000; Dampney et al., 2002; Stauss, 2002). Autonomic ganglia relevant to this body of work include the superior cervical ganglia (SCG) which provide sympathetic input

into brain vasculature (Tsai et al., 1985; Edvinsson et al., 1978; Rosendorff et al., 1976).

3.2 Special Characteristics of Cerebral Vasculature

The anatomy of a blood vessel in the periphery is quite different from that of a blood vessel in the brain. General capillary anatomy is comprised of fenestrated endothelial cells containing numerous mitochondria and a basement membrane. These peripheral endothelial cells also contain contractile proteins which respond to substances such as histamine, serotonin, and NE, changing capillary permeability (Meryrick and Brigham, 1984; Ikeda et al., 1999; Majno et al., 1969; Bottaro et al., 1986). Functionally, these capillaries allow exchange of substances between plasma and tissue through pinocytosis and inter-endothelial spaces. In contrast, capillaries in the brain are much more restrictive to substance exchange due to the presence of the "blood-brain barrier" (BBB), except in circumventricular organs which are more permissive. The BBB is restrictive because brain microvascular endothelial cells are connected by tight junctions, have lower pinocytotic capabilities, fewer fenestrations, lack contractile protein, and are surrounded by astrocytic endfeet (Parent, 1996, chap. 1; Fig. 4) which further restrict molecular exchanges. Typically, astroglial endfeet are in close contact with the vessel wall and are enclosed within vascular basement membrane (White et al., 1981). Autonomic innervation of brain vasculature can occur via neuronal-endothelial cell interactions (Mitchell and Harris, 1981). However, it is more common for neuronal fibers to synapse on astrocytes surrounding and contacting endothelial cells (Chedotal et al., 1994; Cohen et al.,

1997). In cases where smooth muscle cells surround endothelial cells, there is evidence that autonomic fibers directly innervate SMCs (Frank et al., 2003; Marron et al., 1996; Kotecha and Neild, 1995). Vasoactive substances released from autonomic neurons, as well as other types of cells including interneurons (Tong and Hamel, 2000; Cauli et al., 2004) and astrocytes (Paulson and Newman, 1987), can elicit either vasoconstrictive or vasodilatory effects based on receptor subtype and location of brain vasculature (Angus and Korner, 1977; Su and Kubo, 1984).

3.3 Contsrictor and Dilatory Responses to Vasoactive Substances

Typical vascular responses include NE-mediated constriction through the α2-noradrenergic (α2-NA) receptor, NPY-mediated constriction through its Y1 receptor, serotonin (5-HT)-mediated constriction through the $5HT_{1B}$ receptor (as cited in Hamel 2006), and endothelin (ET-1)-mediated constriction through the endothelin-A (ETA) receptor (MacLean et al., 1994). Vasoconstriction mediated by the α2-NA receptor has been shown to function through the G_i pathway (Spitzbarth-Regrigny et al., 2000). Binding of NE to its α2-NA receptor leads to PI3K and PKC activation (Yamboliev and Mutafova-Yambolieva, 2005), resulting in vasoconstriction (Fig. 5). Since the release of NE from sympathetic varicosities can travel some distance from the site of release, it can bind to its receptors located on other cell types that reside in the area including immune cells (Hasko et al., 1995), providing further support for the interaction between the sympathetic nervous system, vascular system, and immune system.

Vasodilation has been shown to be mediated through the β-NA receptor, the gaseous neurotransmitter nitric oxide (NO), Ach activation of the M5 receptor, VIP activation of VPAC1, and prostaglandin E_2 (PGE_2) binding to its receptor EP4 (as cited in Hamel 2006). Some of these vasoactive receptors have been localized to different cell types comprising the neurovascular interface including endothelial cells, smooth muscle cells, and astrocytes (Cohen et al., 1996). Vasodilation mediated by the β-noradrenergic (β-NA) receptor has been shown to function through multiple pathways. One mechanism of vasodilation involves the binding of NE to its β-NA receptor which leads to hyperpolarization of smooth muscle cells, presumably through the factor known as endothelial derived hyperpolarizing factor (EDHF), by decreasing intracellular calcium levels (Bolz et al., 1999; Fig. 5). Cholinergic vasodilation is also mediated by the activation of endothelial nitric oxide synthase (eNOS; Meng et al., 1996; Kullo et al., 1997) which converts L-arginine into nitric oxide (NO; Palmer et al., 1988). NO then diffuses from the EC into neighboring SMCs (Gold et al., 1990) and increases intracellular cyclic guanosine monophosphate (cCMP; Meng et al., 1998), causing smooth muscle relaxation (Ignarro, 1991; Faraci and Heistad, 1998; Fig. 5). Cholinergic vasodilation can also occur through the arachadonic acid pathway (AA) which utilizes cyclooxygenase-1 to convert AA into PGI_2. PGI_2 in turn increases intracellular cyclic adenosine monophosphate (cAMP) in SMCs, resulting in relaxation (Ignarro et al., 1985).

4. Neurogenic Control of the Immune Response

One major function of the vascular system is to deliver nutrients and remove waste from cells all over the body, while another important function is communication between body systems. Two classes of blood cells flow through the vasculature: red blood cells (RBCs) and white blood cells (WBCs). RBCs are involved in oxygen and carbon dioxide transport, while WBCs use the vasculature as a conduit to deliver immune signals between affected areas and effector organs. The activity of WBCs can be modulated both by the vasculature and the nervous system.

4.1 Immune Cells

The function of the immune system is to mount an appropriate response to insults against the body, whether introduced by internal or external factors. Immune cells originate in the bone marrow and, after maturation, circulate throughout the blood and lymphatic system. There are several different types of immune cells, all of which subserve different functions in the immune response.

Immune cells derived from a common myeloid progenitor include monocytes/macrophages, mast cells, and granulocytes. Monocytes continually circulate in the blood and differentiate into macrophages after extravasation into tissues, where they act as one of the two types of phagocytic cells in the immune system. Mast cells also differentiate in tissue, residing mainly around small blood vessels, and release substances affecting vascular permeability (Janeway, 1999, chap. 1). Polymorphonuclear leukocytes, also known as granulocytes, include neutrophils, eosinophils, and basophils, and all contain densely staining granules

in their cytoplasm. Of the three, neutrophils are the most important and numerous cellular component of the innate immune response since they are often the first effector cells to arrive at the site of insult. In addition to macrophages, neutrophils are the second phagocytic cell of the immune system (Janeway, 1999, chap. 1).

Immune cells derived from a common lymphoid progenitor cell include T cells and B cells. T cells originate in the bone marrow but travel to the thymus for maturation, as opposed to B cells which fully mature in the bone marrow. T cells differentiate into two main classes after activation: cytotoxic T cells which mediate cell death and T cells which activate other cells such as B cells and macrophages (Janeway, 1999, chap. 1). B cells, when activated, differentiate into plasma cells which secrete antibodies.

4.2 Leukocyte Activation and Extravasation

Under normal physiological conditions, leukocytes, or white blood cells (WBCs), circulate through the blood in a state of inactivation until they are signaled to mount an inflammatory response. In addition to circulating immune cells, there are inflammatory cells which normally reside in tissue including microglia, the resident brain macrophage (Giulian, 1987). Several mechanisms which can activate leukocytes include signaling of cytokines such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α (TNF-α; Farrar et al., 1980; Luger et al., 1989; Larrick et al., 1987); and neurotransmitter or hormone release (Livnat et al., 1987; Paavonen et al., 1981). An important system which works in conjunction with leukocytes to direct them to the area of insult is the circulatory

system. Endothelial cells lining blood vessels are able to initiate leukocyte rolling and sticking of immune cells to the vessel surface, enabling them to extravasate from blood circulation into affected tissue (Janeway, 1999, chap. 10; Fig. 6). These interactions are mediated through a variety of cell adhesion molecules including proteins from the selectin family, the immunoglobulin superfamily, and the integrins. The initial sticking and rolling of WBCs along vessel walls is mediated by interactions between e-selectin and p-selectin on the EC surface with glycoproteins on leukocyte surfaces (Olofsson et al., 1994; Kanwar et al., 1995). Firm adhesion of WBCs is mediated, in part, by inter-cellular adhesion molecule-1 (ICAM-1) expressed on ECs which binds with leukocyte-function associated antigen-1 (LFA-1) expressed on leukocytes (Yoong et al., 1998). Trans-endothelial migration of WBCs, also known as diapedesis, is then accomplished by platelet endothelial cell adhesion molecule (PECAM; CD31), localized to both endothelial cell junctions and leukocyte surfaces (Albelda et al., 1990; Watt et al., 1993), pulling the WBC between ECs and breaking down surrounding basement membrane (Muller et al., 1993), allowing the entry of WBCs into affected tissue.

4.3 Autonomic Regulation of the Immune System

The inflammatory response is tightly regulated by local and global release of pro-inflammatory substances into the circulation. In addition to this humoral control of inflammation, the central nervous system can also influence the expression of immunity through the autonomic nervous system (Tracey, 2002; Elenkov et al., 2000; Pavlov et al., 2003). Autonomic efferents not only innervate

target organs such as blood vessels but also innervate immunomodulatory organs and cells including the thymus and tissue macrophages (Pavlov and Tracey, 2004). Activation of sympathetic fibers can cause suppression of pro-inflammatory cytokine production, mediated by β-NA receptors (Farmer and Pugin, 2000). Stimulation of the vagus nerve can also inhibit inflammatory responses through activation of α-nACh receptors (Wang et al., 2003). In addition to centrally mediated anti-inflammatory effects, CNS mechanisms can also enhance the inflammatory response. For example, sympathetic activation can lead to α2-NA receptor mediated increases in TNF synthesis, leading to pro-inflammatory downstream effects (Elenkov et al., 2000; Hasko and Szabo, 1998). Though the inflammatory effects of CNS activation could be mediated by indirect activation of immune cells, there is evidence to support direct interactions between sympathetic fibers and leukocytes. For instance, both α- and β-NA receptors have been identified on immune cells such as macrophages, on which catecholamines can exert a direct effect *in vitro* (Spengler et al., 1990; Kees et al., 2003; Ali et al., 1994).

Although the immune system does not develop until after birth, it is still dependent on the neurotrophin-mediated development of the sympathetic nervous system. The major organ of the immune system, the thymus, is heavily innervated by sympathetic nerves which follow the path of vasculature supplying the organ (Williams et al., 1981). Animals in which innervation of the thymus has been interrupted show decreased development of T-lymphocytes (Kasahara et al., 1977; Li et al., 2004). T-lymphocytes express receptors for NE (Sanders et

al., 1997) as well as neurotrophic factors and their receptors, including BDNF and trkB (Rost et al., 2005; Vega et al., 2003; Noga et al., 2002).

Results such as these indicate that the sympathetic nervous system and the immune system have the capability to modulate one another as a result of changes to either system independently.

5. Neurotrophins and Immune Regulation

BDNF has the potential to play a role in the immune response, as indicated by its expression on several different inflammatory cell types (Kerschensteiner et al., 1999) such as macrophages (Rost et al., 2005). Neurotrophin receptors have been found in key immune organs, such as the spleen and thymus, as have mRNAs for the neurotrophins themselves (Vega et al., 2003). Inflammatory cells, such as monocytes and macrophages, express TrkA, TrkB, and TrkC as well as NGF, BDNF, NT-3,and NT-4/5 (Rost et al., 2005; Vega et al., 2003; Noga et al., 2002). During T cell development the trkB receptor is expressed and is inversely related to degree of maturation stage. That is, expression of trkB declines as T cells begin to differentiate into mature forms (Maroder et al., 1996). After maturation, T cells constitutively express the truncated trkB receptor (Besser and Wank, 1999). The release of NGF and BDNF leads to changes in cytokine profiles of blood mononuclear cells and T cells (Bayas et al., 2003). Expression of these sympathetic mediators on inflammatory cells supports the idea of cross-talk between the sympathetic nervous system and the immune system.

While both the sympathetic nervous system and the immune system function independently and in response to independent factors, there is a complex interaction between the two systems that may contribute to disease states, such as immune disorders, in which both systems are dysregulated.

6. Immune Disorders

In order for the immune system to function properly, it must be tightly regulated by autocrine and paracrine mechanisms. If any of these feedback mechanisms become dysregulated, i.e. impaired or enhanced, it can lead to immune dysfunction. Of particular importance to this work is the development of autoimmunity. Under normal circumstances, the immune system destroys inflammatory cells which recognize cellular material originating in the body, designated "self-antigens." Autoimmune disease occurs when an immune response is mounted, in error, against self-antigens. The origins of many autoimmune diseases are unknown, though evidence has emerged showing viral or cell-damage mediated initiation of autoimmunity (Flaegstad et al., 1988; Moens et al., 1995; Itoh et al., 1993). Some common autoimmune diseases in which mechanisms of immune dysfunction have been identified include insulin-dependent diabetes mellitus (IDDM), multiple sclerosis (MS), and systemic lupus erythematosus (SLE).

6.1 Autoimmunity – T Cell Mediated Diseases

Patients with IDDM produce T cells which attack β-islet cells of the pancreas, leading to insulin deficiency (Atkinson and Maclaren, 1993). MS has been shown to have a similar mechanism whereby T cells attack the myelin

sheath encircling neurons, eventually leading to impaired signaling and paralysis (Noseworthy, 1999). Theories about the origin of MS have mainly focused on viral infections where viral antigens mimic human self-antigens, leading to erroneous T cell recognition of myelin protein as a viral antigen (Dandekar et al., 2001). Recently however, a new theory about the origin of MS has been put forth consisting of non-immune mediated neuronal dysfunction initiated by dysregulation of intracellular molecular transport (Tsunoda and Fujinami, 2002).

6.2 Autoimmunity – Immune Complex Mediated Diseases

In contrast to IDDM and MS, SLE is not mediated by T cell destruction of tissue. Instead, SLE is an autoimmune disease in which antibodies are produced against self-antigens including common intracellular molecules such as DNA, histones, and ribosomes (Swaak and Smeenk, 1987; Rubin and Waga, 1987; Fritzler et al., 1982; Reichlin et al., 1999). After these antibodies recognize antigens in the body, they form an immune-complex which can then be deposited in key organs including the kidneys, blood vessels, and joints, eventually leading to dysfunction such as glomerulonephritis, vasculitis, and arthritis (Gilboa et al., 1977; Napirei et al., 2000; Ansari et al., 1986). Another disease in which there is some evidence of autoimmune etiology is systemic sclerosis or scleroderma (SSc). Scleroderma is a relatively rare disease that primarily affects women (Schuna, 2002). According to the American College of Rheumatology, early changes seen in patients with SSc include immune system activation, affecting the vasculature, which may eventually lead to skin, lung and other organ dysfunction.

6.3 Diagnosis of Autoimmune Disease - Scleroderma

SSc is difficult to diagnose because there is no definitive autoantibody which determines the disease. Though not seen in all SSc patients, typical autoantibodies that have been detected include anti-scl70 (anti-topoisomerase1) and anti-dsDNA, among other antibodies against self-antigens (Kato et al., 1993; Kubota and Kanai, 1989; Renaudineau et al., 2001). Therefore other symptoms must accompany autoantibodies in order for a patient to be diagnosed with SSc. One common feature which is used to help identify patients with SSc is impairment in blood flow to the digits (Keberle et al., 2000), which typically manifests as Raynaud's Phenomenon (RP; LeRoy et al., 1971; Fitzgerald et al., 1988). RP is a disorder characterized by vasospastic attacks in the extremities in response to cold or emotional stress (Whitaker and Kelleher, 1994), and is classified as primary Raynaud's if the patient has no accompanying disease, or secondary Raynaud's if the patient has another concurrent disease such as SSc or SLE.

6.4 Autonomic Dysregulation in Autoimmunity

Patients with Primary Raynaud's show increased sympathetic tone (Olsen et al., 1987), which is hypothesized to play a role in the pathophysiology of this disease. This sympathetic overactivation is in contrast to patients with Secondary Raynaud's, due to Systemic Sclerosis, who show hypotonic activity during orthostatic stimulation (Pancera et al., 1999). Patients with other autoimmune diseases, including SLE and IDDM, also show dysregulation in autonomic function. For example, patients with SLE show reduced heart rate

variablility and a larger drop in systoic blood pressure upon standing, compared to controls (Laversuch et al., 1997; Liote and Osterland, 1994). IDDM patients show decreased vasoconstrictor responses to cold-stress tests (Wilson et al., 1992), indicating impairment in blood flow regulation. Dysfunction of the sympathetic nervous system is thought to contribute to vasospasms associated with cold exposure and stress in Primary Raynaud's (Ho and Belch, 1998). Vessel spasms, especially in such areas as the digits, ears, and nose are associated with focal ischemia and can lead to ischemia-reperfusion injury of local endothelium (Generini et al., 1996). Blood vessels in scleroderma patients do not function properly due to endothelial cell damage which would impair endothelium-dependent vasodilation (Schlez et al., 2003). Patients with scleroderma have increased arteriole constrictor responses of the α2-adrenoreceptor, the NE receptor on vascular endothelium (Flavahan et al., 2000). Although SSc patients exhibit decreased sympathetic activity, their noradrenergic receptor responses are increased. It may be that disease symptoms are more related to receptor function than dysregulation in sympathetic activity per se.

6.5 Pathology Associated with Sympathetic and Vascular Dysregulation

One major pathology of vasospasms, or dysregulated constrictor and dilator response, is ischemic damage which, if left untreated, can lead to gangrene. As vessels constrict in the afflicted extremities, they become hypoxic and angiogenesis occurs. This angiogneic effect is mediated by vascular endothelial cell growth factor (VEGF) which is upregulated in low oxygen

environments (Schoch et al., 2002 and Howell et al., 2003). VEGF-mediated angiogenesis causes edema, extravasation of inflammatory molecules, and fibrous leak (Dobrogowska et al., 1998). VEGF has been implicated as a pro-inflammatory cytokine (Croll et al., 2004; Ishida et al., 2003) causing upregulation of inflammatory molecules such as inter-cellular adhesion molecule-1 (Miyamoto et al., 2000). Besides the upregulation of VEGF through hypoxia, studies have also shown that both BDNF and NGF can stimulate the production of VEGF in neuroblastoma cells and peripheral sensory neurons, respectively (Nakamura et al., 2006; Calza et al., 2001).

Primary Raynaud's may precede the onset of autoimmune diseases such as SSc and SLE by many years, but it can be distinguished from Secondary Raynaud's by some hallmark features. The presence of antinuclear antibodies (ANA) and increased levels of endothelial injury byproducts (Ho and Belch, 1998) usually appear when autoimmune disease is present. Additionally, patients with secondary disease show increased white blood cell trans-endothelial cell migration and activation around affected areas (Prat et al., 20002; Lau et al., 1992a; Lau et al., 1992b), reduced fibrinolysis (Ho and Belch, 1998), and diffuse tissue fibrosis (Wigley, 1996). Fibrosis, or the formation of fibrous tissue within an organ, can be caused by many factors including injury, inflammation, and infection. Accumulation of fibrous tissue within an organ, independent of etiology, can eventually lead to organ dysfunction and even organ failure (Intengan and Schiffrin, 2001). Patients with RP secondary to autoimmune diseases show fibrotic damage to many different organs including lung, kidney,

skin, and vasculature (Herbai, 1978; Intengan and Schiffrin, 2001; De Heer et al., 2000).

6.6 Vascular Abnormalities in Autoimmune Diseases

Studies have begun to identify evidence of vascular abnormalities or injury in patients with autoimmune diseases, which may be useful in diagnosing autoimmune disease even before overt signs of autoimmunity are present. For instance, patients with RP secondary to SSc show endothelial cell damage and increased endothelial cell antibodies (Hebbar et al., 1997; Negi et al., 1998). These same patients also have impaired vasodilation, increased arteriole constrictor responses of the α2-adrenoreceptor, and show a loss of EC dependent vascular tone (La Civita et al., 1998; Keberle et al., 2000; Flavahan et al., 2000; Livi et al., 2001). In addition to signs of vascular dysregulation and injury, SSc patients also show elevated levels of VEGF, ICAM-1, endothelin-1, e-selectin, and NOS (Kuryliszyn et al., 2005; Anderson et al., 2002; Del Papa et al., 2004), some of which are associated with VEGF-induced angiogenesis (Miyamoto et al., 2002; Croll et al., 2004). Despite the increased expression of VEGF and ICAM-1 in scleroderma, patients typically show a lack of newly formed vessels (Konttinen et al., 2003), capillary abnormalities (Scheja et al, 1996; Kabasakal et al., 1996), and evidence of ischemia in affected areas (Kaye et al., 1994).

7. Neurotrophins and Autoimmunity

In addition to vascular injury by-products in autoimmune patients, evidence of altered neurotrophin levels has also been detected.

7.1 Nerve Growth Factor (NGF)

In the experimental form of multiple sclerosis, experimental allergic encephalomyelitis (EAE), an upregulation of NGF and p75 mRNA has been seen in the central nervous system, as well as an increase in NGF protein in the brain (De Simone et al., 1996; Micera et al., 1998). In addition to NGF, an upregulation of the NGF receptor, trkA, has also been seen in astroglia in the spinal cord of rats during the acute phase of EAE (Oderfeld-Nowak et al., 2003). In patients with MS, NGF levels are elevated in cerebral-spinal fluid (CSF; Laudiero et al., 1992) and its low-affinity receptor, p75, is upregulated in glial cells during plaque formation (Dowling et al., 1999). A proposed role for nerve growth factor in the etiology of multiple sclerosis is that overexposure to NGF during development can result in dysfunctional sympathetic vascular innervation, leading to vascular dysregulation, ischemia, and subsequent neuronal damage (Olsen, 1998). Altered NGF levels have also been seen in systemic lupus erythematosus (SLE) and scleroderma (SSc) whereby patients have higher serum levels of NGF compared to controls or within specific subtypes of the particular autoimmune disease (Aalto et al., 2002; Bracci-Laudiero et al., 1993; Matucci-Cerinic et al, 2001). Additionally, circulating levels of NGF in SLE patients correlate with disease severity (Aalto et al., 2002).

7.2 Brain-Derived Neurotrophic Factor (BDNF)

BDNF, in addition to NGF, may play a role in autoimmunity. In EAE rats and in mice immunized against myelin proteolipid protein, T-cells reactive to myelin basic protein express BDNF mRNA (Muhallab et al., 2002) and B-cells

express BDNF protein, respectively (Edling et al., 2004). Patients with relapsing-remitting MS show increases in BDNF mRNA in circulating white blood cells compared to healthy subjects (Gielen et al., 2003). In addition to increased BDNF mRNA, white blood cells in MS patients also show increases in BDNF protein levels which correlate with disease state (Sarchielli et al., 2002). In the brain, neurons and reactive astrocytes surrounding MS plaques express the truncated form of the BDNF receptor, trkB, while the number of immune cells immunopositive for BDNF correlate with demyelinating activity (Stadelmann et al., 2002).

8. The Sympathetic Nervous System, Immune System, and Neurotrophins

Several disease states, including Raynaud's phenomenon, show abnormal sympathetic function (Olsen et al., 1987). Dysregulation of vascular tone, due to sympathetic dysfunction, can lead to abnormal dilation and constriction of vasculature (vasospasms). These vasospasms have been shown to contribute to local tissue damage including upregulation of inflammatory markers such as ICAM-1, extravasation of white blood cells (WBCs) and deposition of proteins into local tissue (Ogawa et al., 2000; van Laar and Tyndall, 2003; Kahaleh, 1990). Abnormal inflammatory activity is hallmark in certain connective tissue diseases including Scleroderma (SSc) and Lupus. Raynaud's phenomenon is one of the primary symptoms of connective tissue diseases such as lupus or, most prominently, scleroderma. Patients with RP secondary to SSc show increased levels of soluble inflammatory markers including ICAM-1 and e-selectin (Brevetti et al., 2000), and evidence of vascular abnormalities (Houtman

et al., 1986) including immune-mediated destruction of endothelial cells (Kahaleh et al., 1979). As a long-term consequence of vascular damage, endothelium-dependent vasodilation is often severely compromised (Schlez et al., 2003), further worsening vasoconstriction. As a result of RP, vessels constrict in the afflicted extremities and become ischemic, potentially leading to the blood vessel damage observed in SSc patients. Experimentally-induced ischemic conditions leads to increases in NE levels in key immune organs (Sperlagh et al., 2000). Ischemia-reperfusion injury has also been shown to cause migration of inflammatory cells into affected tissue (Hawkins et al., 1996), subsequently leading to severe tissue damage (Berlanga et al., 2002).

In addition to vascular injury and dysregulation, ischemia has been shown to upregulate neurotrophic factors such as BDNF (Tokumine et al., 2003; Zeng et al., 2001; Stadelmann et al., 2002; Miyake et al., 2002; Ferrer et al., 2001). It is unclear if the release of these neurotrophic factors is neuroprotective or not under pathophysiological conditions, however, upregulation of these factors could lead to an increase in inflammation surrounding the damaged area. Levels of BDNF and NGF are higher in patients with autoimmune disease (Petereit et al., 2003; Oderfeld-Nowak et al., 2001) and therefore, they may be an important link in either the pathogenesis or expression of inflammatory responses in such diseases.

Chapter 2: SPECIFIC AIMS

It is clear that there is a complex relationship between the sympathetic nervous system, neurotrophic factors, the immune system, and vasculature. However, the precise mechanism of these interactions is not understood. Previous data collected in our laboratory showed the unexpected result that BDNF and the NGF inhibitor, trkA-Fc, when co-infused into the hippocampus, caused perivascular inflammation. We were interested in whether or not the interaction between BDNF and NGF inhibition could explain the complex inter-relationship between neurotrophins, inflammation, and subsequent vascular changes.

Specific Aim 1: To characterize the vasculature, its sympathetic innervation, and BBB compromise, if any, in animals given BDNF + TrkA-Fc.

Signaling of the neurotrophins occurs through dimerization of the high-affinity receptors. Neurotrophin receptor body traps consist of two ligand-binding domains of specific trk receptors held together by the Fc portion of human IgG1 (Glass et al., 1996). TrkA-Fc is designed to act as a false receptor for NGF and therefore prevent binding of the neurotrophin to endogenous receptors. Both BDNF and the trkA ligand, NGF, enhance survival and growth of neurons (Achesone et al., 1995; Levi-Montalcini, 1951). Removing NGF from circulation can lead to atrophy of sympathetic ganglia (Gorin and Johnson, 1980) as well as an increase in experimentally induced inflammation (Reinshagen et al., 2000). In addition to its role as a neurotrophic factor and anti-inflammatory agent, NGF

may also support the survival and function of endothelial cells (Tanaka et al., 2004; Moser et al., 2004) and, BDNF has been shown to act as a pro-inflammatory molecule (Bayas et al., 2003). The removal of NGF from circulation, in combination with BDNF, may synergize their individual effects and lead to a combination of decreased sympathetic and vascular survival or function as well as increased inflammation.

<u>Specific Aim 2</u>: To determine if the sympathetic nervous system plays a role in the abnormalities seen in animals given BDNF + TrkA-Fc by administering BDNF concurrently with surgical interruption of sympathetic fibers innervating brain vasculature.

NGF is a potent growth and survival factor for sympathetic neurons (Levi-Montalcini, 1951). Surgical damage to sympathetic neurons, e.g. nerve transection, leads to a dramatic upregulation of NGF mRNA within the injured nerve and in surrounding cells, indicating the induction of an NGF-mediated survival mechanism (Heumann et al., 1987a). Upregulation of NGF mRNA and NGF protein levels is biphasic, with the first peak occurring immediately after transection and the second peak occurring around 4 days after injury (Heumann et al., 1987b). The level of NGF production achieved by the injured nerve is only a fraction of normal levels of NGF provided by peripheral targets (Heumann et al., 1987a), in effect creating a local environment of transient and phasic NGF deprivation. The combination of surgically-induced sympathetic damage and local delivery of BDNF may lead to neuronal, vascular, and inflammatory abnormalities similar to those seen in animals given BDNF + TrkA-Fc.

Specific Aim 3: To determine if norepinephrine plays a role in the vascular abnormalities seen in animals given BDNF + TrkA-Fc.

Long term NGF deprivation leads to a profound decrease in norepinephrine levels in target tissues in addition to neuronal degeneration (Gorin and Johnson, 1980). However, sympathetic neurons deprived of NGF *in vitro* show an efflux of neurotransmitter before degeneration has occurred (Tolkovsky and Buckmaster, 1989). *In vivo,* nerve transection leads to a transient increase in norepinephrine synthesis (Anden, 1977) before NE levels decrease. The transient increase in norepinephrine levels may be a necessary component for the development of neuronal, vascular, and inflammatory abnormalities seen in animals treated with BDNF + TrkA-Fc.

Specific Aim 4: To determine if specific sympathetic damage can be induced by autoimmune induction and, when combined with BDNF treatment, cause perivascular inflammation.

NGF deprivation can be induced in developing animals by immunizing pregnant adult animals with NGF protein, leading to the production of circulating maternal NGF antibodies which result in damage to fetal tissue (Gorin and Johnson, 1980). The immune response to NGF is potentiated by combining it with complete Freund's adjuvant (CFA). CFA is an emulsion of *mycobacterium tuberculosis,* a highly immunogenic bacterium, in oil. CFA has been used in autoimmune induction models including experimental autoimmune myelitis (EAE), the experimental form of multiple sclerosis. When animals are exposed to a mixture of CFA and spinal cord tissue or myelin sheath proteins, immune

mediated destruction of the myelin sheath ensues and subsequent paralysis occurs in treated animals (Hashim et al., 1980; Feurer et al., 1985). Exposure to NGF antibodies during development leads to sympathetic ganglia atrophy and a reduction in sympathetic neuron number (Gorin and Johnson, 1980). In order to make this model as physiologically relevant as possible, we will induce autoimmunity against sympathetic ganglia instead of NGF protein itself. Sympathetic ganglia contain NGF protein (Korsching and Thoenen , 1983) and therefore, autoimmunity against these ganglia may include antibodies against NGF. However, patients with autoimmune disorders such as scleroderma or lupus exhibit antibodies to a variety of proteins, some which are present in sympathetic ganglia, and do not exclusively produce anti-NGF antibodies. Therefore we will use autoimmune induction against entire sympathetic ganglia as our model for sympathetic damage. Thus, the combination of autoimmune-induced sympathetic damage and BDNF delivery may result in neuronal, vascular and inflammatory abnormalities similar to those seen in animals treated with BDNF + TrkA-Fc.

Chapter 3: GENERAL METHODS

Stereotaxic Surgeries (specific aims #1-4)

Male and female adult Sprague-Dawley or Lewis rats 8-10 weeks old (275-375g, Charles River Laboratories, Kingston, NY) were housed in groups of two or three in standard cages and maintained on a 12 hour light/dark cycle (lights on 06:00) with water and food available *ad -libitum*. Animals were anesthetized using the pre-anesthetic chlorpromazine (3mg/kg) followed by ketamine (210 mg/kg). Animals were then shaved and treated with povidine-iodine solution. Incisions were made in the scalp and, after placing two anchor screws into the skull, a 4 mm indwelling cannula (Plastics One, Roanoke, Virginia) was placed -2.6 mm ML and -3.7 mm AP from bregma (Paxinos and Watson, 1984) into the left hippocampus. The cannulae was attached to heat-sealed polyvinyl catheters filled with sterile phosphate-buffered saline (PBS) and cemented to the skull using dental acrylic. The incision was sutured with 3-0 nylon suture (Henry Schein, Melville, NY), and a topical antibiotic cream applied. Animals were recovered under heat lamps and monitored until fully awake.

Pump Implantation Surgeries (specific aims #1-4)

Animals were re-anesthetized under 2% isoflurane (Baxter, Deerfield, Illinois) 7 days after placing cannulae into the hippocampus. An incision was made across the nape of the neck and the tubing attached to the cannulae withdrawn. The heat-sealed catheter end was snipped with sterilized scissors. We then attached a 14 day, 0.5 µL/hour osmotic mini-pump (Alza Corporation, Mountain View, California) to the catheter end. Rats received one of the

following infusions: BDNF (12 µg/day) + TrkA-Fc (30 µg/day), BDNF + TrkB-Fc (30 µg/day), BDNF + TrkC-Fc (30ug/day), BDNF + NE (24 ng/day), BDNF alone, TrkA-Fc alone, PBS alone, and or NE alone. All solutions were dissolved in sterile PBS, and not all treatments were used in all experiments. We then placed the pump subcutaneously along the back, closed the incision with 3-0 nylon suture, and applied topical antimicrobial cream to the wound.

Sympathectomies (specific aim #2)

At the same time as pump surgery (surgery #2), we removed the superior cervical ganglion (SCG) bilaterally from some rats receiving BDNF only or PBS only. Rats in the same experiment received a sham surgery. Briefly, we made an incision across the neck exposing the muscles and thyroid gland. The thyroid gland was then separated from underlying tissues and wrapped in sterile, saline-soaked gauze above the incision site. We next dissected down to the right superior cervical ganglion, isolated it from surrounding fascia and blood vessels, and used microscissors to remove it completely. We then repeated this procedure for the left superior cervical ganglion. After completing the dissections, the thyroid gland was put back in place, the incision closed and the animal recovered as previously described. Sham animals received the same procedure, except that superior cervical ganglia were not removed after isolation.

Autoimmune Induction (specific aim #4)

Animals (Lewis rats) were anesthetized under 2% isoflurane (Baxter, Deerfield, Illinois) and had their left hindpaw cleaned with 70% alcohol followed by povidine-iodine solution (Henry Schein, Melville, NY). A mixture of 100µL of

one of the following treatments was injected subcutaneously into the ventral aspect of the hind paw with a 25G needle: 400μL complete Freund's adjuvant (CFA, 1mg/ml in oil; Sigma, St. Louis, MO) emulsified with 400μL sterile saline or 100μL CFA emulsified with 20mg/ml SCG in PBS taken from donor animals.

Cold Stress Test (specific aim #4)

Animals (Lewis rats) were wrapped in a towel with one hind paw exposed. The hindpaw color opposite to the paw of injection was measured against a graded color scale consisting of pure white (0) to deep red (19) (Fig. 7). Animals were then placed on a cooled platform (0ºC) for 1 minute. The animal was again wrapped in a towel with the contralateral paw exposed in order to get a post-ice exposure reading. Color values after cold exposure were normalized to values obtained before ice exposure and expressed as a proportion color change. Foot color will be measured 1 day prior to foot injection and every other day, subsequent to experimental treatment. Color changes will be used as an indirect measure of sympathetic function such that hindpaw blanching after cold-exposure indicates an abnormal response.

Tissue Collection (specific aims #1-4)

On day 3 or 12 after pump surgery or 21 days after footpad injections, we deeply anesthetized rats using a pentobarbital-based euthanasia solution, and perfused them transcardially with 4% paraformaldehyde in the following manner. After animals no longer responded to painful stimuli (toe pinch), an incision was made under the xyphoid process and tissue was dissected out to expose the heart. A 20 G blunt needle was inserted into the left ventricle and an incision

made in the right atrium. A perfusion pump (VWR Model #61161-354) was used to exsanguinate the animal by an infusion of 100 mL of ice-cold heparinized saline (0.9%). This was followed by 200 mL of 4% paraformaldehyde in acetate buffer (pH 6.5) and then 200 mL of 4% paraformaldehyde in borate buffer (pH 9.5). We then collected the brains, cut them on a freezing stage sliding microtome into 40μm sections, and stored them in a cryoprotectant solution (Watson et al., 1986) at -20ºC.

Tissue Staining (specific aims #1-4)

Sections were stained using a protocol previously published in the following manner. Briefly, we washed the tissue in potassium phosphate buffered saline three times for 15 minutes each wash followed by a blocking solution of 4% goat or horse serum (Vector Laboratories, Burlingame, CA), 1% bovine serum albumin (BSA; Sigma, St. Louis, MO), and 0.2% triton-x (TX; Sigma, St. Louis, MO) and incubated it with the following antibodies: tyrosine hydroxylase at 1:2000 (Chemicon, Temecula, CA, OX-1 at 1:10,000 (Serotec, Raleigh, NC), fibrinogen at 1:20,000 (Dakocytomation, Carpinteria, CA), smooth muscle actin at 1:500 (SMA; DAKOcytomation, Carpinteria, CA), rat endothelial cell antigen at 1:100 (RECA; Serotec, Raleigh, NC) and glial fibrilary acidic protein at 1: 60,000 (GFAP; DAKOcytomation,Carpinteria, CA). We used biotinylated secondary antibodies (1:1500, Vector Laboratories, Burlingame, CA), including goat-anti-rabbit for tyrosine hydroxylase, GFAP, and fibrinogen, and horse-anti-mouse for OX-1, RECA, and SMA. The signal was amplified using the Vectastain Elite ABC immunoperoxidase kit (Vector Laboratories), visualized

using a diaminobenzidene chromagen, mounted on gelatin coated glass slides, and coverslipped using DPX mounting media (Sigma, St. Louis, MO). Staining with GFAP was conducted as a double stain with RECA in order to look at the contacts that activated astroglial endfeet make with cerebral vasculature. GFAP was stained black as described above and RECA was stained brown by omitting the nickel-intensification step described above. All other steps were the same. Additionally, we conducted a Nissl stain using cresyl violet on brain sections.

Histological Quantification (specific aims #1-4)

Vascular Diameter

Observers blind to treatment group examined the stained tissue using a Nikon Eclipse E400 microscope (Morrell Instruments, Melville, NY). Images were captured using a SPOT digital camera and vessel diameter analyzed with the public domain NIH image program (http://rsb.info.nih.gov/nih-image/). We evaluated vascular diameter on Nissl-stained sections by measuring the shortest distance across the vessel lumen for vessels located in the hippocampal fissure (Fig. 8) within 1mm on either side of the cannula track. Measurements were taken from 2 sections containing cannula track, the mean vascular diameter was determined for each group and analyzed using either one-way or factorial ANOVAs.

Inflammatory Infiltrate and Fibrinogen Deposition

Observers blind to treatment group used a subjective rating scale (where 0 is the least and 4 is the most; Fig. 9) to semi-quantitatively analyze amounts of inflammatory infiltrate. In addition, we measured the presence of inflammation

localized to the perivascular space by counting the number of “cuffed vessels”. This term was defined as all vessels displaying a ring of inflammatory cells. We then converted this count into a proportion of cuffed vessels out of total number of vessels in the hippocampal fissure. In order to measure fibrinogen leak we converted images of fibrinogen stained sections into density threshold images using NIH image and measure the amount of pixelated space in each section.

Quantification of Sympathetic Fibers

The sympathetic nerves innervating vasculature were measured by observers blind to treatment group using TH-stained tissue in both a semi-quantitative and quantitative way. Initially, we rated TH-positive fibers at 60X oil, 1.4NA lens, on a scale which describes the properties of the fiber (fine, medium, coarse; tortuous, not tortuous) and the number of contacts TH-positive fibers make with vasculature in the hippocampal fissure. Fiber contacts were defined as the number of times a TH-positive fiber touched or came within a few microns of a cross-sectional vessel wall. The properties measured were then converted into a percent score including percent vessels innervated, percent coarse, medium, or fine fibers, and percent tortuous fibers. Our second measure of sympathetic fiber properties was done on the Neurolucida system (Microbrightfield, Williston, VT). A computer assisted image analysis system including the Olympus BX51 microscope, an LEP computer controlled *x-y-z* motorized stage, an Optronics Microfine camera system, an Intel Pentium 4 computer, Neurolucida and Neuroexplorer software (Microbrightfield, VT) was used to capture images and quantify TH-positive neuronal properties. TH-

positive fibers innervating vasculature in the hippocampal fissure were traced using a 60X oil, 1.4NA lens, giving us quantitative measures of fiber volume and number of vessel contacts. The number of TH fibers contacting vasculature was also converted into the proportion of vasculature innervated by sympathetic fibers. For all measurements, an average value was calculated for each animal and this number was used for statistical analysis.

Quantification of smooth muscle cells

Since smooth muscle cells are normally wrapped in a relatively continuous fasion around blood vessels, we used SMA stained tissue, indicating arteries, to determine the characteristics of smooth muscle cells in our experiments. Observers blind to treatment group sampled all vessels in the hippocampal fissure from sections containing the cannula tract. We placed SMA positive cells into two different types of categories: wrapped and continuous. The options within each category were “yes”, “no”, or “mixed”. That is “wrapped”, “unwrapped”, or vessels containing “wrapped and unwrapped” smooth muscle cells and “continuous”, “discontinuous”, or vessels containing “continuous and discontinuous” smooth muscle cells. We then analyzed the data as both frequency counts and as the proportion of wrapped vessels and the proportion of discontinuous vessels.

Quantification of vascular astroglial cells

We semi-quantitatively analyzed RECA positive vessels in the hippocampal fissure which had GFAP positive cells in close association with them using a categorical scale (no GFAP positive/RECA positive associations through many

GFAP positive/RECA positive associations). Glial cells were considered to be associated with vasculature when they were within a few microns of, or in contact with, the vessel wall. We then converted frequencies of each category into a percentage. The mean value for each animal was calculated and used for statistical analysis.

Statistics (specific aims #1-4)

We analyzed all quantitative measures using SPSS version 12.0 for Windows (SPSS, Chicago, Illinois). ANOVAs to compare groups were conducted at a $p<.05$ significance level. We applied Hayter-Fisher LSD or Tukey's HSD post-hoc tests to probe pairwise comparisons of any significant results with alpha set to .05. All quantitative data was graphed as mean ± SEM.

Chapter 4: SPECIFIC METHODS

Specific Aim 1: To characterize the vasculature, its sympathetic innervation, and BBB compromise, if any, in animals given BDNF + TrkA-Fc.

TrkA-Fc is a receptor body trap which blocks NGF bioactivity (Shelton et al., 1995). Since NGF is a survival factor for sympathetic neurons (Levi-Montalcini, 1951) we will look at the effects that the lack of NGF has on sympathetic neurons innervating vasculature in the hippocampus. Damage to sympathetic neurons innervating vasculature can lead to fiber retraction (Aberdeen et al., 1991; Cowen et al., 1982) and alterations in vascular tone (Ralevic et al., 1991). The alteration of vascular tone can lead to disruption of the BBB (Kobayashi et al., 1990; Heistad, 1984; Beausang-Linder and Bill, 1981) and result in leakage of plasma protein and inflammatory infiltrates into the surrounding area (Jacobs, 1995). TrkA-Fc, an NGF inhibitor, is expected to lead to sympathetic compromise. Aim one asks if TrkA-Fc with concurrent application of BDNF would further promote the arrival of inflammatory cells into the perivascular space.

Receptor Body Trap Experimental Design

Unilateral implantation of hippocampal cannulae were conducted at day -7. Seven days later, at day 0, mini-osmotic pumps were attached via heat sealed catheter in the following groups:

S.A. #1	Receptor Body Trap Treatment			
Protein Treatment	**Vehicle (PBS)**	**TrkA-Fc**	**TrkB-Fc**	**TrkC-Fc**
Vehicle (PBS)	n=4	n=5	n=5	n=5
BDNF	n=4	n=5	n=5	n=5

Our experimental aim is only concerned with the effects of trkA-Fc in conjunction with BDNF. However, we included other receptor body traps such as trkB-Fc and TrkC-Fc as receptor body trap controls.

Fourteen days after pump implantation, brain tissue was collected as described in the general methods section. A cresyl violet stain and a tyrosine hydroxylase stain were conducted on all brains from this experiment and quantification of vascular diameter, vascular inflammation, and sympathetic fiber morphology were done as described in the general methods section.

Statistical analysis of vascular diameter, vascular inflammation, both semi-quantitative and quantitative measures of sympathetic fibers were done using a factorial ANOVA with α level set to .05.

Specific Aim 2: To determine if the sympathetic nervous system plays a role in the vascular abnormalities seen in animals given BDNF + TrkA-Fc.

Even though NGF is a survival factor for sympathetic neurons (Levi-Montalcini, 1951), it is not clear from specific aim one if damage to the sympathetic neurons, in conjunction with BDNF, causes perivascular inflammation. The second specific aim will ask this question directly by looking at the effect of bilateral surgical sympathectomy of the superior cervical ganglia

(SCG), concurrent with delivery of BDNF into the hippocampus. If sympathetic damage mediates the vascular abnormalities we have seen when animals are given BDNF + trkA-Fc, including changes in cells that contribute to the BBB, smooth muscle cells and astrocytes, we will have more direct evidence that sympathetic damage is involved. There is also a certain window of time within which denervation will be successful. Specifically, after surgical resection of nerves, there is a possibility that the transected pieces can grow back together again (You et al., 2000). In addition, effects of surgical denervation may not be immediate because it takes time for sympathetic terminals to retract and degenerate from target organs after transection (George and Griffin, 1994). Because of these time-course effects seen in transected nerves, we will use a short-term and long-term design by looking at the experimental groups at 3 days and 12 days after treatment, respectively. Aim two asks if sympathetic damage is directly involved in perivascular inflammation seen in animals receiving BDNF + TrkA-Fc.

Bilateral SCG Removal Experimental Design

Unilateral implantation of hippocampal cannulae was conducted at day -7. Seven days later, at day 0, sympathetic surgery was performed and mini-osmotic pumps were attached via heat sealed catheter in the following groups:

S.A. #2	Surgical Treatment			
	Short Term (3 Day)		Long Term (12 Day)	
Protein Treatment	Sham	SCG	Sham	SCG
Vehicle (PBS)	n/d	n/d	n=5	n=5
BDNF	n=5	n=4	n=5	n=5

Surgical procedures were performed as outlined in the general methods section. Three days after pump implantation and sympathectomy surgery, all animals in the 3 day group were sacrificed and tissue collected as described in the general methods section. Twelve days after pump implantation, the rest of the animals, or all animals in the 12 day group, were sacrificed and their tissue collected as described in the general methods section. Tissue from both day 3 and day 12 groups were stained with cresyl violet, tyrosine hydroxylase, SMA, and GFAP/RECA as described in general methods.

Statistical analysis of perivascular inflammation, sympathetic fiber morphology, smooth muscle cells, and glial-vascular interactions were done using a one sample t-test or a factorial ANOVA with α level set to .05.

Specific Aim 3: To determine if norepinephrine plays a role in the vascular abnormalities seen in animals given BDNF + TrkA-Fc.

Damage to sympathetic fibers can lead to dysregulation of vascular tone as well and changes in smooth muscle cell and glial cell morphology (Wecht et al., 2000; Mangiarua and Lee, 1992; Sheng et al., 1993; Steinle et al., 2005). It is not clear how perivascular inflammation after bilateral sympathectomies and

BDNF delivery is mediated by damaged sympathetic terminals. Damaged sympathetics have been shown to over-release NE (Koss et al., 1987). The third specific aim will be addressing the hypothesis that elevated NE is necessary for the expression of perivascular inflammation in conjunction with BDNF delivery.

Norepinephrine Experimental Design

Unilateral implantation of hippocampal cannulae was conducted at day -7. Seven days later, at day 0, mini-osmotic pumps were attached via heat sealed catheter in the following groups:

S.A. #3	**Neurotransmitter Treatment**	
Protein Treatment	**Vehicle (PBS)**	**Norepinephrine**
Vehicle (PBS)	n=5	n=5
BDNF	n=5	n=5

Three days after pump implantation, brain tissue was collected as described in the general methods section. A cresyl violet stain and a tyrosine hydroxylase stain were conducted on all brains from this experiment and quantification of vascular diameter, vascular inflammation, and sympathetic fiber morphology was done as described in the general methods section.

Statistical analysis of vascular diameter, vascular inflammation, and both semi-quantitative and quantitative measures of sympathetic fibers was done using a factorial ANOVA with α level set to .05.

<u>Specific Aim 4</u>: To determine if sympathetic damage can be induced by autoimmune induction and, when combined with BDNF treatment, cause perivascular inflammation.

An abnormal inflammatory response is a key feature in many diseases, especially those of autoimmunological etiology (Potter et al., 2003; Litherland et al., 1999). Dysfunction associated with these types of diseases is not limited to only the inflammatory system. Indeed, other abnormalities can be seen in the vascular system and nervous system (Lambert et al., 1997; Khan et al., 2000; Rajagopalan et al., 2004; Black et al., 2000; Axford et al., 2001). While the above experiments possibly demonstrate the interaction between damaged sympathetic fibers, NE, and perivascular inflammation, they do not address the etiology of these real autoimmune phenomena. The fourth aim will try to create a more physiologically relevant mechanism leading to the development of sympathetic damage, or autoimmune attack against sympathetic ganglia. It is proposed that this more realistic model of sympathetic damage, when combined with BDNF delivery, will result in similar phenomena as those discovered in the previous aims.

Studies have shown that autoimmune diseases can be induced experimentally by combining an antigen of interest, e.g. myelin basic protein (MBP), with a highly immunogenic substance, e.g. Freund's Complete Adjuvant (a mixture of *mycobacterium tuberculosis* in oil), producing an experimental model of autoimmunity, e.g. multiple sclerosis (Elliot et al., 1996; Tuohy et al., 1988). Several studies have also shown that certain autoimmune diseases are

accompanied by abnormalities of the autonomic nervous system, e.g. Sjogren's Disease and scleroderma (Sorajja et al., 1999; Shimoyama et al., 2002; Ferri et al., 1997; Cozzolino et al., 2002). There are many different types of autoimmune diseases but many of them present with similar symptoms including skin disorders (Saito et al., 2002; Chan et al., 1999; Chan et al., 2001). Since all of our previous aims were carried out in brain tissue, we will continue to utilize this organ as a background for our phenomenon, especially since development of autoimmunity, even when experimentally induced, is not limited to the site of induction (Elliot et al., 1996; Tuohy et al., 1988).

Autoimmune Induction of Sympathetic Damage Experimental Design

Unilateral implantation of hippocampal cannulae was conducted at day -7. Seven days later, at day 0, mini-osmotic pumps were attached via heat sealed catheter in the following groups:

S.A. #4	**Autoimmune Treatment**	
Protein Treatment	**CFA alone**	**CFA + SCG**
Control (BSA)	n=5	n=5
BDNF	n=5	n=5

Beginning on the day of surgery, animals were assessed for sympathetic function by measuring hindpaw response after exposure to ice. A full description of the cold stress test procedure is given in general methods. Readings were taken every other or every third day until the twenty first day after pump implantation, when brain tissue was collected as described in the general methods section. A

cresyl violet stain and a fibrinogen stain were conducted on all brains from this experiment and quantification of vascular inflammation was done as described in the general methods section.

Statistical analysis of cold stress test data was analyzed using a factorial ANOVA with alpha set to .05 for the day identified in which color change is at maximal (or minimal) capacity. In addition, vascular inflammation was analyzed using a factorial ANOVA with α level set to .05.

Chapter 5: RESULTS

Specific Aim 1: TrkA-Fc Studies

Vascular Inflammation

While looking at the role of neurotrophins in the brain, we serendipitously discovered an inflammatory infiltrate localized around the vasculature in some animals. Animals co-infused with both BDNF and TrkA-Fc showed obvious vascular cuffing (Fig. 10D, F), characterized by focal leukocyte extravasation, as shown by OX-1 staining (Fig. 10H). Neither cuffing nor OX-1 positivity was observed with either BDNF or TrkA-Fc alone, nor with the co-infusion of BDNF with any other protein (Fig. 10A-C, E, G). In order to rule out inflammatory effects due to the possibility of bacterial contamination from production of recombinant protein in E. coli, all reagents were checked for endotoxin levels. Endotoxin was very low, and approximately equivalent for the three receptor body proteins, with TrkA-Fc (0.8 ppm) being the median between TrkB-Fc (0.6 ppm) and TrkC-Fc (1.2 ppm), neither of which produced this phenomenon.

Vascular Diameter

Anecdotal observation of the brain sections suggested differences in vascular diameters between groups. Therefore, vascular diameters were measured at the site of infusion. The mean diameter for SMA positive vessels in the BDNF+TrkAFc combined group was significantly smaller than vessels in the control group and the BDNF+control group, and marginally smaller than the TrkA-Fc only group ($F(3,31)=6.027$, $p<.002$, Hayter-Fisher LSD; Fig. 11). We then measured diameters for SMA negative vessels using the same technique

described previously and found no significant difference between treatment groups ($F(3,28)=2.23$; $p>.05$; Figure 12).

Sympathetic Innervation of Vasculature

Because TrkA-Fc is an inhibitor of NGF, and NGF is a potent trophic factor for the sympathetic nervous system, we hypothesized that the vessels in the area of infusion were vasoconstricted due to abnormal sympathetic activity. We visualized sympathetic nerve terminals innervating vessels ipsilateral to the infusion site by tyrosine hydroxylase immunostaining. Terminals in the PBS only and all BDNF control groups (BDNF+hFc shown) had a normal morphology, characterized by fine processes extending to, and around, vascular lumens (Fig. 13A, D). Sympathetic fibers in animals receiving TrkA-Fc (both TrkA-Fc alone and BDNF+TrkA-Fc) were significantly more coarse than those receiving PBS ($F(1,14)=6.989$;$p<.019$; Fig. 13B, C, D), although those in the BDNF co-infused group had the highest percentage of coarse fibers innervating vasculature ($HSD(2,14)=8.75$;$p<.05$; Fig. 13D). In order to further characterize sympathetic fibers, sections were analyzed using the Neurolucida system. Similar to the subjective rating scale, sympathetic fibers in the TrkA-Fc group showed a significant increase in fiber volume ($F(1,12)=6.411$;$p<.026$; Fig. 14), although those in the BDNF co-infused group had a significantly larger volume than both the PBS alone and BDNF alone groups ($HSD(2,12)=4.51$;$p<.05$). There was no significant difference between PBS + TrkA-Fc and BDNF +TrkA-Fc groups ($HSD(2,12)=4.51$;$p>.05$; Fig. 14D). In addition to neuronal volume, fiber diameter, tortuosity, and number of contacts per vessel were analyzed on the

Neurolucida system. There was no significant difference in fiber diameter between treatment groups ($F(1,11)=1.061;p=.325$; Fig. 15). However, for all animals receiving BDNF, there was a trend towards increased fiber diameter ($F(1,11)=3.537;p=.087$). Additionally, there was no significant difference in tortuosity and number of contacts per vessel between treatment groups ($F(1,11)=0.26;p=.62$ and $F(1,11)=0.13;p=.72$, respectively; Fig. 16, 17)

Specific Aim 2: Sympathectomy Studies

Vascular Inflammation

To further assess the role of sympathetic neurons in vascular cuffing, we surgically removed the superior cervical ganglia of animals infused intrahippocampally with either BDNF or PBS using mini-osmotic pumps. Animals receiving sham surgery, and then infused with BDNF or PBS, showed no evidence of vascular cuffing at 3 or 12 days after sham sympathectomy (Fig. 18E, F). Three days after SCG removal, animals given PBS showed no evidence of perivascular inflammation (Fig 18A). In contrast, animals given sympathectomy surgery and infused with BDNF had increased perivascular cellularity and edema at day 3 (Fig. 18C). Twelve days after sympathectomy, animals did not show the same effect. That is, no perivascular inflammatory cells were observed in either PBS or BDNF infused groups 12 days after SCG removal (Fig. 18B, D).

Sympathetic Innervation of Vasculature

Three days after sympathectomy, perivascular sympathetic fibers were still present in the hippocampus (80% of vasculature innervated by TH positive

fibers), whereas 12 days after sympathectomy, fewer fibers were present (40% of vasculature innervated by TH positive fibers) ($F(1,4)=8.021;p<.047$; Fig. 19). Animals given BDNF and sympathectomy surgery, at 12 days after surgery, show significantly fewer vessels innervated compared to those three days after surgery ($HSD(2,4)=43.89$; $p<.05$; Fig. 19). A similar result was found on the neurolucida system whereby 12 day sympathectomy animals given BDNF have significantly fewer fiber contacts per vessel ($F(1,5)=43.79$; $p<.001$ $HSD(2,5)=.488;p<.05$; Fig. 20). These results suggested that the presence of sympathetic terminals was a necessary condition for the development of BDNF-induced inflammatory pathology.

Vascular Smooth Muscle Cells

Dysregulation of sympathetic vascular innervation can lead to changes in smooth muscle cells surrounding the vasculature (Dimitriadou et al., 1988). Under normal physiological conditions, smooth muscle cells are wrapped around vascular endothelial cells. Twelve days after sympathectomy, animals given BDNF show no significant differences in the percent wrapped (Fig. 21A), unwrapped (Fig. 21B), or continuous (Fig. 21D) vascular smooth muscle cells ($F(1,13)= 468.63$; $p=0.363$; $F(1,13)= 36.61;p=0.615$). However, there is a significant increase in the percent of mixed smooth muscle cells in animals 12 days after sympathectomy and BDNF treatment (i.e. vessels containing a mix of both wrapped and unwrapped smooth muscle cells, $F(1,13)=206.94;p=.066$; $HSD(2,13)=10.94;p<.05$; Fig. 21C, 22). The changes seen in smooth muscle

cells 12 days after SCG removal is not present 3 days after sympathectomy ($t(5)=.721;p=.435$; Fig 23A mixed, B continuous).

Glial Cells Associated with Vasculature

Dysregulation of sympathetic fibers can also lead to changes in astroglial cells (Krassioukov and Weaver, 1996). Similar to changes seen in vascular smooth muscle cells, glial cells also show changes 12 days after SCG removal concurrent with BDNF delivery. Specifically, vessels show increased investment with activated astrocytes (Fig. 24). This increase was statistically significant when the number of activated glia per vessel was quantified ($F(1,13)=5.414;p<.037$; $HSD(2,13)=19.25;p<.05$; Figure 25). This increase in glial-endothelial associations is not present at 3 days after SCG removal in animals receiving BDNF. In fact, BDNF infusion, in conjunction with either 3 day SCG removal or sham surgery, results in the same number of activated glial-endothelial associations in the hippocampus ($t(5)=.675;p=.530$; Fig 26).

Specific Aim 3: Norepinephrine Studies

Vascular Inflammation

Previous research has shown that, in the early phases of sympathetic damage, vasculature engages in a compensatory oversensitivity to norepinephrine (NE) (Rizzoni et al., 2000, Slovut et al., 2004). To determine if this increase in noradrenergic responsiveness might contribute to the inflammatory pathology observed in our studies, we co-infused BDNF with NE into the hippocampus of naïve rats for 3 days. Animals infused with PBS alone and BDNF+PBS showed no perivascular inflammation, but animals

infused with BDNF+NE and PBS+NE showed increased cellularity and perivascular edema, with BDNF + NE showing the greatest amount of inflammation (Fig. 27B, D as compared to 27A, C). There was also a non-significant decrease in vascular diameter in animals receiving PBS + NE compared to all other groups ($F(3,22)=.869$; $p=.472$; Fig. 28).

For both studies using sympathetic manipulation to induce perivascular inflammation (3 days of NE or 3 days after SCG removal), we analyzed the proportion of cuffed vessels on the side of infusion. Both the BDNF+NE and BDNF+SCG groups showed a significant increase in the proportion of cuffed vessels relative to control animals ($F(2,30)=8.12$, $p<.002$; Fig 29).

Sympathetic Innervation of Vasculature

In order to determine if both NE and 3 day SCG treatment, concurrent with BDNF infusion, were inducing sympathetic abnormalities similar to that seen in animals receiving BDNF +TrkA-Fc, TH fibers were analyzed across all groups. There were no significant differences in any measures taken on TH positive fibers between BDNF +TrkAFc, BDNF + SCG (3 day) removal, BDNF + NE, and BDNF sham (3 day) ($F(3,10)=3.31$;$p=.066$; Figure 30), suggesting that all three manipulations resulted in equivalent sympathetic fiber abnormalities. However, since the p value indicated a trend in axonal volume, a post-hoc analysis revealed that animals treated with BDNF and trkA-Fc had a significant increase in axonal volume compared to those treated with BDNF + sham operations and those treated with BDNF + NE (LSD, $p<.02$ for both pairwise comparisons).

However, the BDNF + trkA-Fc were not significantly different than BDNF + SCG animals, reflecting the intermediate values of the latter group.

Vascular Leak

Because perivascular pathology accompanied by sympathetic overactivity has been reported in some autoimmune diseases, especially systemic sclerosis, and these diseases are characterized by deposition of plasma proteins such as fibrin, we stained rat brains for fibrinogen. All control animals showed little or no evidence of parenchymal fibrinogen staining. However, animals treated with either BDNF+SCG removal (3 days) or BDNF+NE showed increased fibrinogen deposits. While both BDNF+SCG (3 day) and BDNF+NE groups were increased over control, only the BDNF+NE group was significantly increased ($F(2,37)=7.23$; $p<.002$, Hayter-Fisher LSD; Fig. 31).

Specific Aim 4: Autoimmune Studies

Different autoimmune diseases, such as lupus and scleroderma, share common symptoms including excessive inflammation, sympathetic damage, vascular abnormalities, and protein deposition in tissues. These characteristics have been evident in our experimental studies looking at the interaction between sympathetic damage and BDNF. In order to make our model more physiologically relevant, we injected SCG tissue in complete Freund's adjuvant, a manipulation previously shown to induce autoimmunity against specific antigens. Some animals in this experiment also received BDNF. Female Lewis rats were used in this experiment because this strain of rats is susceptible to the induction of autoimmunity.

Sympathetic Damage

In order to determine if autoimmune induction of sympathetic damage was successful, we measured the animals' blood-flow response to a cold-stress test. All values greater than one represent redder foot color after exposure to ice and all values less than one represent whiter foot color after exposure to ice. All groups of animals receiving Freund's adjuvant exhibited no change in blood flow or a decrease in blood flow in which their foot color became whiter after exposure to ice (CFA alone, CFA + SCG; $F(2,16)=6.15$; $p<.01$; LSD saline vs. CFA, $p<.005$; LSD saline vs. CFA/SCG, $p<.017$; Fig. 27), except for the group of animals given no CFA (saline; Fig. 32). These control animals showed an increase in blood flow, in which their foot color became redder after exposure to ice.

Vascular Inflammation

In contrast to prior results, perivascular inflammation was seen in animals given BDNF and CFA, both with and without the presence of a specific antigen (BDNF + CFA alone and BDNF + CFA/SCG; Fig. 33D, F). Additionally, perivascular inflammation could also be seen in the absence of BDNF delivery, but only when the specific antigen (SCG) was delivered (BDNF + CFA/SCG; Figure 33E). When CFA was delivered alone, without a specific antigen and without BDNF (BSA + CFA alone; Fig. 33C) there was little evidence of inflammation. Subjective inflammatory ratings on nissl-stained brain tissue revealed a significant difference in inflammation between foot injections ($F(2,9)=8.708$; $p<.008$; Fig. 34A) such that animals given CFA/SCG together

showed a significant increase in inflammation over saline or CFA alone. However, animals given BDNF + CFA/SCG clearly had the highest inflammation score (LSD saline vs. CFA/SCG, $p<.002$, CFA vs. CFA/SCG, $p<.03$). While animals given BDNF + CFA/SCG clearly showed the highest degree of inflammation, this difference was not evidence in the proportion of cuffed vessels. In fact, animals treated with CFA/SCG, both with and without BDNF, showed a significantly greater proportion of cuffed vessels compared to animals treated with saline ($F(2,11)=4.63$; $p<.035$; LSD saline vs. CFA/SCG $p<.012$; Fig. 34B), but no significant difference in proportion of cuffed vessels compared to animals given CFA alone.

Vascular Leak

Since this experiment used a model of autoimmune-mediated damage, and several autoimmune diseases present with fibrin deposition in tissue, we measured the levels of fibrinogen deposits in brain. While there was no significant difference in fibrinogen area between the groups, animals treated with BSA + CFA/SCG and BDNF + CFA alone showed a tendency toward increased parenchymal fibrinogen staining ($F(2,12)=1.96$; $p=.183$; Fig. 35)

Chapter 6: DISCUSSION

In the current experiments, brain sympathetic dysfunction induced by surgical sympathectomy or inhibition of the sympathetic survival factor NGF was insufficient to lead to inflammatory changes in brain vasculature. In addition, norepinephrine alone, which simulated sympathetic overactivity, did not lead to inflammatory changes in brain. The combination of sympathetic abnormality and BDNF led to striking perivascular compromise characterized by edema, leukocyte extravasation, and fibrinogen deposition. BDNF has been given alone numerous times to adult hippocampal tissue, including in the present experiment, and has never been observed to induce tissue compromise or inflammation. Interestingly, autoimmune induction of sympathetic damage by itself in autoimmune-prone animals was able to elicit inflammatory changes in brain vasculature, suggesting that autoimmune-prone animals can exhibit aspects of this phenomenon even in the absence of BDNF.

1. The Role of Sympathetics in Perivascular Inflammation

In our experiment, animals receiving trkA-Fc, an inhibitor of the sympathetic neuron survival factor NGF, displayed abnormal sympathetic fiber morphology, characterized by increased fiber coarseness and volume. SNS development and survival is guided, in part, by nerve growth factor (NGF). Nerve growth factor appears to be vital for sympathetic integrity even in the adult (Aloe et al., 2000). For example, experimentally-induced decreases in circulating NGF levels in adult animals lead to atrophy of sympathetic ganglia, reduced numbers of sympathetic neurons, decreased length of sympathetic dendrites, and

decreased levels of norepinephrine in certain organs (Gorin and Johnson, 1980; Ruit et al., 1990). Overexpression of NGF leads to increased innervation of vasculature in the hippocampal fissure which can subsequently be decreased by surgical resection of superior cervical ganglia (Kawaja and Crutcher, 1997).

Fibers in trkA-Fc treated animals appeared to have increased coarseness and increased volume compared to controls. However, when BDNF was combined with trkA-Fc, fibers showed a further increase in coarseness and volume compared to animals given only trkA-Fc. In addition to changes seen in fiber coarseness and volume, there was a trend towards increased fiber diameter in all animals treated with BDNF. This was not an unexpected result because BDNF has been implicated as a trophic factor for sympathetic neurons (Kobayashi et al., 1994), and addition of BDNF to nerve fibers has been shown to result in increased axonal diameters (Moir et al., 2000). Although sympathetic fibers appeared morphologically abnormal, there were no changes in the number of contacts the fibers made onto vasculature in any treatment groups in the trkA-Fc experiments.

Interestingly, the opposite phenomenon occurred in sympathectomized animals. At both time points, three days or twelve days after bilateral SCG removal with concurrent BDNF infusion, there was no evidence of morphological abnormalities in sympathetic fibers. However, at twelve days after BDNF infusion and SCG removal, fewer vessels were innervated with sympathetic fibers and, of those innervated, there were fewer contacts between fibers and vessels.

Although animals treated with BDNF + trkA-Fc and those treated with BDNF + 3 day sympathectomies both showed evidence of perivascular inflammation, there were differential effects seen in sympathetic fibers. These differences may be explained by the mechanism of sympathetic damage. By removing NGF from local tissue, we removed the target-derived survival factor. Adult sympathetic neurons are not as dependent on NGF for survival as developing sympathetic neurons, nonetheless, removal of NGF leads to decreases in perivascular axons (Isaacson and Crutcher, 1995). In addition, removing NGF has been shown to cause changes in sympathetic fibers including upregulation of trkA mRNA, as well as increases in neuropeptide protein and mRNA (Mearow and Kril, 1995; Shadiack et al., 2001), which could account for the increase in fiber volume seen in animals treated with BDNF + trkA-Fc. At the same time, reactive increases in NGF protein may explain the lack of changes seen in number of fibers contacting vasculature. The relative increase in NGF may have, in effect, rescued sympathetic fibers from NGF deprivation-induced fiber loss since increases in endogenous NGF have been shown to increase perivascular axons (Isaacson and Crutcher, 1998). It is possible that, if given for a longer period of time, trkA-Fc treatment would have eventually led to a decrease in the percent vessels innervated and number of contacts sympathetic fibers made on vasculature, as this treatment blocks endogenous NGF. While not significant, our data suggest that animals receiving trkA-Fc do have, on average, fewer sympathetic fibers contacting vessels in the hippocampus. Our inability to detect a significant difference in vessel innervation may be due to high

variability observed in our treatment groups. Indeed, all treatment groups showed high variability which could be a function of the measurement itself. Typically, as vascular diameter increases, sympathetic innervation increases (Hejtmancik and Su, 1981). However, it is possible that this relationship changes when sympathetic fibers are compromised.

Animals treated with BDNF + sympathectomies exhibited sympathetic characteristics opposite to those seen in fibers of animals treated with BDNF + trkA-Fc. Specifically, sympathectomy animals displayed no changes in fiber volume but showed decreases in the percent vessels innervated and decreased contacts between fibers and vasculature twelve days after surgery. Successful transection of sympathetic fiber disrupts axonal transport of molecules including NGF protein (Wu et al., 1993; Nagata et al., 1987), and therefore would prevent the accumulation of new protein in perivascular fiber terminals, possibly accounting for lack of changes in fiber volume. At the same time, surgical transection and chemical destruction of nerve fibers can lead to retraction of perivascular terminals (Koistinaho et al., 1990; Zochodne et al., 1989), thus accounting for the decreases seen in sympathetic innervation of vasculature twelve days after surgery. It is also possible that compensation in NGF production began in sympathetic fibers after day three and before day twelve and therefore may no longer be evident at the timepoints studied. Indeed, although it was not significant, three days after SCG removal and BDNF infusion, sympathetic fibers had a tendency towards increased volume. This could be the result of increased production of neurotransmitter mRNA and protein in fiber

axons, which has shown to be upregulated after sympathetic denervation (Zigmond et al., 1996). Alternatively, the tendency towards increased axonal volume could be the result of fiber retraction, a normal consequence of fiber transection (Koistinaho et al., 1990).

One confound of these data is that we do not know the morphological characteristics of sympathetic fibers three days after surgery alone, i.e in the absence of BDNF. Although we saw no sympathetic fiber changes in animals receiving BDNF + short-term sympathectomies, it is possible that BDNF itself is preventing the expression of sympathetic fiber changes in some unknown way. However, this is an unlikely scenario since we do see sympathetic changes in animals receiving BDNF + long-term sympathectomies. BDNF could also be acting as a temporary inhibitor of sympathetic changes and therefore, our timepoint would not be long enough to observe sympathetic changes in amials receiving BDNF + long-term sympathectomies. In addition, while BDNF is a trophic factor which can affect sympathetic neurons, it has a more prominent role in non-sympathetic neuronal survival and function (Glebova and Glinty, 2004; Acheson et al., 1995).

It is important to note that it was only during the short-term (3 day) sympathectomy and BDNF infusion that we saw evidence of perivascular inflammation. At the long-term (12 day) timepoint, there was no evidence of vascular inflammation. Based on the short-term results, it is likely that the long-term animals had inflammation at an earlier timepoint but all abnormal inflammatory activity resolved by the later timepoint.

All of the abnormalities seen at 12 days post sympathectomy and BDNF infusion, which are not seen 3 days post sympathectomy and BDNF infusion, suggest that sympathetic fibers must be present, and in an active state of injury, in order for perivascular inflammation to develop in the presence of BDNF. However, once sympathetic fibers are decreased or damaged beyond a certain degree, the inflammation may no longer present in the same way, if at all, as that seen in response to BDNF, i.e. vascular cuffing.

2. <u>The Interaction Between Sympathetic Fibers and Immune Cells</u>

Our results indicate that both elevated levels of BDNF and abnormalities of sympathetic fibers must occur at approximately the same time, and within a certain time-frame, in order to elicit an inflammatory response. If these two processes occur independently or outside the optimal timecourse, inflammation may not occur. Further, it supports the idea of complex interactions between the sympathetic nervous system, neurotrophic factors, and the immune response.

Studies have shown that normal sympathetic function is important in the regulation of inflammatory responses. Indeed, sympathetic input is important in mediating production of pro-inflammatory cytokines including IFN-γ (Swanson et al., 2001), as well as mediating cellular responses to pro-inflammatory cytokines such as histamine (Garrity et al., 1985). In accordance with the importance of sympathetics in normal immune responses, abnormal sympathetic function has been associated with inflammatory abnormalities. For example, stroke patients exhibiting decreased sympathetic vasoconstrictive functioning show increased inflammatory responses to histamine (Tarkowski et al., 1995). Patients with

rheumatoid arthritis show decreased responsiveness of β-NA receptors on T cells, resulting in decreased NE-mediated immunosuppression and an enhanced inflammatory response (Baerwald et al., 1987).

While our results show that sympathetic abnormalities are necessary for the development of perivascular inflammation in this model, sympathetic dysregulation alone is not sufficient. One possibility is that sympathetic abnormalities potentiate the immune response such that the addition of other exacerbating factors, e.g. elevated BDNF levels would lead to full expression of perivascular inflammation.

3. Contribution of Norepinephrine to Perivascular Inflammation

It is clear that the presence of abnormal sympathetic fibers, in conjunction with BDNF, is necessary for the development of perivascular inflammation in this model. Based on our previous experiments, we were not able to determine what, if any, changes in neurotransmitters occur as a result of abnormal sympathetic activity. In fact, one confound in these data is that we do not know if SCG removal results in changes in NE, the primary sympathetic neurotransmitter. However, based on previous studies showing that nerve transection leads to transient increases in NE levels (Anden, 1977; Koss et al., 1987) we would predict similar effects in our experiments.

Perivascular Inflammation

In order to determine what role the sympathetics, in particular NE, may play in this phenomenon, we infused NE in conjunction with BDNF into brain. All animals infused with NE showed an increase in cellularity. However, animals

infused with NE and BDNF showed the greatest increase in cellularity, with many of the cells concentrated around vasculature. This manipulation resulted in less overall perivascular cellularity than that seen in the previous experiments using trkA-Fc and sympathectomies.

Our results indicate that this type of perivascular inflammation is dependent, in part, on the interaction between BDNF and NE. However, NE may not be the sole sympathetic component in the development of inflammation. In fact, it may be a combination of sympathetic mediators, such as NE and NPY, in conjunction with BDNF, which contribute to the inflammatory, sympathetic, and vascular changes seen in our previous experiments. Indeed, BDNF itself induces NPY expression in neurons both *in vitro* and *in vivo* (Takei et al., 1996; Croll et al., 1994; Nawa et al., 1993; Nawa et al., 1994). In addition, both BDNF and NPY have been shown to be upregulated after nerve transection, with increases in NPY occurring after increases in BDNF levels (Li et al., 1999).

Vasoconstriction

NE alone can act as a vasoactive substance, either inducing constriction or dilation depending on its binding with the α-NA or β-NA receptors, respectively (as cited in Hamel 2006). Application of NE into the brain at high concentrations is likely to be vasoconstrictive (Marin et al., 1982). Additionally, NE alone has been shown to induce sympathetic damage in a dose-dependent manner (Albino et al., 1989). In our NE treated animals, either with or without BDNF, there was a non-significant decrease in vascular diameter and no evidence of altered sympathetic fiber morphology even though there was evidence of perivascular

inflammation. It is possible that our NE infusion was too long for us to observe significant changes in vascular diameter, which normally results from acute physiological effect of NE. Especially because chronic treatment with NE can lead to changes in vascular noradrenergic receptor properties, making vessels refractory to NE-mediated vasoconstriction (Kiuchi et al., 1992). Alternatively, it might not be possible to accurately measure physiological vasoconstriction in response to NE in our tissue preparation. However, it is not an unexpected result that the addition of NE did not lead to changes in sympathetic fibers because the exogenous NE would act predominantly on post-synaptic receptors, i.e. vascular noradrenergic receptors. While there are pre-synaptic NE receptors on sympathetic fibers, binding of NE to these receptors causes changes in neurotransmitter release, not changes in fiber morphology (Todorov et al., 2001; Martinez and Adler-Graschinsky, 1980). It is also possible that we did not see significant vasoconstriction due to NE because under normal circumstances, NE is co-released from sympathetic terminals with NPY (as cited in Hamel, 2006), which itself has vasoconstrictive properties (Oellerich and Malik, 1993). NE alone might not have been enough to induce significant vasoconstriction in our model.

In our experiments, animals receiving trkA-Fc did not exhibit vasoconstrictive changes. In fact, it was only when trkA-Fc was combined with BDNF that abnormal vasoconstriction occurred in the surrounding blood vessels. In our hands, BDNF alone had no effect on vascular tone, though overexpression of BDNF has been shown to result in increased sensitivity of small arteries to

vasoconstrictive substances (Springer et al., 2004). NE alone has also been shown to decrease vascular diameter. However, in our study neither NE alone nor NE combined with BDNF resulted in decreased vascular diameter. In addition, there was no decrease in vascular diameter seen in animals twelve days after bilateral SCG removal alone, or when three or twelve day sympathectomy was combined with BDNF. This is an interesting finding since fewer fibers and fiber contacts were found associated with vessels at the long-term (12 day) timepoint.

4. Perivascular Abnormalities

The immune system, sympathetic nervous system, and vascular system have a complex relationship under normal physiological conditions. Innervation of sympathetic fibers onto vasculature is important in the regulation of vascular tone (McCulloch and McGrath, 1998). In part, peripheral vascular tone is mediated by the effects of vascular smooth muscle cells, which are also innervated by sympathetic fibers (McCulloch and McGrath, 1998). In brain, vascular tone is mediated, in part, by cells comprising the neurovascular unit: vascular endothelial cells, smooth muscle cells and astrocytes. These three cell types contribute to the formation of the blood-brain barrier (BBB) in cerebrovasculature (Parent, 1996, chap. 1). The BBB is an interface between endothelial cells, smooth muscle cells, and astroglial cells which all work together to restrict access of many substances to the brain. The integrity of all three cell types is important for the maintenance of the BBB and, if breached, leak can occur into brain tissue. Disruption in normal functioning in any of these cells can

have an effect on the permeability of cerebrovasculature to blood plasma proteins and inflammatory cells (Parent, 1996, chap. 1). Studies have shown that damage to sympathetic fibers can lead to alterations in vascular resistance and fluid homeostasis in distal areas affected by the injury (Johansson, 1979).

Astroglia

Studies have shown that after sympathetic damage, other cells in the area, specifically glial cells, will begin to produce NGF mRNA (Schwartz and Nishiyama, 1994); possibly in response to changing NE levels since astroglia express functional α-NA and β-NA receptors (Hirata et al., 1983; Schwartz and Mishler, 1990). This compensatory mechanism may explain the changes we found in perivascular glial cells where at twelve days after sympathectomy and BDNF infusion, there was an increase in the number of activated glial cells associated with cerebrovasculature. Interestingly, these glial changes are not seen in animals given long-term sympathectomies alone, i.e without BDNF. It is therefore possible that BDNF itself is having an effect on glial cell activation since the BDNF receptor, truncated trkB, is upregulated in glial cells after injury (Widenfalk et al., 2001) and, activation of glial truncated trkB has been shown to have a direct signaling role (Rose et al., 2003). However, in our experiments it is unlikely that BDNF alone is responsible for the changes seen in perivascular glial cells since animals receiving BDNF infusion and sham surgery did not show a significant increase in glial cell activation.

Smooth Muscle Cells

In these experiments, animals who were given long term (12 day) sympathectomies in conjunction with BDNF show abnormalities in smooth muscle cells associated with brain vasculature. In particular, these animals exhibit an increase in "mixed" smooth muscle cells, such that they are less wrapped. These effects do not occur in any other treatment groups and do not occur in animals given BDNF concurrently with short-term (3 day) sympathectomies. These findings may reflect evidence of sympathetic dysregulation since studies have shown that sympathetic input is necessary for the proper associations between endothelial cells, smooth muscle cells and glial cells (Adams et al., 2004; Lo et al., 2003). In addition, these finding concur with our other finding that at 12 days after BDNF and sympathectomy, there is a decrease in the number of innervated vessels and a decrease in the number of contacts on those vessels that are innervated. Interestingly, at day 3 after sympathectomy and BDNF infusion, there is an increase in the number of innervated vessels and number of contacts on those vessels. Studies have shown that transection of sympathetic fibers can lead to compensatory sprouting of intact sympathetic fibers (Handa et al., 1991) as well as a shift in the type of neurotransmitter produced and released from damaged fibers (Zigmond et al., 1996), which could protect vasculature from secondary damage.

These results indicate that the mechanistic basis of perivascular inflammation may lie within the precise interaction between BDNF and time-sensitive responses to sympathetic injury. Specifically, perivascular inflammation

occurring in conjunction with BDNF and short-term sympathetic damage, but not BDNF and long-term sympathetic damage, could be mediated by the transient increase in NE levels seen shortly after fiber transection (Anden, 1977). Additionally, our results may indicate that sympathetic dysfunction, as a necessary component in the development of perivascular inflammation, precedes the development of perivascular inflammation, which itself, precedes the development of vascular changes. These findings are important because they may elucidate possible etiological milestones in the development of immune diseases.

5. Role of BDNF and NE in Perivascular Inflammation

Both BDNF alone and NE alone have the ability to act as pro-inflammatory molecules. Full-length trkB is expressed on eosinophils and is probably functional since binding of BDNF leads to phosphorylation of the trkB receptor in these cells (Noga et al., 2002). The truncated form of the BDNF receptor has been found on inactivated T cells (Besser and Wank, 1999). However, this expression shifts to the fully functional BDNF receptor, trkB, after antigenic stimulation of T cells (Besser and Wank, 1999). Stimulation of the trkB receptor on immune cells can lead to increased expression of immunomodulatory cytokines such as interferon gamma (Bayas et al., 2003). In addition to expressing trkB receptors, T cells can produce BDNF in response to antigenic stimulation (Braun et al., 1999; Barouch et al., 2000; Kerschensteiner et al., 1999), indicating both autocrine and paracrine functions for BDNF in immune-mediated responses. Norepinephrine also has direct effects on immune cells. In

fact, a normal antibody response from B cells is dependent, in part, on signaling through the β-NA receptor (Kohm and Sanders, 1999). Additionally, NE has chemotactic and chemokinetic effect on antigen presenting cells in lymph organs, though this effect is mediated through the α-NA receptor (Maestroni, 20000).

6. Autoimmune Diseases Presenting with Sympathetic and Vascular Abnormalities

The ability of both BDNF and NE to interact with both T and B cells may contribute to the development of different types of autoimmune disease. Autoimmune diseases are classified in terms of their etiology, i.e. what type of immune cells mediate pathophysiological responses in the body. T-cell mediated autoimmune diseases such as insulin-dependent diabetes mellitus (IDDM) and multiple sclerosis (MS) are characterized by T-cell destruction of pancreatic cells and destruction of the myelin sheath surrounding axons, respectively (Atkinson and Maclaren, 1993; Noseworthy, 1999). Immune-complex mediated autoimmune disease such as systemic lupus erythematosus (SLE) and systemic sclerosis (SSc), are characterized by depositions of abnormal protein complexes within organ systems, possibly leading to organ dysfunction and failure (Gilboa et al., 1977; Napirei et al., 2000; Ansari et al., 1986).

Autoimmune diseases of both T-cell mediated and immune-complex mediated origins are debilitating illnesses that preferentially affect women. Symptoms of autoimmunity vary widely among the different types of diseases, and even present differently within subtypes of specific autoimmune diseases. Many autoimmune disorders present between 30 and 50 years of age. It is

unclear what factors trigger the development of autoimmunity, though development of these diseases most likely depends on the convergence of many factors, both environmental and genetic. Two autoimmune diseases which are relevant to this dissertation are systemic lupus erythematosus (SLE) and systemic sclerosis (scleroderma; SSc).

Symptoms of lupus vary widely but include: a butterfly shaped rash across the bridge of the nose, photosensitivity, pleuritis, pericarditis, psychosis, arthritis, vasculitis, nephritis, and Raynaud's phenomenon. The cause of SLE is unknown but genetic linkage studies suggest that polymorphisms in certain genes are risk factors for the disease. For example, patients with SLE who are homozygous for the *Fas* gene, a gene encoding a transmembrane glycoprotein involved in apoptosis, show increased photosensitivity (Nakajima et al., 1997). Recently, studies have shown that mice lacking DNAse1, an enzyme which removes cellular debris, develop lupus-like symptoms 6-8 months after birth (Napirei et al., 2000).

In addition to genetic risk factors, hormonal and environmental factors have been proposed to contribute to the development of SLE. For example, female patients with SLE tend to have flares just before menstruation, and higher levels of hormones correlate with increased mortality risk (Rood et al., 1998) indicating that hormones may also play a role in the pathology of lupus. Patients with SLE also show abnormal immune responses to UV radiation as well as seropositive results for Epstein-Barr viral infections (Golan et al., 1992; James et

al., 1997) , indicating a possible role for environmental factors in the development of SLE.

Symptoms of scleroderma can also vary widely among patients but can include fibrosis of the skin, lungs and other organs, vasculitis, and Raynaud's phenomenon. Similar to SLE, the cause of scleroderma remains unknown, yet linkage studies on families with SSc have shown that genetics play an important role in the etiology of this autoimmune disease. Choctaw Indians, who have an abnormally high incidence of SSc, and Japanese patients with SSc show specific polymorphisms in the fibrillin1 gene (*FBN1*), indicating that this gene may be a risk factor for developing scleroderma (Tan et al., 2000). The fibrillin gene encodes for an extracellular matrix (ECM) glycoprotein (Siracusa et al., 1996) and disruption of ECM proteins may contribute to fibrosis seen in these patients. The tight skin mouse (tsk1), which contains a duplication of the *FBN1* gene, is used as an animal model of scleroderma. Tsk1 mice develop some scleroderma-like symptoms, but not all, indicating that the etiology of scleroderma is multi-factorial.

Similar to SLE, patients with SSc exhibit abnormalities in response to environmental factors in addition to genetic factors. For example, patients with SSc show abnormal cardiovascular responses to cold exposure compared to patients with primary Raynaud's and normal controls (Engelhart, 1990; Long et al., 1986). Cold-induced vasoconstrictive changes have been shown to be dependent on a specific subtype of the α2-NA receptor located on vascular smooth muscle cells (Chotani et al., 2000). In fact, the α2-NA receptor has been

shown to be upregulated in patients with scleroderma compared to controls (Flavahan et al., 2000), though it is not clear if the upregulation is in response to cold exposure or if the expression pattern is constitutive in these patients.

While scleroderma and lupus are autoimmune diseases, presumably of different etiology, they have many overlapping symptoms. The commonalities between scleroderma and lupus which are of particular importance in this dissertation include an exaggerated inflammatory response, sympathetic dysfunction, and vascular abnormalities. Since both of these diseases are multi-factorial, it is possible that the abnormalities seen in both scleroderma and lupus occur in a particular order and are dependent upon one another for the expression and maintenance of disease symptoms.

7. Clinical Relevance of Sympathetic and Vascular Dysfunction in Immune Disease

The SNS and vascular functioning show abnormalities in several disease states in which sympathetic tone is too high (Olsen et al., 1987). For example, sympathetic tone is hypothesized to play a role in the pathophysiology of Raynaud's, which is characterized by blanching of the extremities (Generini et al., 1996). Raynaud's phenomenon is one of the primary symptoms of connective tissue diseases such as SLE or, most prominently, scleroderma (systemic sclerosis (SSc)). Patients with SSc, for instance, have been shown to have increased sympathetic activity, e.g. heart rate ratio from lying to standing, compared to normal age-matched controls (Cozzolino et al., 2002). SSc patients exhibiting sympathetic and vascular abnormalities show increased arteriole

constrictor responses of the a2-adrenoreceptor, the NE receptor on vascular endothelium (Flavahan et al., 2000). Changes in NE receptors may be a consequence of sympathetic dysfunction, not autoimmunity per se, since sympathetic denervation leads to enhanced NE-mediated smooth muscle contraction (Abdel-Latif and Zhang, 1991; Hogestatt et al., 1988). As vessels constrict in the afflicted extremities of patients with sympathetic dysfunction, e.g. Raynaud's, they become hypoxic, potentially leading to the blood vessel damage observed in SSc patients. As a long-term consequence of this damage, endothelium-dependent vasodilation is often severely compromised (Schlez et al., 2003), further worsening the vasoconstriction.

Primary Raynaud's may precede the onset of autoimmune diseases such as SSc and SLE by many years, but it can be distinguished from Secondary Raynaud's by some hallmark features. The presence of antinuclear antibodies (ANA) and increased levels of endothelial injury byproducts (Ho and Belch, 1998) usually appear when autoimmune disease is present, suggesting a progression from sympathetic dysfunction to vascular damage. Additionally, patients with secondary disease show increased white blood cell activation around affected areas (Lau et al., 1992a; Lau et al., 1992b). In addition to alterations in the inflammatory response, patients also show evidence of reduced fibrinolysis (Ho and Belch, 1998) and diffuse tissue fibrosis (Wigley, 1996), potentially reflecting endothelial cell dysfunction.

The sympathetic, vascular, and inflammatory changes seen in patients with SSc or SLE demonstrate a complex relationship between these systems.

Our studies looking at the interaction between sympathetic dysfunction and the neurotrophic factor BDNF may elucidate the etiology of these types of autoimmune diseases.

8. Autoimmune-prone Animals as a Model to Induce Perivascular Inflammation

In the current experiment, female Lewis rats were used to induce autoimmunity instead of Sprague-Dawleys because Lewis rats are highly susceptible to autoimmune diseases. Studies have shown that autoimmune susceptibility in Lewis rats is mediated by genetic factors since cross breeding these rats with autoimmune resistant strains leads to a reduction in the expression of autoimmune diseases (Roth et al., 1999; Waxman et al., 1981). Some differences seen in autoimmune-prone Lewis rats include enhanced expression of pro-inflammatory molecules such as MHC class II and TNFα (Massa et al., 1987; Chung et al., 1991), decreased responsiveness of the hypothalamic-pituitary-adrenocortical axis (Stefferl et al., 1999), and decreased expression of the vasoactive substance nitric oxide (Staykova et al., 2005), which is important in regulating vasodilation.

In addition to using Lewis rats instead of Sprague-Dawley rats for autoimmune induction, we also switched to female rats. Most autoimmune diseases preferentially affect women. It is unclear why this preference exists, though hormones are considered to be a primary factor. For example, induction of EAE in naïve animals, using myelin-reactive T cells from donor animals, is more severe when T cells donated from female mice are used to induce

autoimmunity (Bebo et al., 1999), indicating a role of androgens in inflammatory responses. Indeed, estrogen receptors have been found on immune cells (Tornwall et al., 1999), and activation of these receptors leads to upregulation of cytokines such as IL-6 (Yang et al., 2006).

In the current study, we observe that female Lewis rats treated with autoimmune induction of sympathetic damage and BDNF show perivascular white blood cell infiltrates. Interestingly, evidence of inflammation is also present in animals treated with autoimmune-induced sympathetic damage alone, exposure to CFA alone, and exposure to CFA and BDNF combined. These results suggest that a genetic predisposition to autoimmunity alone may be enough to elicit abnormal inflammatory responses directed against self-antigens. That is, a genetic predisposition to abnormal inflammatory responses may result in low levels or sub-clinical manifestations of autoimmune disease. However, when genetic predisposition interacts with either elevated levels of BDNF or abnormal sympathetic activity, it may further enhance the abnormal inflammatory response and therefore contribute to either the pathogenesis or full clinical expression of autoimmune disease.

In the current study we also observed that animals treated with autoimmune-induced sympathetic damage show increased fibrinogen deposition in tissue in the absence of BDNF. Similar to the result seen with perivascular inflammation, animals treated with the combination of CFA alone and BDNF also show evidence of increased fibrinogen deposition in tissue. Interestingly, when CFA combined with a specific sympathetic antigen, SCG, is given with BDNF,

fibrinogen deposition decreases to a level below that of saline controls. It is possible that induction of autoimmune-mediated damage of sympathetic fibers in autoimmune-prone animals is sufficient to cause vascular abnormalities even in the absence of exogenous BDNF. However, when exogenous BDNF is present, vascular abnormalities may occur in autoimmune-prone animals even in the absence of specific autoimmunity. That is, it is not necessary for the immune system to errouneously recognize a self-antigen. Instead, the immune system may already be primed, due to genetic factors, such that high levels of BDNF potentiate the immune response to a non-specific stimulus resulting in vascular abnormalities. One interesting finding is the low level of vascular leak seen in animals receiving BDNF and autoimmune-induced sympathetic damage. It may be that in our model, BDNF is acting as a survival factor for sympathetic fibers undergoing active autoimmune-mediated damage. A possible outcome of these trophic actions of BDNF on sympathetic neurons could be to prevent the dysregulation of vascular tone, therefore resulting in decreased vascular leak. If BDNF is acting in this capacity to prevent sympathetic damage, it still may be able to act as a pro-inflammatory molecule, resulting in extravasation of white blood cells without active leak occurring. However, it is more likely that the low level of vascular leak seen in animals receiving BDNF and autoimmune-induced sympathetic damage is due to edemic pressure on vasculature, which could prevent leak of plasma protein independently of permeability to inflammatory cells. This speculative mechamism could explain the disparate results we found in our model where animals receiving BDNF and autoimmune-induced

sympathetic damage show evidence of perivascular inflammation in the absence of vascular leak.

Alternatively, BDNF itself could be acting as an anti-inflammatory agent. Studies have shown that NGF can act in this manner but typically, the actions of BDNF have been shown to be pro-inflammatory (Kerschensteiner et al., 1999; Vega et al., 2003). However, it is unlikely that BDNF is acting in an anti-inflammatory capacity since animals receiving BDNF and CFA alone showed evidence of both increased perivascular inflammation and vascular leak.

7. Limitations in Interpretation

While our results show that the combination of BDNF and sympathetic dysfunction results in perivascular inflammation and vascular abnormalities, it does not address the mechanisms underlying this phenomenon.

It is still unclear how BDNF and sympathetic dysfunction interact to produce inflammation but it is possible that this phenomenon would not occur in more physiologically relevant contexts. The levels of all reagents used in these experiments were very high and might not have been representative of levels seen in the body even during pathophysiological conditions. In addition, many autoimmune diseases present in organs other than the brain. In fact, with the exception of neuropsychiatric lupus and multiple sclerosis, autoimmune diseases typically do not affect the brain. Common organs which do show abnormalities in autoimmune diseases include the lungs, kidneys, and skin. It will be important to determine if the interaction between BDNF and sympathetic dysfunction manifests in similar ways in other systems beside brain.

Another important limitation to consider is the fact that BDNF has not been studied extensively in the context of autoimmunity. Many studies have looked at correlations between the levels of NGF in CSF or serum and disease activity (Laudiero et al., 1992), and some studies have found NGF to be anti-inflammatory (as reviewed in Viloslada and Genain, 2005). The literature on neurotrophins in autoimmunity does not extensively cover BDNF in this capacity, either because no correlations were found between these two factors, or because there have been little data generated supporting the idea that BDNF and autoimmune diseases are linked in any way. The role BDNF plays in perivascular inflammation in the presence of sympathetic abnormalities may be epiphenomenological or only partially contributory to the phenomenon. However, based on the potentiation of inflammation by BDNF in all of our experiments, it seem parsimonious to conclude that this neurotrophin is likely to play a role in the development of inflammatory disease subsequent to sympathetic abnormalities.

8. Speculations

Sympathetics and Neurotrophins

The pathology of both Primary Raynaud's and Scleroderma consists of dysregulation of the sympathetic nervous system. Nerve growth factor and brain-derived neurotrophic factor have trophic effects on sympathetic neurons (Glebova and Ginty, 2004). Levels of BDNF and NGF are higher in patients with autoimmune disease (Petereit et al., 2003; Oderfeld-Nowak et al., 2001) and therefore may be an important link in either the pathogenesis or expression of inflammatory responses in such diseases. Elevated levels of neurotrophins, and

in particular BDNF, may be etiological, contributory or epiphenominological to the development of inflammatory diseases with concurrent sympathetic abnormalities.

It is unclear at this point why certain neurotrophin levels are higher in patients with autoimmune diseases. However, there are several possibilities including both pathological and genetic factors. BDNF has been found to be upregulated after ischemia, which is particularly relevant in SSc patients who exhibit vascular abnormalities and insufficiency, typically manifested as Raynaud's Phenomenon (Tokumine et al., 2003; Zeng et al., 2001; Stadelmann et al., 2002; Miyake et al., 2002; Ferrer et al., 2001). Although BDNF is not typically associated with sympathetic neuron survival, overexpression of BDNF in experimental models has been shown to lead to enhanced sympathetic innervation of target organs (Botchkarev et al., 1998) and increased vasoconstrictor responses of small arteries (Springer et al., 2004). While BDNF plays an important role in the pathogenesis of perivascular inflammation seen in our experiments, it is possible that BDNF potentiates this inflammation in our models by upregulating other factors. In particular, BDNF is a potent inducer of NPY (Croll et al., 1994), a sympathetic neurotransmitter, which itself has been implicated in the development of inflammatory disease (Reibel et al., 2000; Hassani et al., 2005).

Our results show that inhibition of NGF binding to its endogenous receptors, when combined with BDNF, leads to abnormalities in the morphology of sympathetic fibers as they contact brain vasculature as well as abnormal

perivascular inflammation. Additionally, our results also demonstrate that sympathetic damage, induced either surgically or by an autoimmune-mediated mechanism, leads to similar abnormal inflammatory activity and vascular leak when combined with BDNF. Overall, these results indicate that both sympathetic abnormalities and elevated levels of BDNF must be present in order to produce perivascular inflammation.

Sympathetic abnormalities in patients susceptible to autoimmune diseases may occur through several mechanisms: 1) damage may be genetically predetermined such that SNS abnormalities are present throughout the lifespan, but consequences of these abnormalities only surface later in life; 2) damage may be induced by erroneous recognition of sympathetic fibers by immune cells, leading to subsequent dysfunction of sympathetics; and 3) damage may be induced by environmental factors such as exposure to cold temperatures and/or stress, also leading to damage and subsequent dysfunction of sympathetics.

Abnormally high BDNF levels in patients prone to autoimmune diseases may also occur through several possible mechanisms including: 1) patients prone to autoimmune disease could have a higher constitutive expression of BDNF, mediated by genetic, transcriptional, or translational regulatory abnormalities; 2) if patients prone to autoimmune disease exhibit sympathetic abnormalities leading to dysregulation in vasculature, they may undergo a multitude of ischemic events leading to elevated levels of BDNF and subsequent inflammatory disease; and 3) expression of BDNF in response to ischemic events may be elevated in people prone to autoimmunity, compared to

autoimmune-resistant people, therefore leading to the expression of inflammatory disease in response to events that would not normally elicit abnormal inflammatory responses.

9. Conclusion

In the present study we tested the effect of sympathetic dysregulation, in conjunction with BDNF infusion, on inflammation in adult rat brain. We found that perivascular leak and inflammation occurred only when BDNF was infused in conjunction with any manipulation leading to sympathetic abnormalities. Neither sympathetic abnormalities alone, nor BDNF alone, caused any discernable inflammation. Our observations suggest the possibility that the difference between those patients with isolated sympathetic pathologies, such as Primary Raynaud's, versus those who progress to inflammatory disease is the presence of high levels of BDNF. That is, patients who either have higher basal levels of BDNF, or have increased ischemic induction of BDNF, may have a greater chance of developing inflammatory disease as a consequence of their sympathetic abnormalities. Future experiments should help to further elucidate the pathological relevance of this phenomenon for autoimmune disease, as well as uncover the precise mechanism of this interaction.

FIGURE CAPTIONS

Figure 1. Neurotrophins and their receptors

Figure 2. Schematic of the autonomic nervous system

Figure 3. Schematic of arteries, arterioles and veins depicting the different investment of endothelial cells, smooth muscle cells, and basement membrane in each type of vessel (adapted from www.people.virginia.edu/ ~dp5m/phys_304/pix.html).

Figure 4. Differences between peripheral capillaries and brain capillaries. Brain capillaries have tight junctions between endothelial cells, fewer fenestrations, and an investment of astroglial endfeet all working together to limit the exchange of substances between the circulation and brain tissue.

Figure 5. Different pathways of vasoconstriction and vasodilation in vasculature

Figure 6. Extravasation of white blood cells from the circulation, through the endothelial cell wall, into tissue

Figure 7. Cold stress test color chart. A score of 0 represents pure white and a score of 19 represents pure red.

Figure 8. Representative photomicrograph of hippocampal figure at low magnification. Blow up segment of blood vessels in hippocampal fissure as a representation of where quantification of different cell types was done. Low magnification scale bar = 100μm. High magnification scale bar = 50μm.

Figure 9. Subjective inflammatory rating scale. A score of 0 represents no inflammation and a score of 4 represents maximum inflammation. Scale bar = 50μm.

Figure 10. Photomicrographs showing cuffed vessels, comprised of inflammatory cells, in animals treated with BDNF + TrkA-Fc. Nissl stained sections of **(A)** PBS alone, **(B)** BDNF alone and **(C)** TrkA-Fc alone treated animals with normal vasculature in the hippocampal fissure. Nissl stained section of **(D)** BDNF + TrkA-Fc treated animal showing vascular cuffing. Scale bar (A-D) = 100μm. Low magnification nissl stained **(E)** control brain reveals normal vasculature in hippocampal fissure. Low magnification **(F)** BDNF + TrkA-Fc brain shows evidence of vascular cuffing throughout hippocampal fissure. OX-1 stained section showing **(G)** control brain with no evidence of OX-1 positive inflammatory cells around vasculature in hippocampal fissure. Inset shows high magnification of single vessel. OX-1 stained section of **(B)** BDNF + TrkA-Fc brain with vascular cuffing staining positive for OX-1 cells, indicating inflammatory cells surrounding vasculature. Inset shows high magnification of single vessel. Scale bar (E-H) = 100μm.

Figure 11. Animals treated with BDNF +TrkA-Fc show a significantly decreased vascular diameter in SMA positive vessels compared to all other control groups, $p<.002$

Figure 12. There is no significant difference in vascular diameter in SMA negative vessels in any treatment groups, $p<.05$

Figure 13. TH stained sections show that animals treated with BDNF + TrkA-Fc show significantly more coarse fibers than control groups. **(A)** PBS alone **(B)** TrkA-Fc alone **(C)** BDNF + TrkA-Fc. $p<.019$. Scale bar A-C=40μm

Figure 14. Neurolucida tracings show that fibers in animals treated with TrkA-Fc have a larger volume than those treated with control. **(A)** PBS alone **(B)** TrkA-Fc alone **(C)** BDNF + TrkA-Fc. $p<.019$. Scale bar = 50μm **(D)** Graph showing that animals treated with TrkA-Fc have higher fiber volume than those treated with controls, $p<.026$. In addition, fibers in the BDNF co-infused group had a significantly larger volume than both PBS alone and BDNF alone groups, $p<.05$. Scale bar=50μm.

Figure 15. No difference seen in mean fiber diameter between treatment groups, $p>.05$. However, animals treated with TrkA-Fc show a trend towards increased fiber diameter compared to controls, $p<.087$

Figure 16. No difference seen in mean fiber tortuosity between treatment groups, $p>.05$

Figure 17. No difference seen in number of fiber contacts per vessel between treatment groups, $p>.05$

Figure 18. Nissl-stained sections showed that infusion of BDNF in animals receiving SCG removal results in vascular cuffing. Sympathectomized animals infused with PBS show no evidence of cuffing at **(A)** 3 days or **(B)** 12 days after sympathectomy. Sympathectomized animals infused with BDNF show abnormal vasculature and increased cellularity at **(C)** 3 days but normal vasculature at **(D)** 12 days. Sham surgery animals infused with either PBS (data not shown) or BDNF show normal vasculature at **(E)** 3 days and **(F)** 12 days. Scale bar=100μm.

Figure 19. Graph showing that animals treated with 12 day sympathectomy have significantly fewer innervated vessels compared to 3 day sympathectomy animals ($F(1,4)=8.021;p<.047$; $HSD(2,4)=43.89$; $p<.05$).

Figure 20. Graph showing that animals treated with 12 day sympathectomy have significantly fewer fiber contacts per vessel compared to 3 day sympathectomy animals ($F(1,5)=43.79$; $p<.001$; $HSD(2,5)=.488;p<.05$).

Figure 21. Graphs showing that animals receiving BDNF + 12 day sympathectomy exhibit changes in vascular smooth muscle cells such that there is a significant increase in the number of mixed smooth muscle cells (a mixture between smooth muscle cells that are wrapped and smooth muscle cells that are unwrapped around blood vessles). **(A)** There is no significant difference in the percent unwrapped vascular smooth muscle cells between groups ($F(1,13)=.266$; $p=.615$). **(B)** There is no difference in the percent wrapped smooth muscle cells between groups ($F(1,13)=.889$; $p=.363$). **(C)** There is a significant increase in the percent mixed vascular smooth muscle cells in animals receiving BDNF and 12 day sympathectomies ($F(1,13)=4.036$; $p<.066$; HSD $(2,13)=10.94$; $p<.05$). **(D)** There is no significant difference in the continuity of vascular smooth muscle cells between any groups ($F(1,13)=3.43$; $p=.087$).

Figure 22. Smooth muscle actin stained photomicrographs, indicating arteries, showing that in animals given long-term (12 day) sympathectomies and BDNF, there appear to be fewer smooth muscle cells wrapped around arterial vasculature. Scale bar = 50μm

Figure 23. Graphs showing that there are no changes in vascular smooth muscle cells after short-term (3 day) sympathectomies. All animals in these groups received BDNF. **(A)** There is no significant difference in percent mixed vascular smooth muscle cells in sham animals and short-term sympathectomy (3

day) animals receiving BDNF (t(5)=.721; p=.435). **(B)** There is no significant difference in percent continuous vascular smooth muscle cells in sham animals and short-term sympathectomy animals (3 day) receiving BDNF (t(5)=1.00; p=.368.

Figure 24. Photomicrograph showing that GFAP-positive activated glial cells associated with vasculature are increased in animals receiving long-term (12 day) sympathectomies and BDNF **(D,** compared to **A-C)**. All animals in this groups are long-term (12 day) sympathectomy animals. Scale bar = 50 μm.

Figure 25. Graph showing that animals receiving 12 day sympathectomy and BDNF had significantly more activated glia associated with vasculature than animals receiving sham surgery and BDNF (F(1,13)=5.414; p<.037; HSD(2,13)=19.25;p<.05).

Figure 26. Graph showing no differences in activated glial cells associated with vasculature in animals receiving sham + BDNF and short-term (3 day) sympathectomies + BDNF (t(5)=.675; p=.530).

Figure 27. Photomicrograph of Nissl-stained sections showing increased cellularity in animals receiving NE. **(A)** Control animals receiving PBS show no evidence of increased cellularity. **(B)** Animals receiving NE alone show a slight increase in cellularity. **(C)** Animals receiving BDNF alone show no evidence of

increased cellularity. **(D)** Animals receiving BDNF + NE show increased cellularity compared to PBS and BDNF controls as well as a further increase in cellularity over animals receiving NE alone. Scale bar=100μm.

Figure 28. Graph showing no significant changes in vascular diameter in animals treated with BDNF alone, NE alone or BDNF + NE ($F(3,22)=.869$; $p=.472$). However, there is a tendency towards decreased vascular diameter in animals receiving NE alone.

Figure 29. Graph showing that animals given both BDNF + short-term (3 day) sympathectomies and BDNF + NE have an increased proportion of cuffed vessels over control animals ($F(2,30)=8.122$; $p<.002$)

Figure 30. Graph showing no significant difference in axonal volume in animals receiving BDNF and any sympathetic manipulation (sham, trkA-Fc, 3 day sympathectomy, or NE). However, there was a statistical trend where animals receiving trkA-Fc show an increases over sham controls and animals receiving BDNF + NE ($F(3,10)=3.31$; $p=.066$)

Figure 31. Mean fibrinogen area shows that animals receiving BDNF + NE have significantly increased leak compared to both controls and BDNF + 3 day sympathectomies. However, those receiving BDNF + 3 day sympathectomies

fall in the middle between controls and those receiving BDNF + NE ($F(2,37)=7.227$; $p<.002$).

Figure 32. Graph showing abnormal sympathetic function in animals receiving CFA alone and CFA + SCG, such that animals receiving CFA (either with or without SCG) show a significant whitening of their foot pad after exposure to ice, compared to control animals receiving saline ($F(2,16)=6.15$;$p<.01$; LSD=saline vs cfa $p<.005$ LSD=saline vs cfa/scg $p<.017$).

Figure 33. Photomicrograph of Nissl-stained sections showing increased inflammation in animals receiving CFA, both with and without BDNF. **(A)** control animals receiving BSA and saline foot injections show normal vasculature. **(B)** control animals receiving BDNF and saline foot injections also show no evidence of vascular inflammation. **(C)** control animals receiving BSA and CFA only show normal vasculature. **(D)** animals receiving BDNF + CFA alone show evidence of very mild inflammation around blood vessels. **(E)** animals receiving BSA + CFA/SCG show perivascular cuffing. **(F)** animals receiving BDNF + CFA/SCG also show evidence of perivascular inflammation. Scale bar = 200μm.

Figure 34. Graphs showing increased inflammation in animals given sympathetic autoimmune damage. **(A)** Graph showing that animals receiving CFA/SCG, either with or without BDNF, have significantly increased inflammation over CFA only and saline controls ($F(2,9)=8.708$; $p<.008$). Animals receiving

both CFA/SCG and BDNF clearly show the highest amount of inflammation (HSD(2,9)=1.7; p<.05). **(B)** Graph showing that animals receiving CFA/SCG, both with and without BDNF, show significantly increased proportion of cuffed vessles compared to saline. Animals receiving BDNF + CFA alone also show increased proportion of cuffed vessels (F(2,11)=4.627; p<.035; LSD saline vs CFA/SCG, p<.012). Notice saline and BSA/CFA alone have no bars because there were no cuffed vessels

Figure 35. Graph showing no significant difference in mean fibrinogen area for any group (F(2,12)=1.96; p=.183).

Figure 36. Speculative mechanism for the interaction between BDNF, sympathetic dysfunction and inflammatory disease

FIGURES

Figure 1

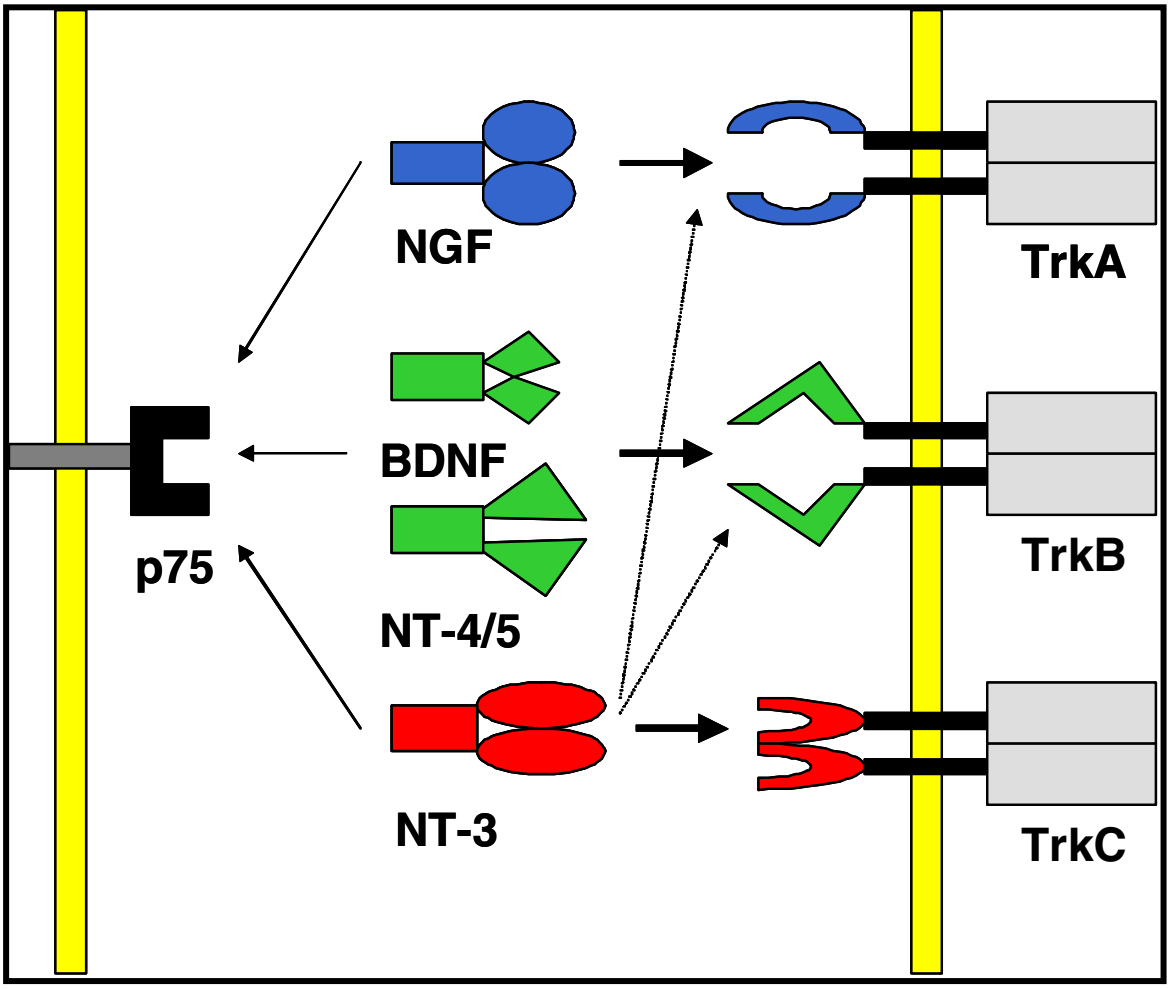


Figure 2

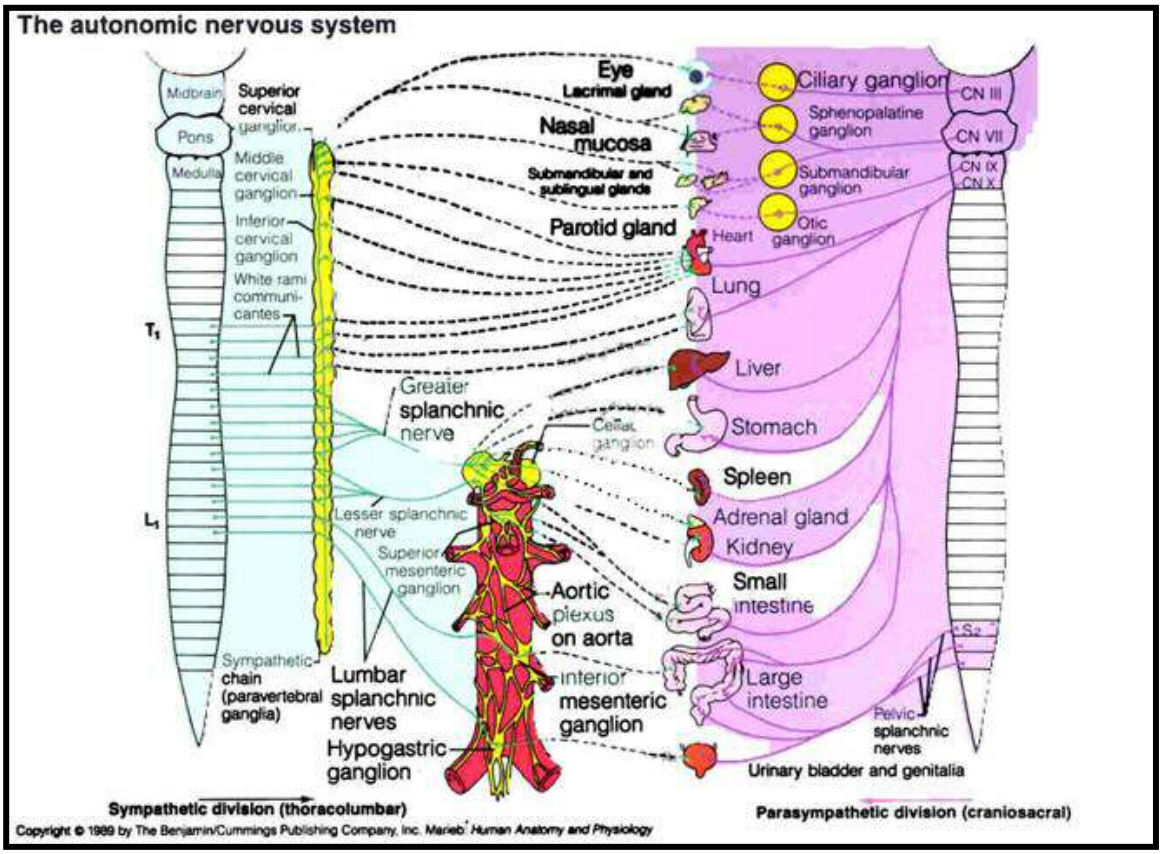

The autonomic nervous system
Midbrain
Pons
Medulla
Superior cervical ganglion
Middle cervical ganglion
Inferior cervical ganglion
White rami communicantes
T1
L1
Eye
Lacrimal gland
Nasal mucosa
Submandibular and sublingual glands
Parotid gland
Heart
Lung
Liver
Stomach
Spleen
Adrenal gland
Kidney
Small intestine
Large intestine
Greater splanchnic nerve
Celiac ganglion
Lesser splanchnic nerve
Superior mesenteric ganglion
Aortic plexus on aorta
Inferior mesenteric ganglion
Sympathetic chain (paravertebral ganglia)
Lumbar splanchnic nerves
Hypogastric ganglion
Ciliary ganglion
Sphenopalatine ganglion
Submandibular ganglion
Otic ganglion
CN III
CN VII
CN IX
CN X
S2
Pelvic splanchnic nerves
Urinary bladder and genitalia
Sympathetic division (thoracolumbar)
Parasympathetic division (craniosacral)
Copyright © 1989 by The Benjamin/Cummings Publishing Company, Inc. Marieb Human Anatomy and Physiology

Figure 3

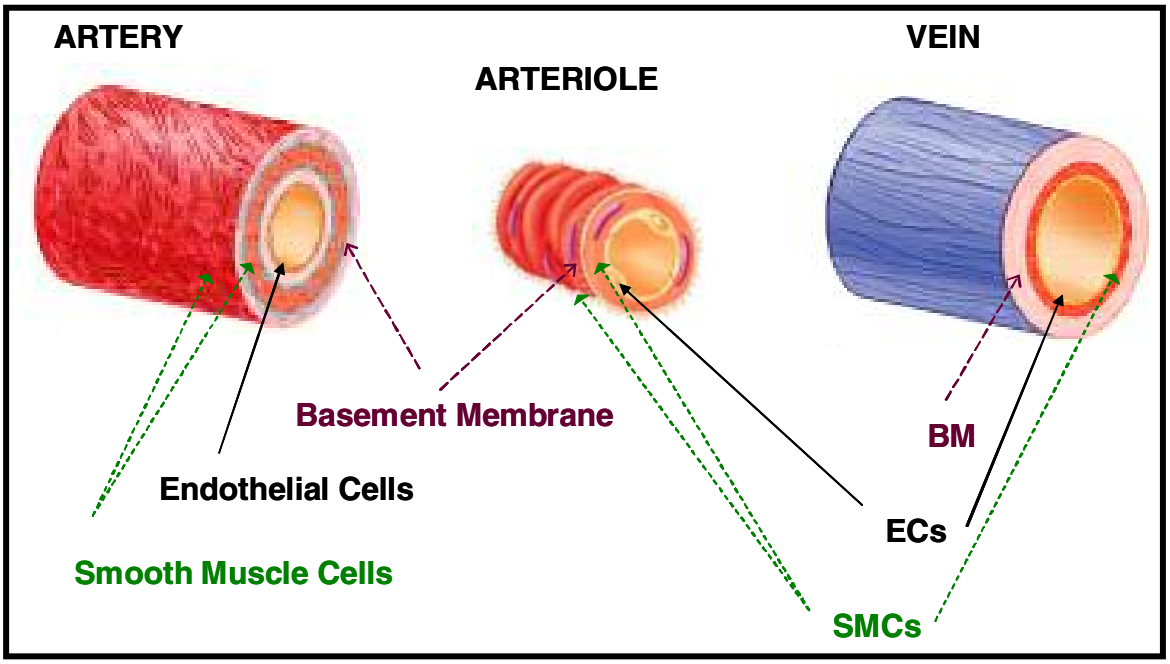

ARTERY
ARTERIOLE
VEIN
Basement Membrane
BM
Endothelial Cells
ECs
Smooth Muscle Cells
SMCs

Figure 4

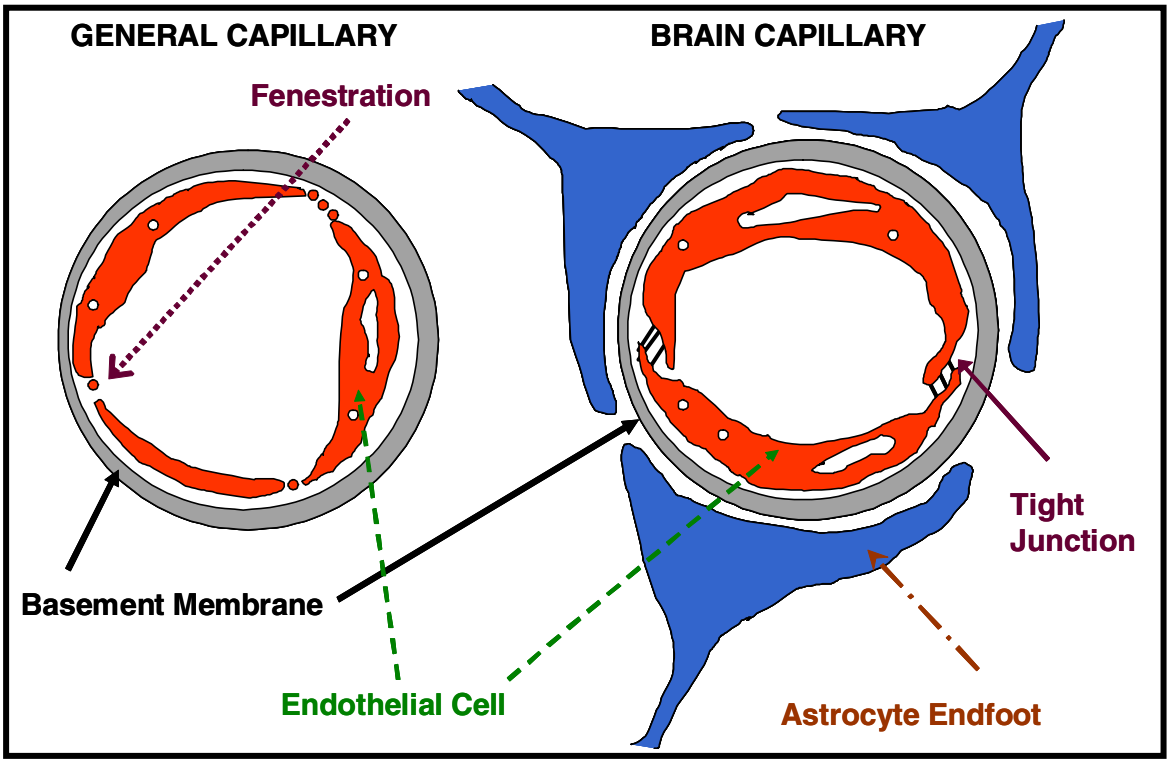

GENERAL CAPILLARY
BRAIN CAPILLARY
Fenestration
Basement Membrane
Endothelial Cell
Tight Junction
Astrocyte Endfoot

Figure 5

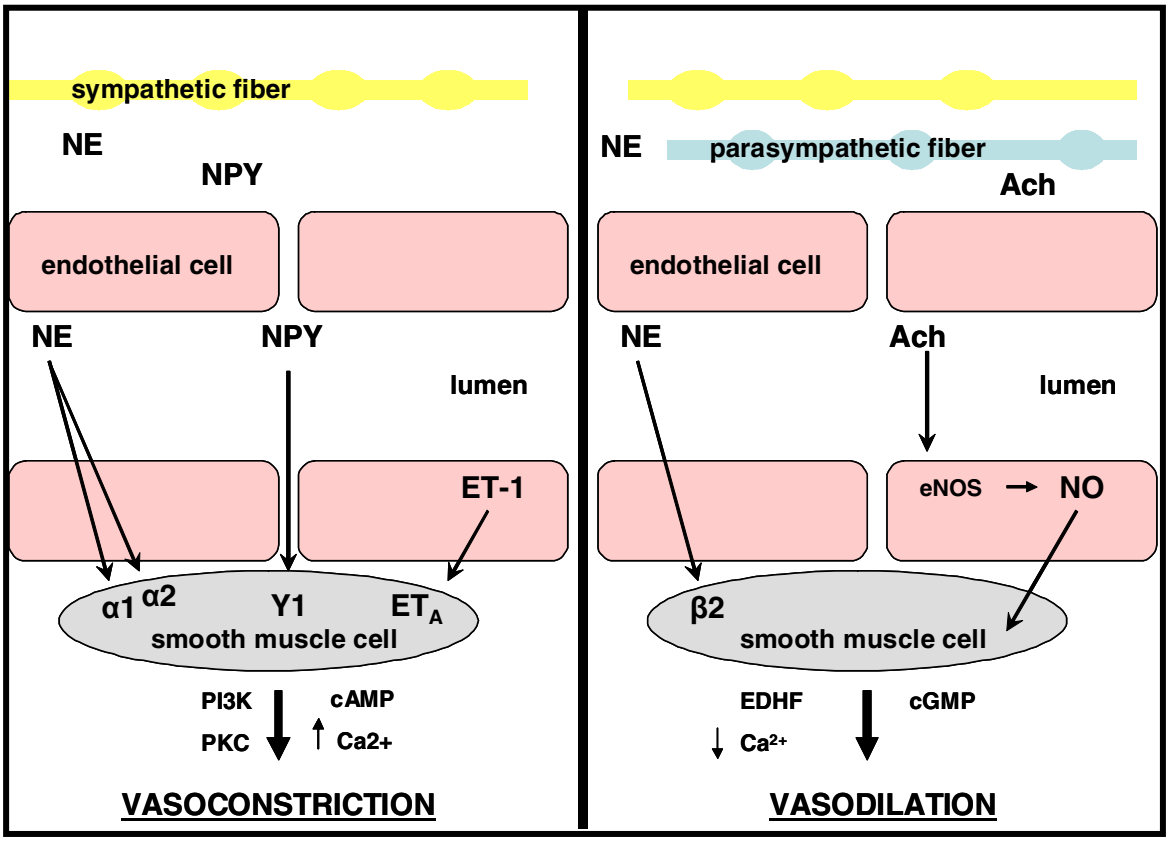

sympathetic fiber
NE
NPY
endothelial cell
NE
NPY
lumen
ET-1
α1 α2
Y1
ET$_A$
smooth muscle cell
PI3K
PKC
cAMP
Ca2+
VASOCONSTRICTION
NE
parasympathetic fiber
Ach
endothelial cell
NE
Ach
lumen
eNOS
NO
β2
smooth muscle cell
EDHF
Ca^{2+}
cGMP
VASODILATION

Figure 6

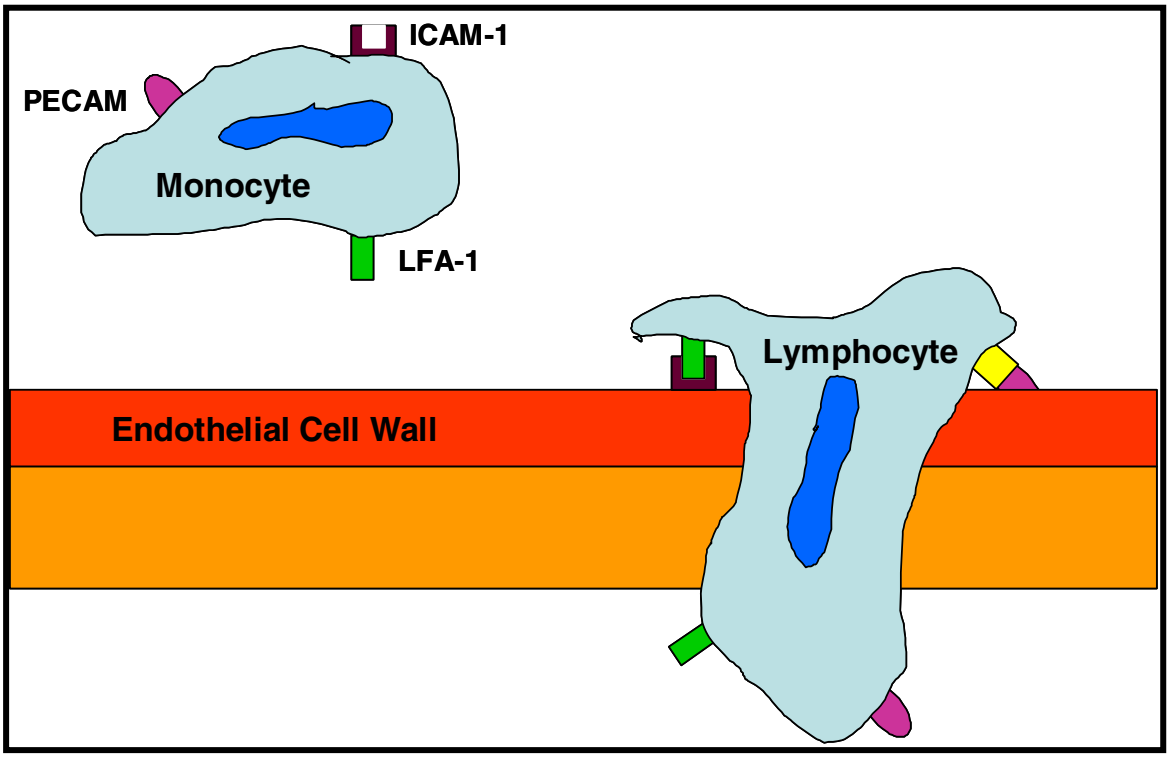

ICAM-1
PECAM
Monocyte
LFA-1
Lymphocyte
Endothelial Cell Wall

Figure 7

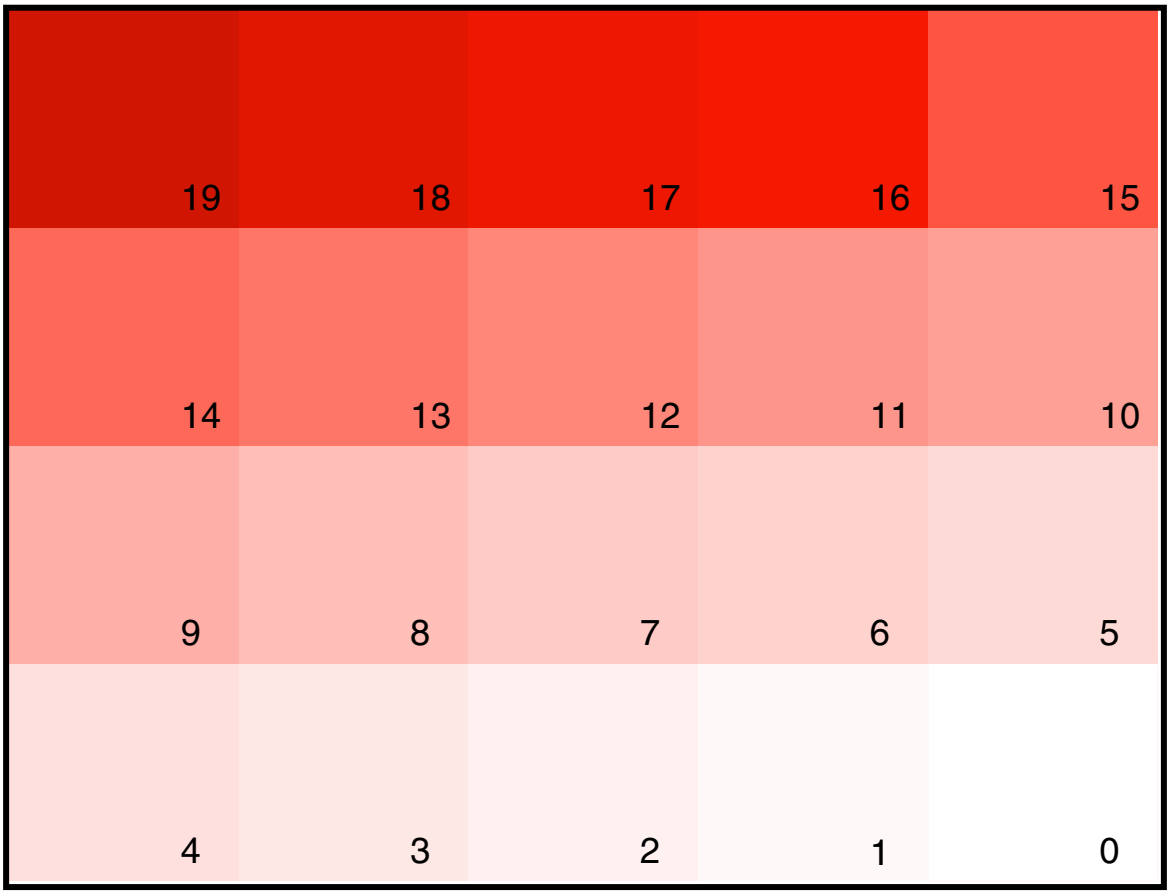


Figure 8

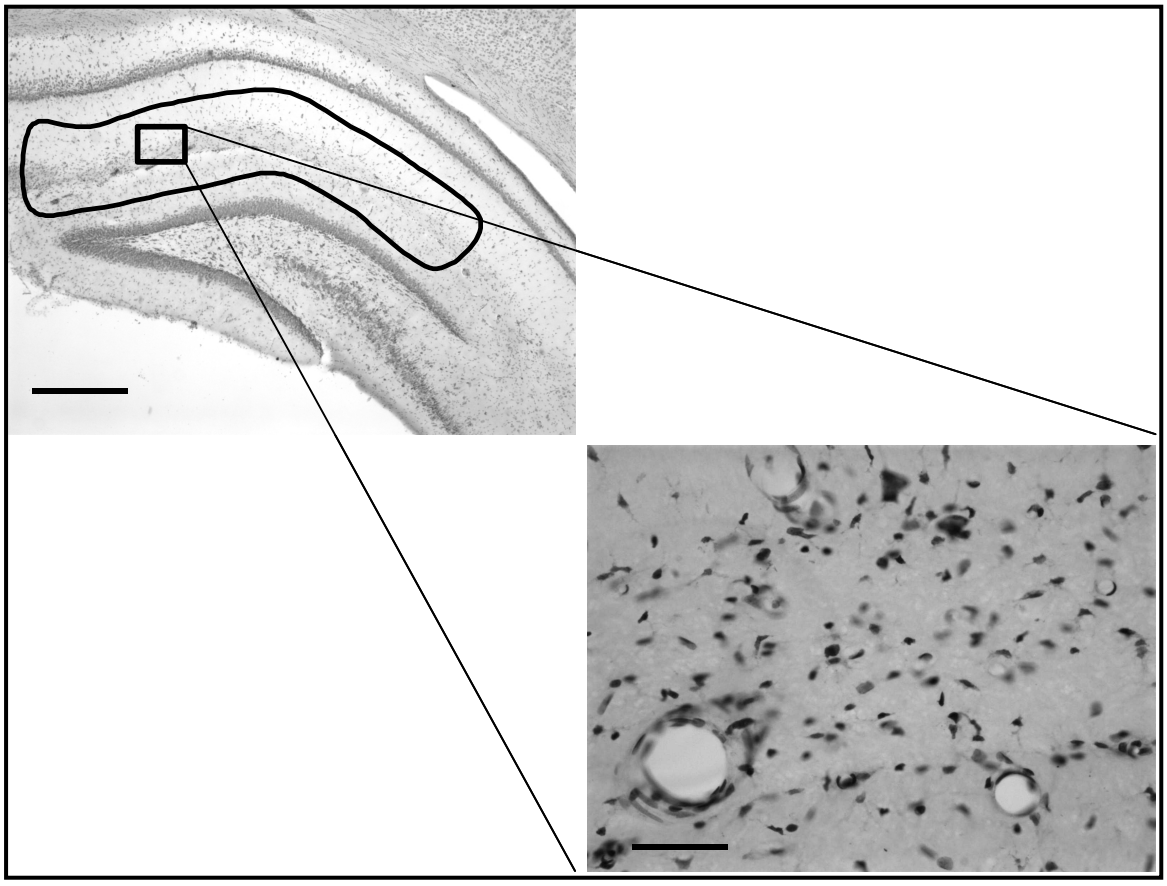


Figure 9

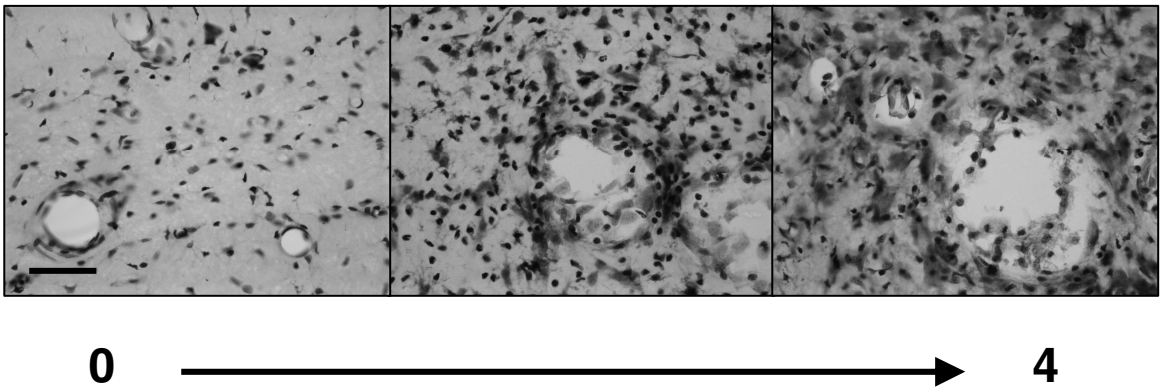


Figure 10

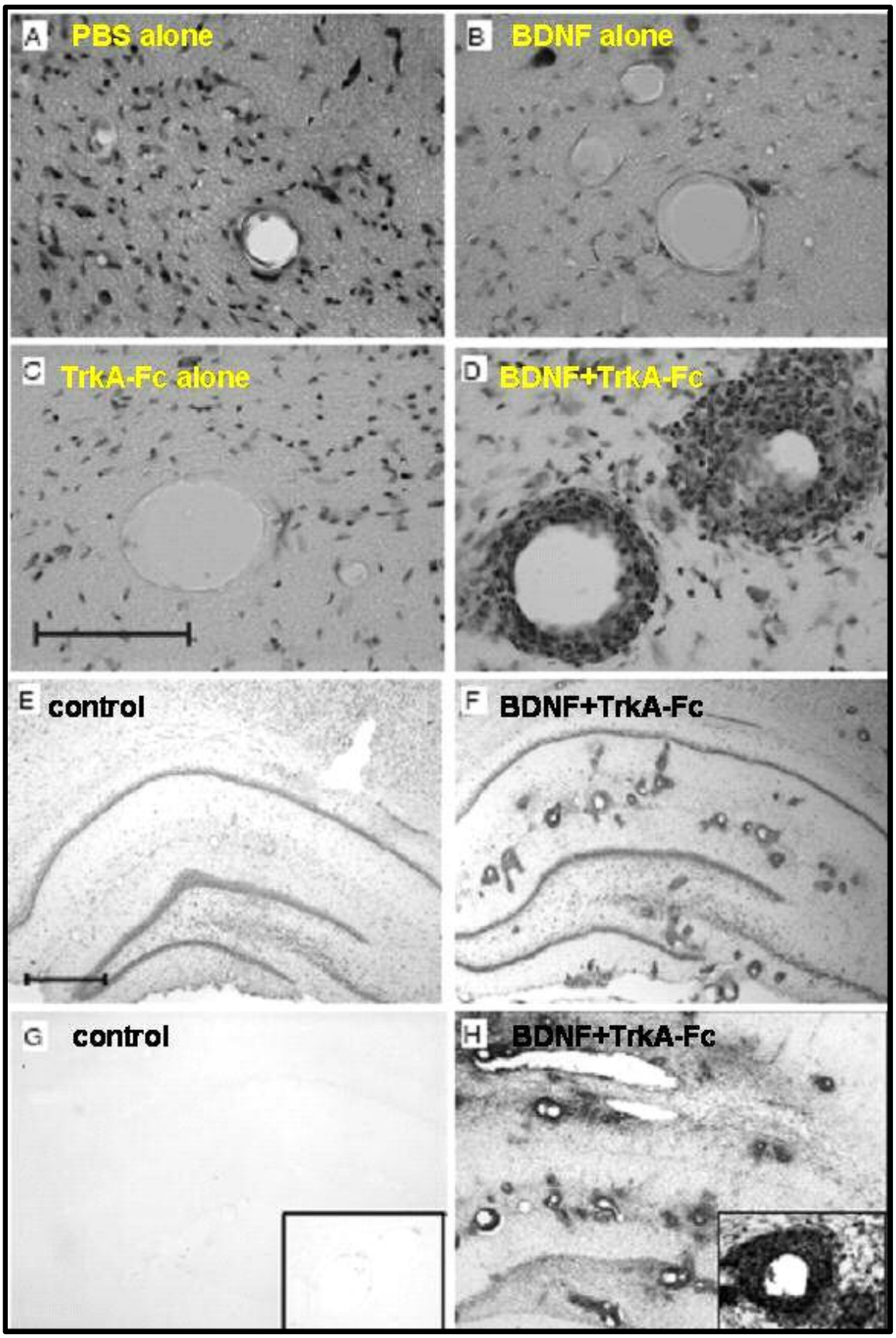

A PBS alone
B BDNF alone
C TrkA-Fc alone
D BDNF+TrkA-Fc
E control
F BDNF+TrkA-Fc
G control
H BDNF+TrkA-Fc

Figure 11

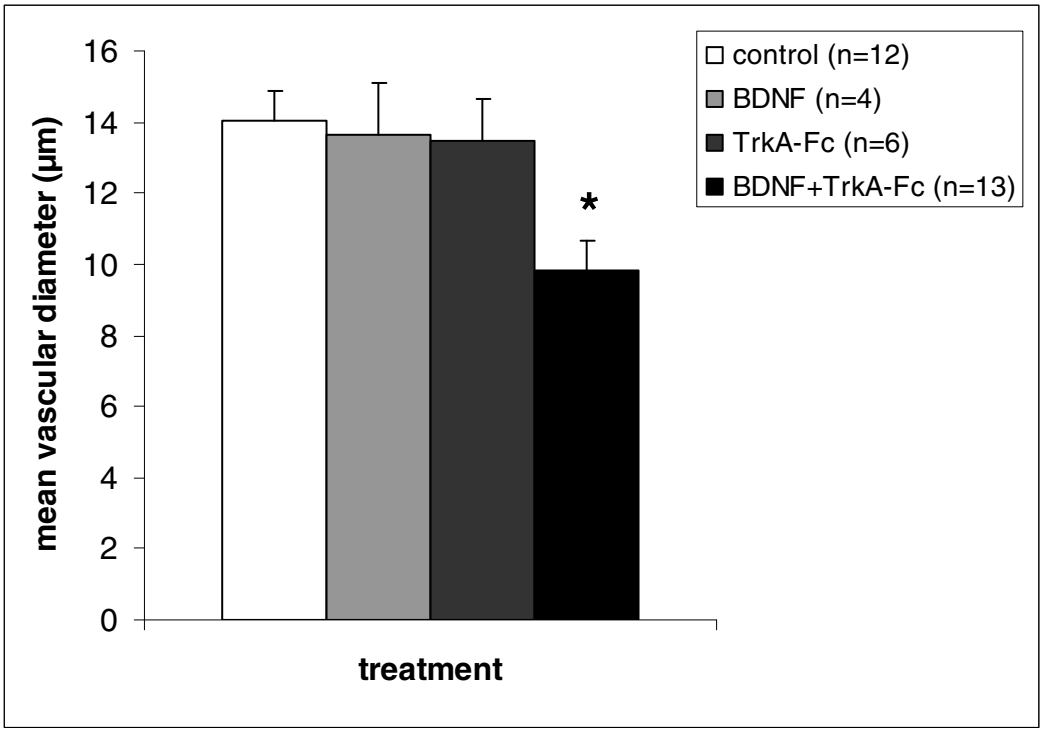

mean vascular diameter (µm)
16
14
12
10
8
6
4
2
0
*
treatment
control (n=12)
BDNF (n=4)
TrkA-Fc (n=6)
BDNF+TrkA-Fc (n=13)

Figure 12

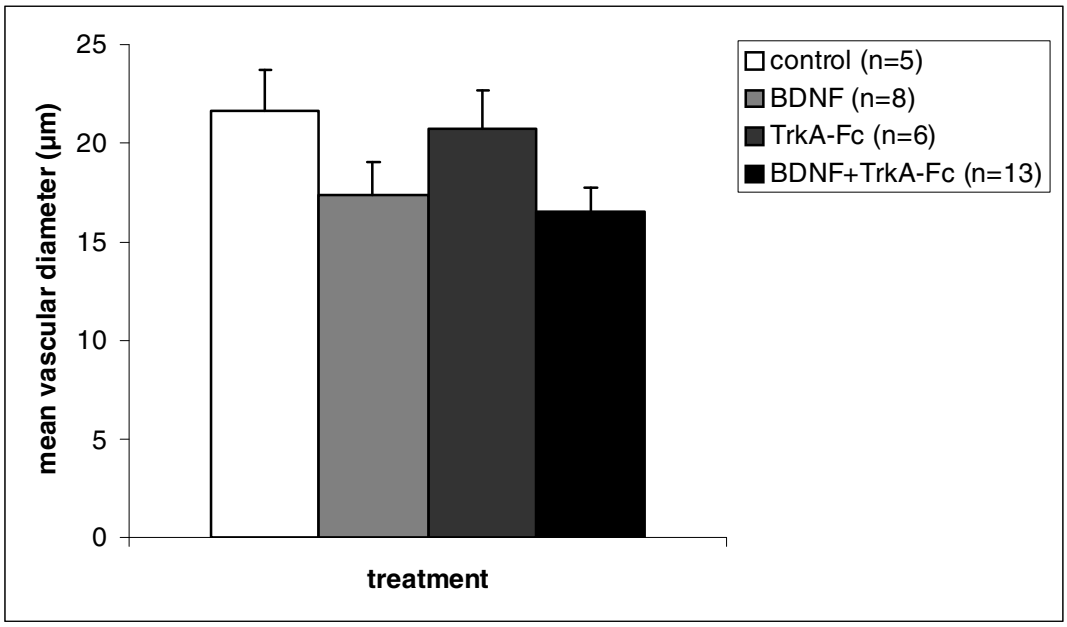

25
20
15
10
5
0
mean vascular diameter (µm)
treatment
control (n=5)
BDNF (n=8)
TrkA-Fc (n=6)
BDNF+TrkA-Fc (n=13)

Figure 13

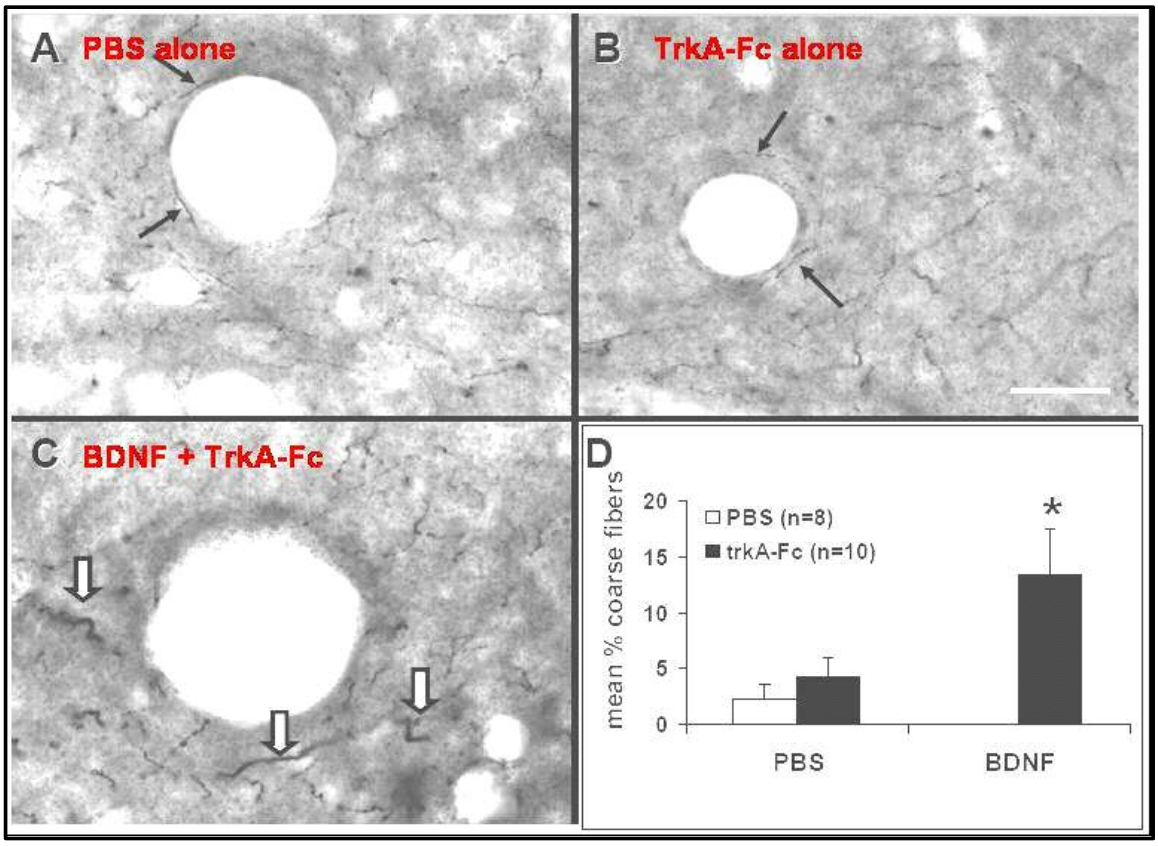

A PBS alone
B TrkA-Fc alone
C BDNF + TrkA-Fc
D
mean % coarse fibers
20
15
10
5
0
PBS (n=8)
trkA-Fc (n=10)
*
PBS
BDNF

Figure 14

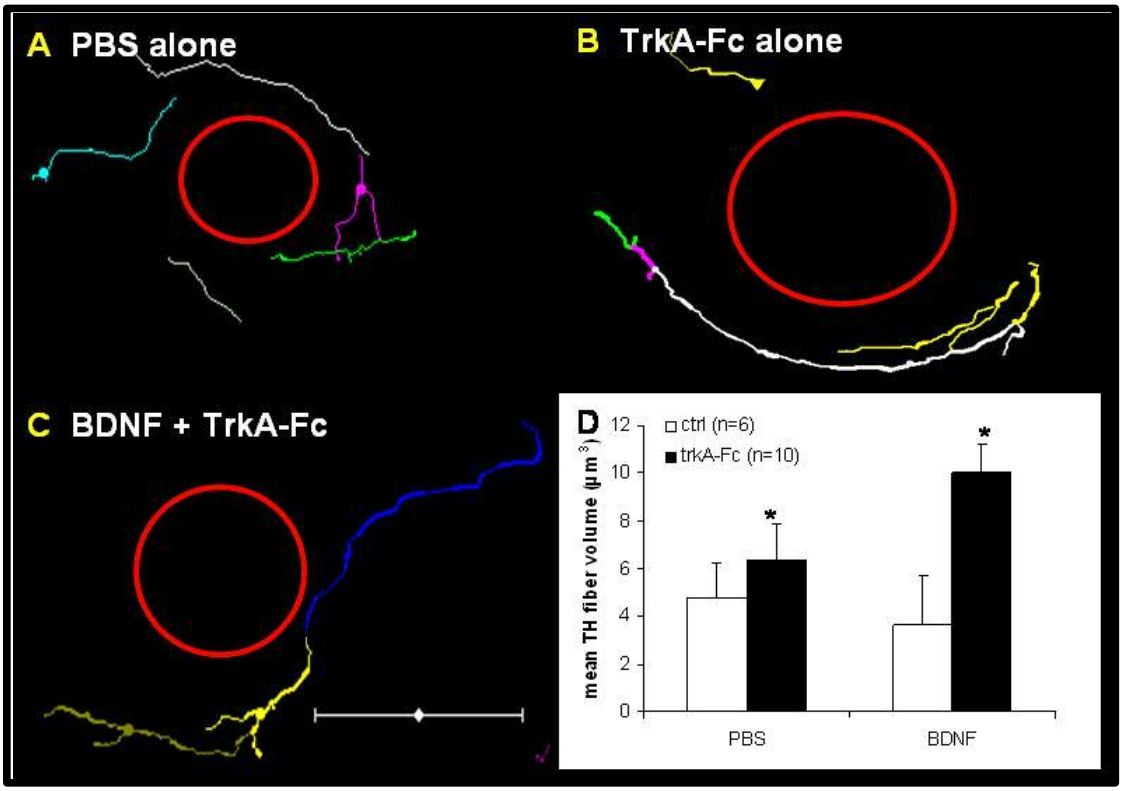

A PBS alone
B TrkA-Fc alone
C BDNF + TrkA-Fc
D
ctrl (n=6)
trkA-Fc (n=10)
mean TH fiber volume (μm³)
0
2
4
6
8
10
12
*
*
PBS
BDNF

Figure 15

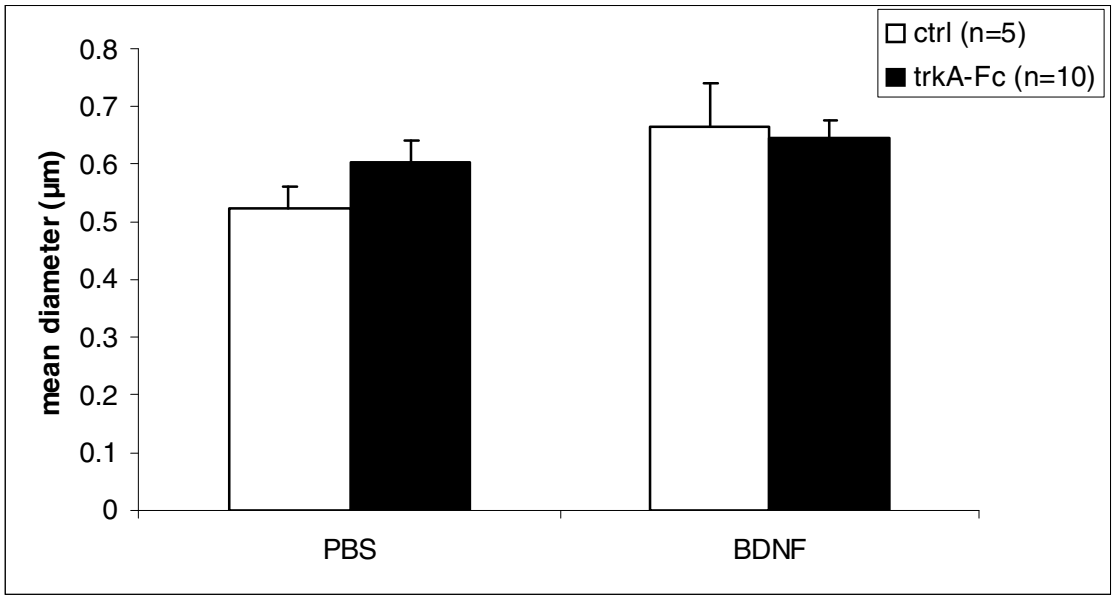

ctrl (n=5)
trkA-Fc (n=10)
mean diameter (µm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
PBS
BDNF

Figure 16

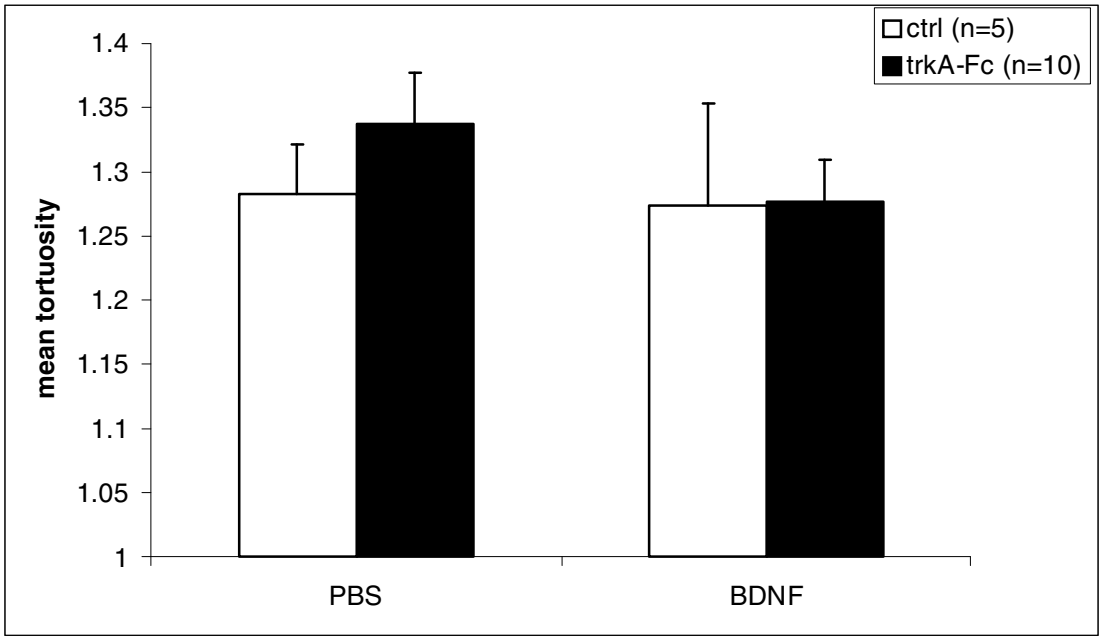

ctrl (n=5)
trkA-Fc (n=10)
mean tortuosity
1.4
1.35
1.3
1.25
1.2
1.15
1.1
1.05
1
PBS
BDNF

Figure 17

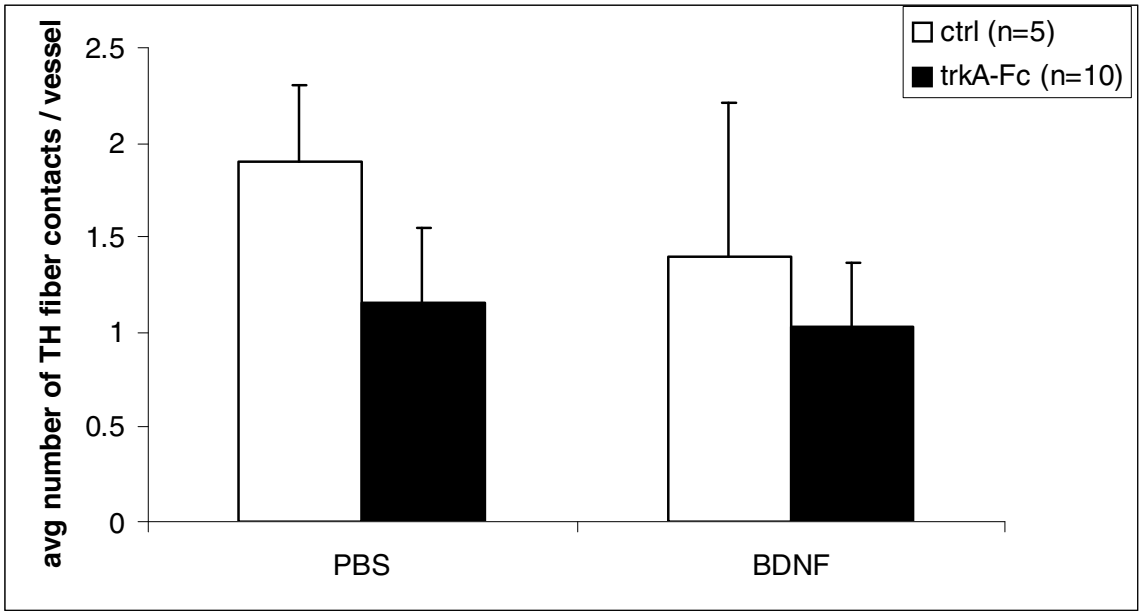

ctrl (n=5)
trkA-Fc (n=10)
avg number of TH fiber contacts / vessel
2.5
2
1.5
1
0.5
0
PBS
BDNF

Figure 18

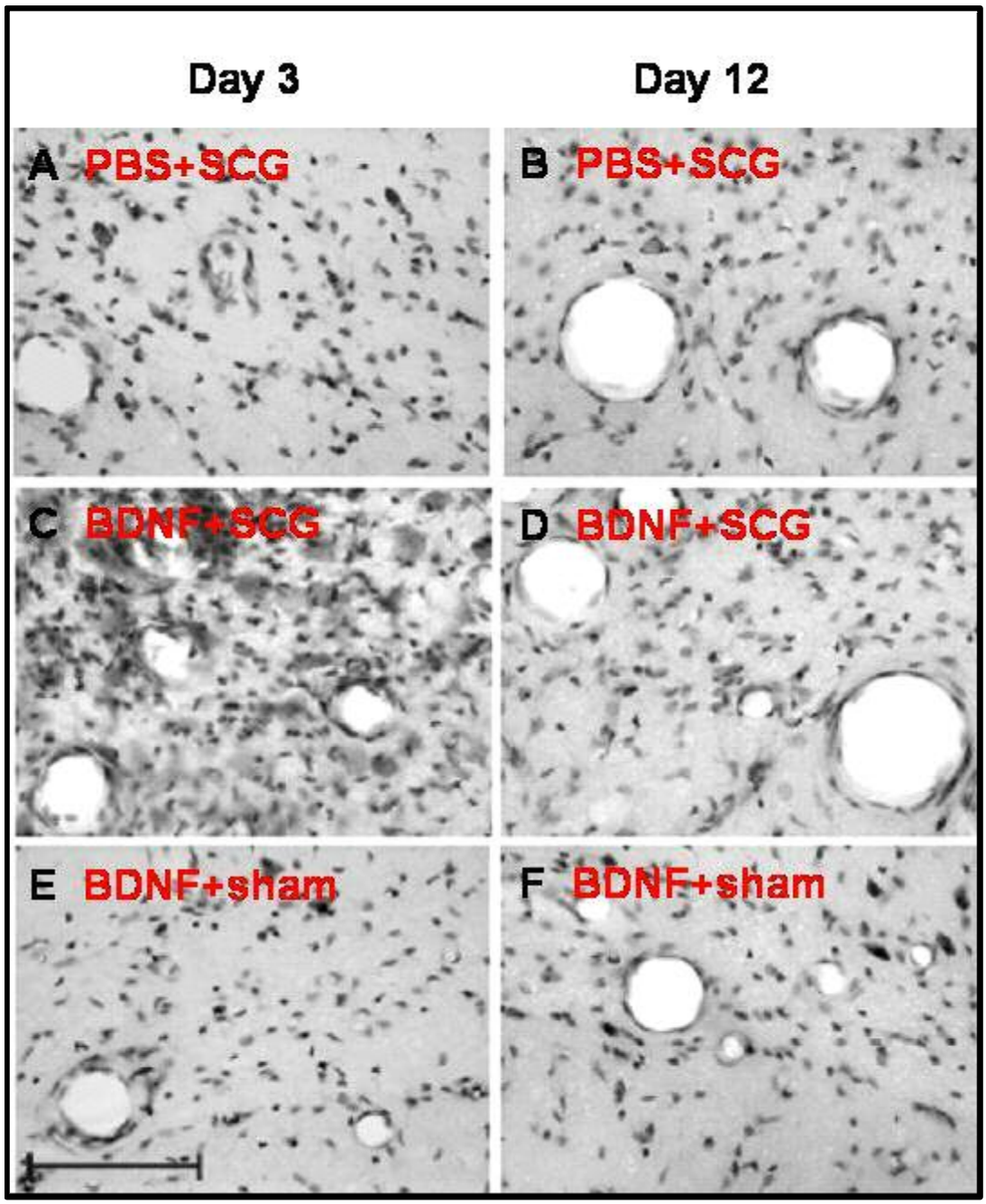

Day 3
Day 12
A PBS+SCG
B PBS+SCG
C BDNF+SCG
D BDNF+SCG
E BDNF+sham
F BDNF+sham

Figure 19

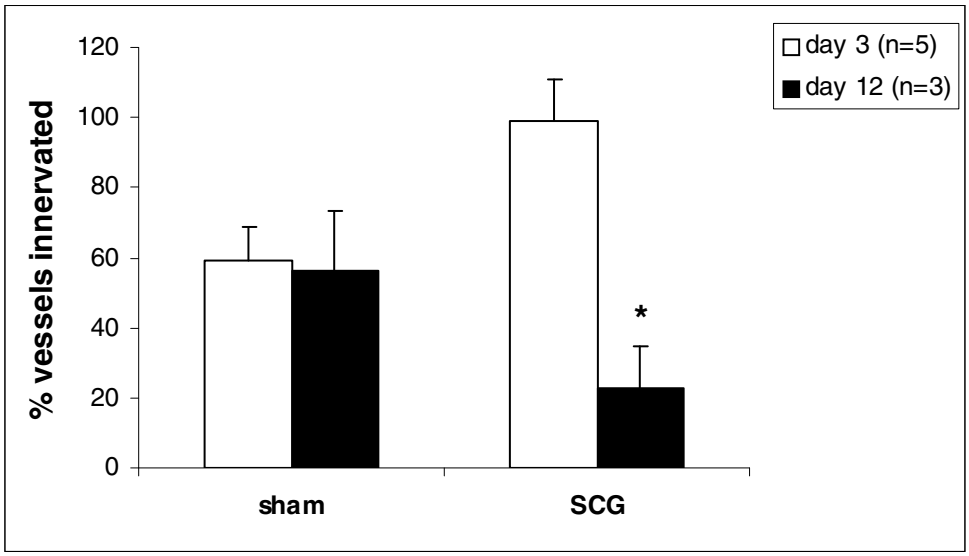

% vessels innervated
120
100
80
60
40
20
0
day 3 (n=5)
day 12 (n=3)
*
sham
SCG

Figure 20

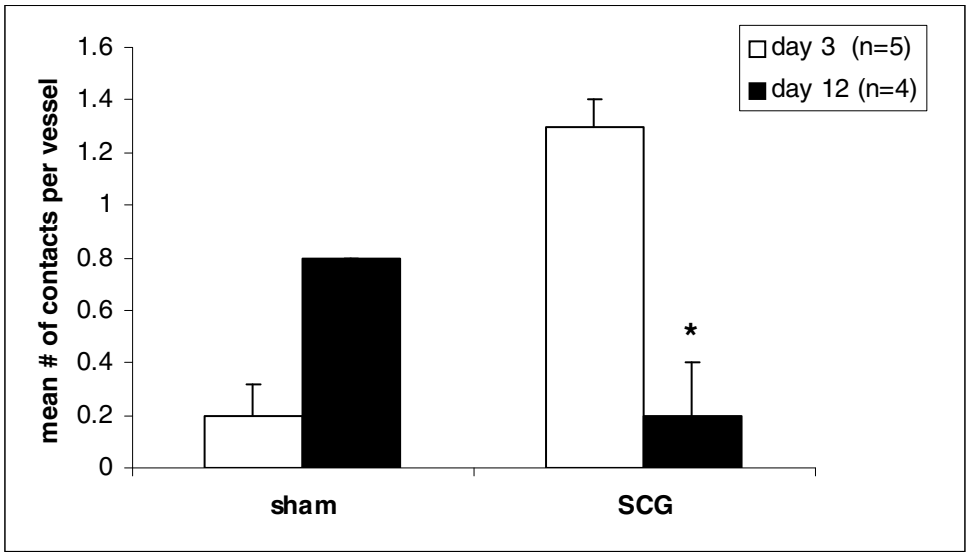

day 3 (n=5)
day 12 (n=4)
mean # of contacts per vessel
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
sham
SCG

Figure 21

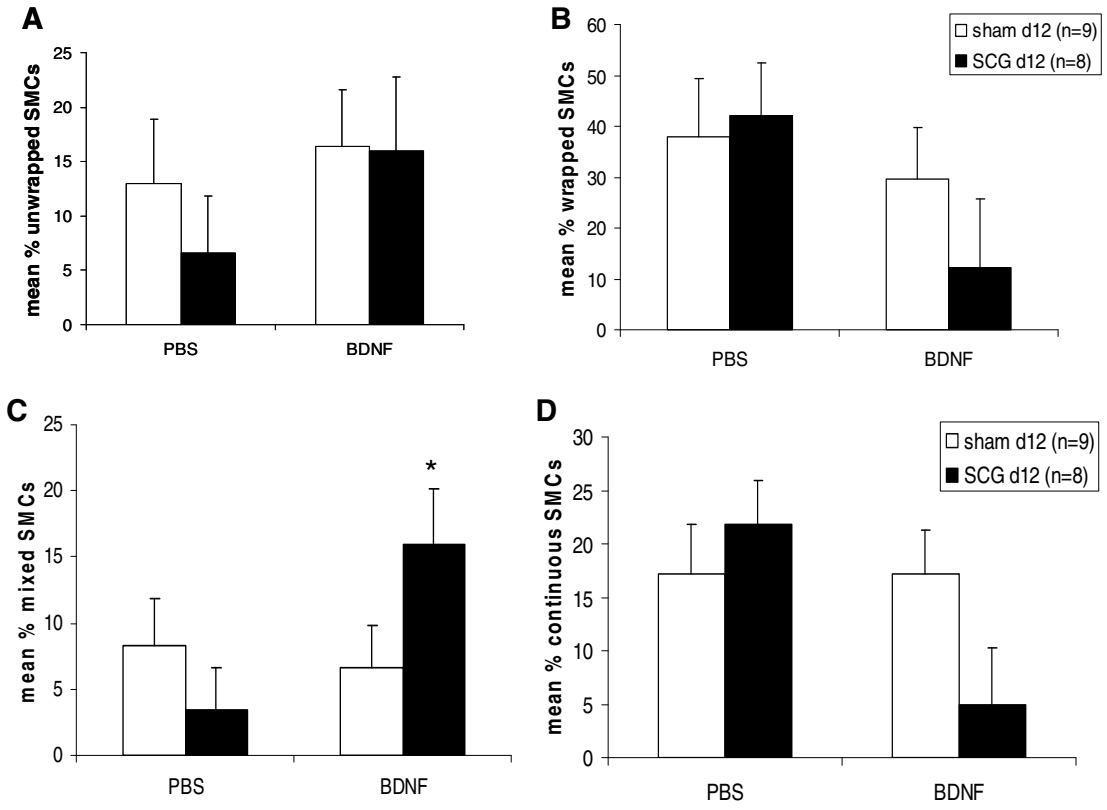

A
mean % unwrapped SMCs
25
20
15
10
5
0
PBS
BDNF
B
mean % wrapped SMCs
60
50
40
30
20
10
0
sham d12 (n=9)
SCG d12 (n=8)
PBS
BDNF
C
mean % mixed SMCs
25
20
15
10
5
0
*
PBS
BDNF
D
mean % continuous SMCs
30
25
20
15
10
5
0
sham d12 (n=9)
SCG d12 (n=8)
PBS
BDNF

Figure 22

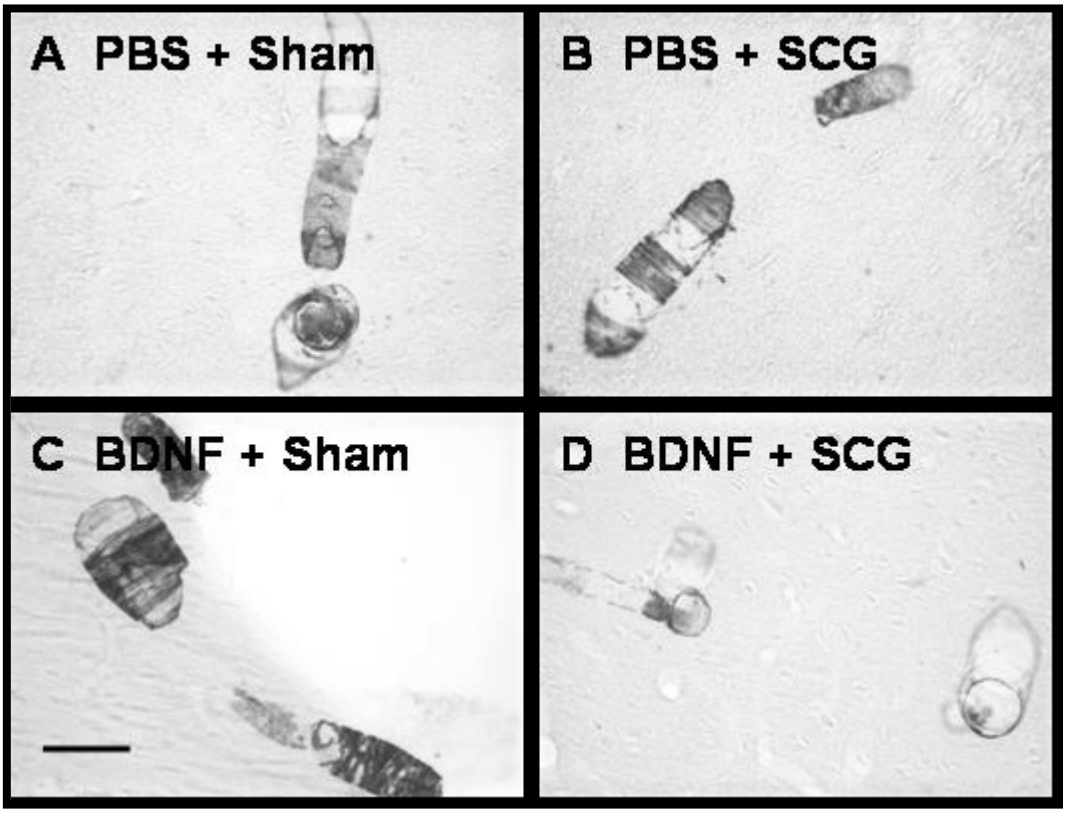


Figure 23

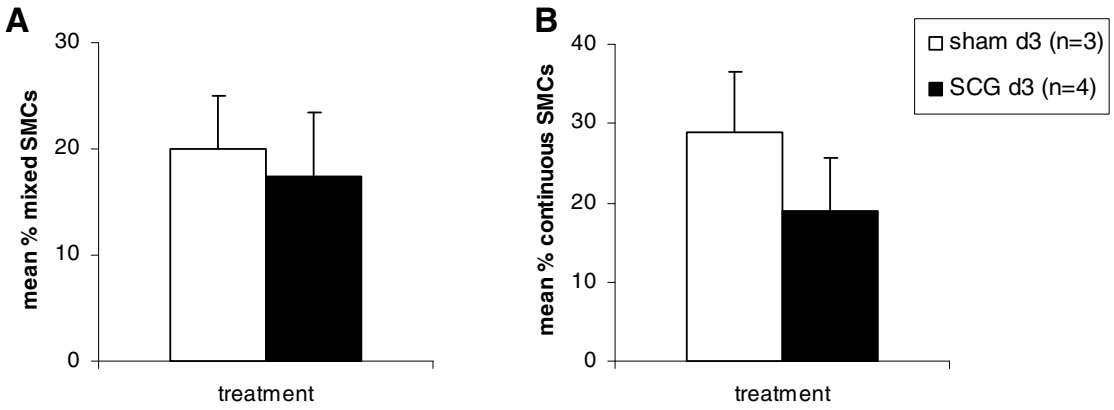

A
mean % mixed SMCs
30
20
10
0
treatment
B
mean % continuous SMCs
40
30
20
10
0
treatment
sham d3 (n=3)
SCG d3 (n=4)

Figure 24

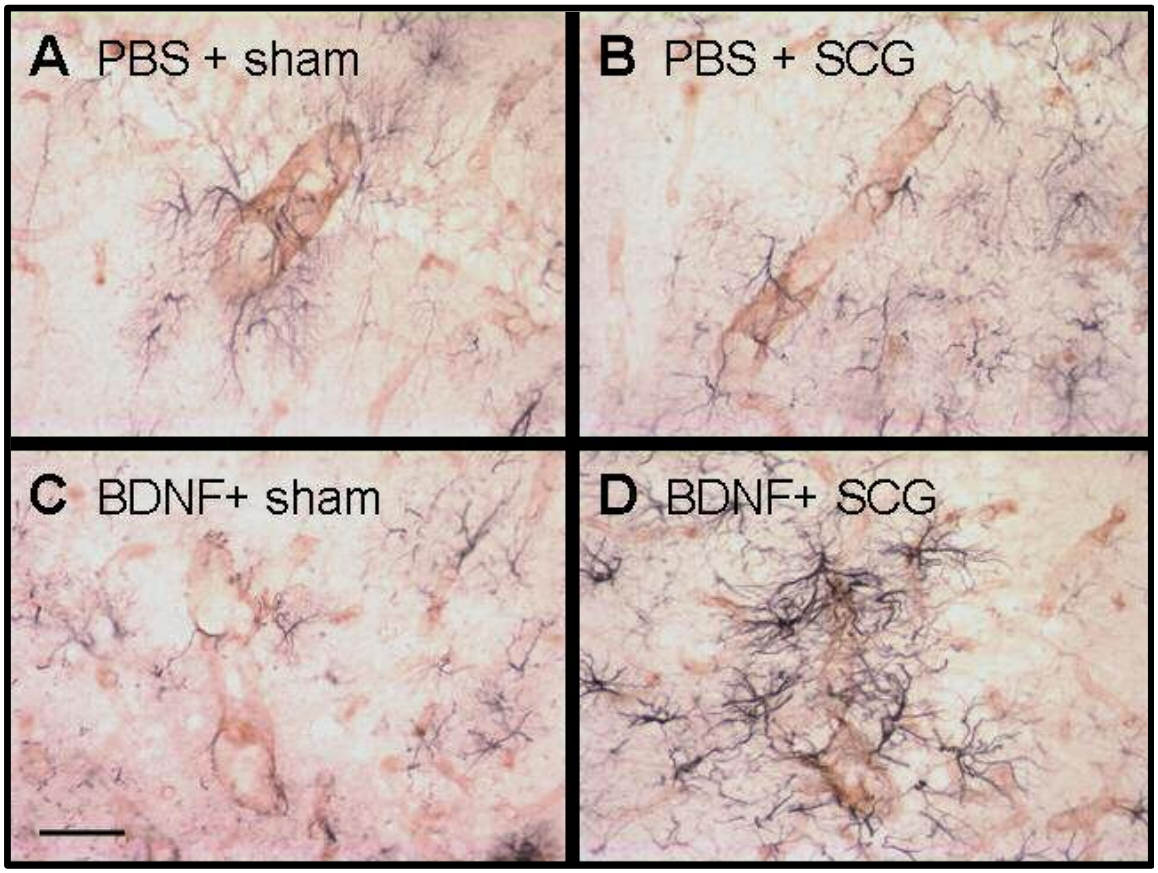


Figure 25

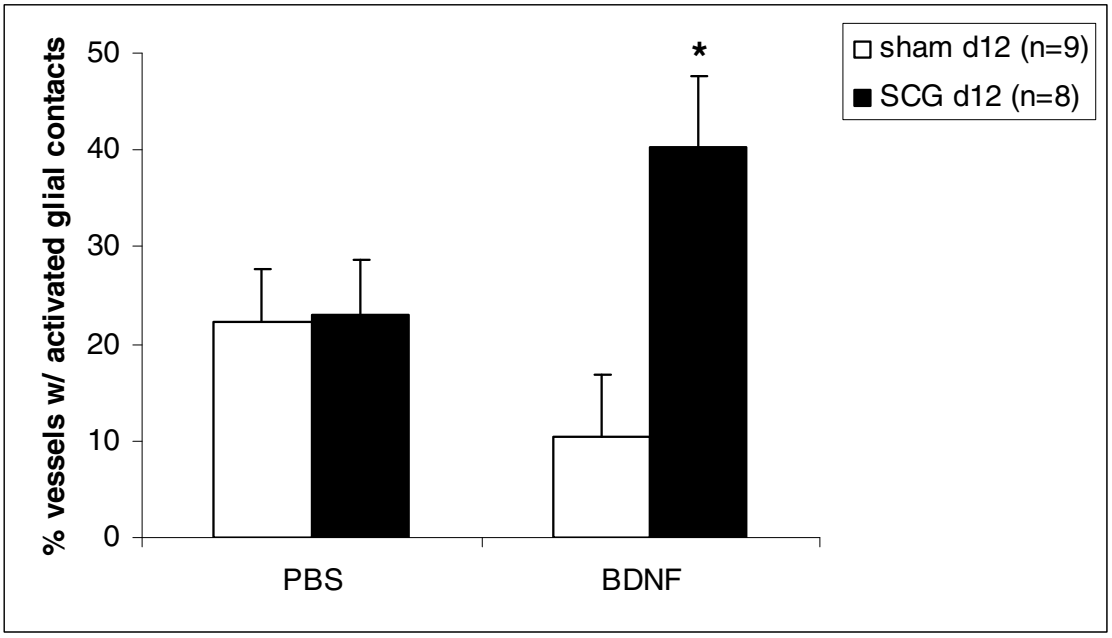

% vessels w/ activated glial contacts
50
40
30
20
10
0
PBS
BDNF
*
sham d12 (n=9)
SCG d12 (n=8)

Figure 26

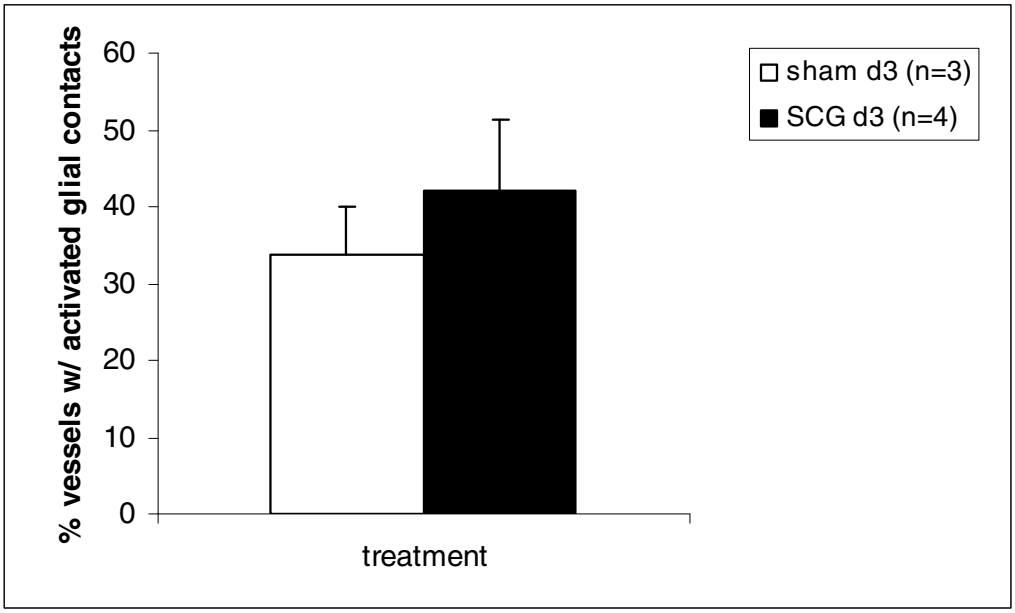

% vessels w/ activated glial contacts
60
50
40
30
20
10
0
treatment
sham d3 (n=3)
SCG d3 (n=4)

Figure 27

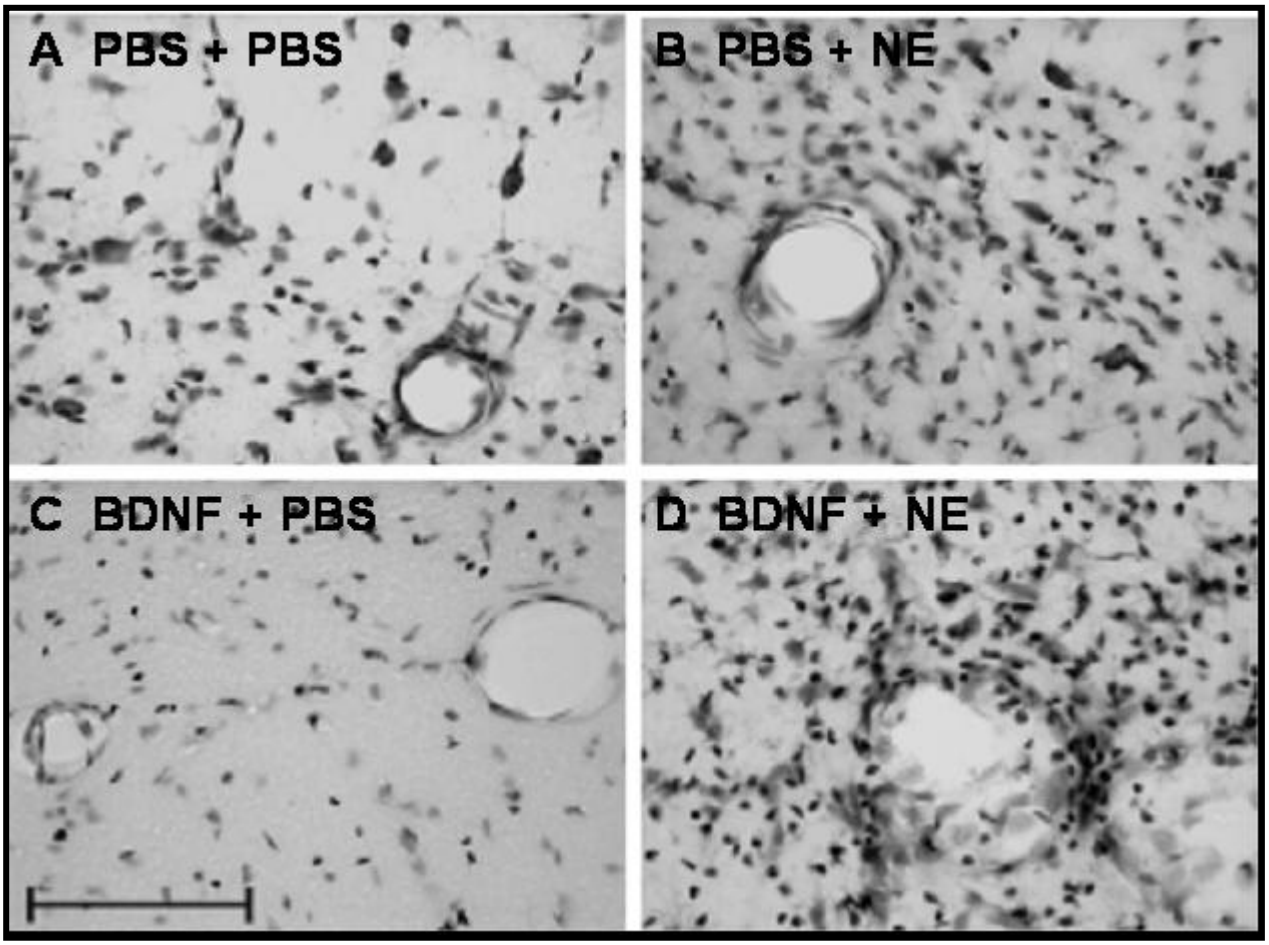


Figure 28

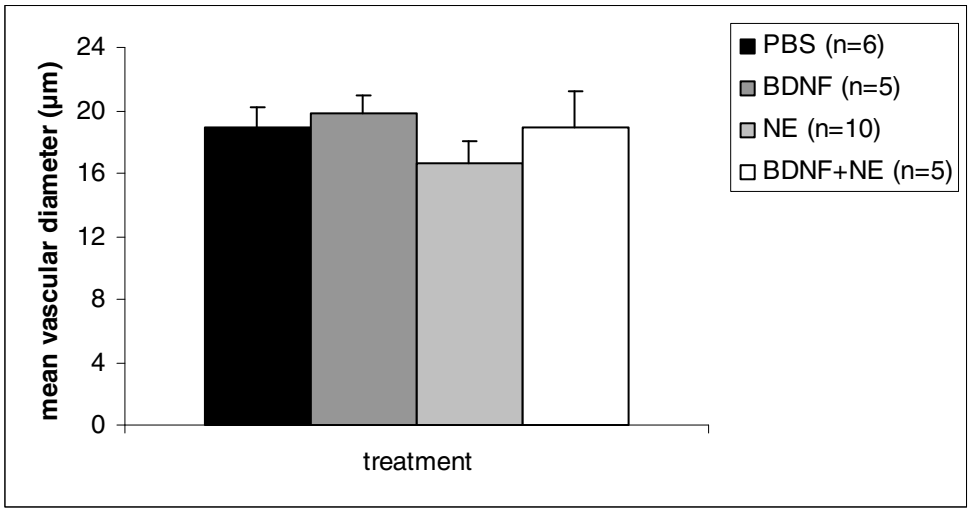

mean vascular diameter (µm)
24
20
16
12
8
4
0
treatment
PBS (n=6)
BDNF (n=5)
NE (n=10)
BDNF+NE (n=5)

Figure 29

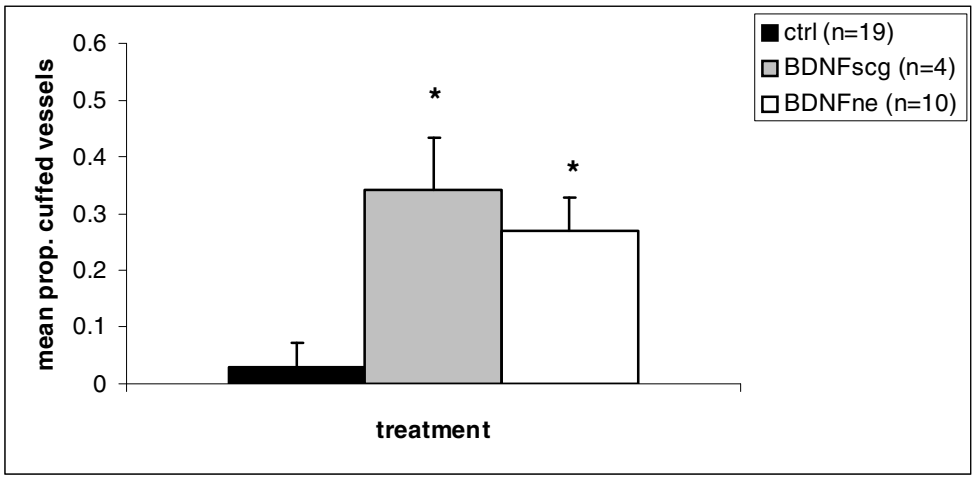

ctrl (n=19)
BDNFscg (n=4)
BDNFne (n=10)
mean prop. cuffed vessels
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
treatment

Figure 30

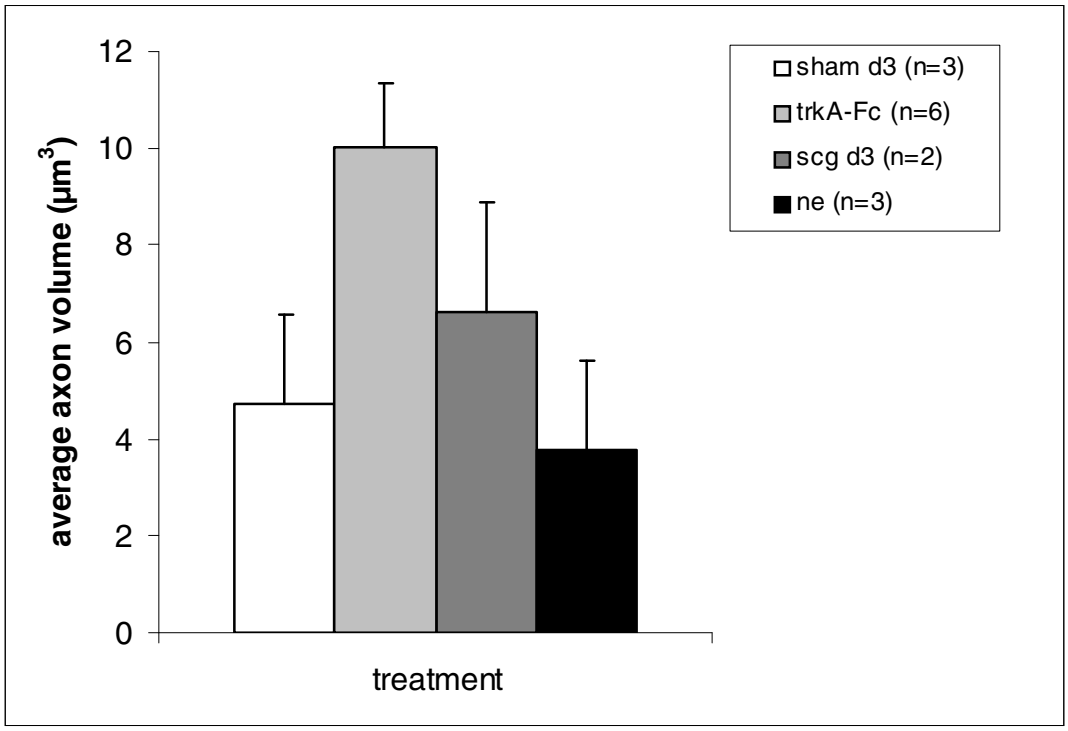

12
10
8
6
4
2
0
average axon volume (µm3)
treatment
sham d3 (n=3)
trkA-Fc (n=6)
scg d3 (n=2)
ne (n=3)

Figure 31

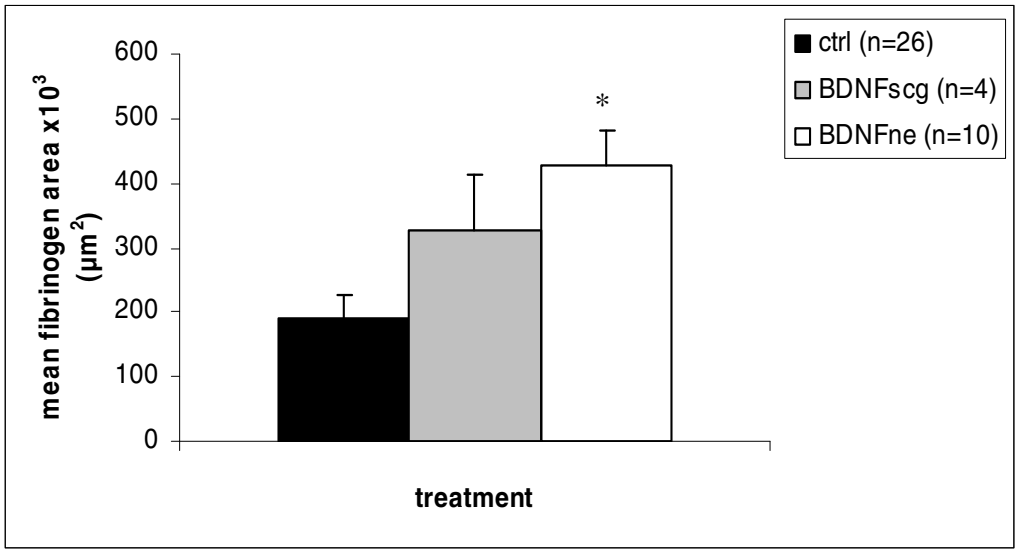

ctrl (n=26)
BDNFscg (n=4)
BDNFne (n=10)
mean fibrinogen area x10^3 (µm^2)
600
500
400
300
200
100
0
*
treatment

Figure 32

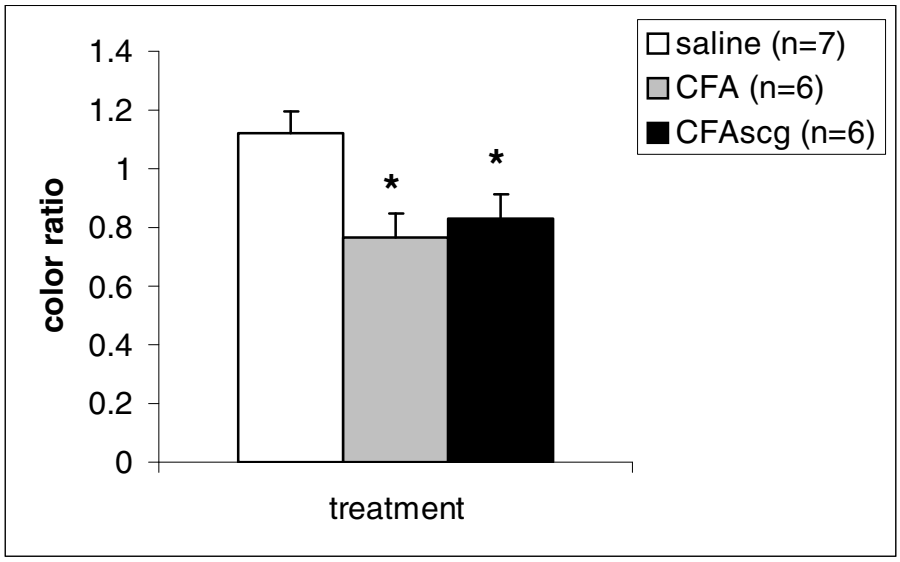

saline (n=7)
CFA (n=6)
CFAscg (n=6)
color ratio
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
treatment

Figure 33

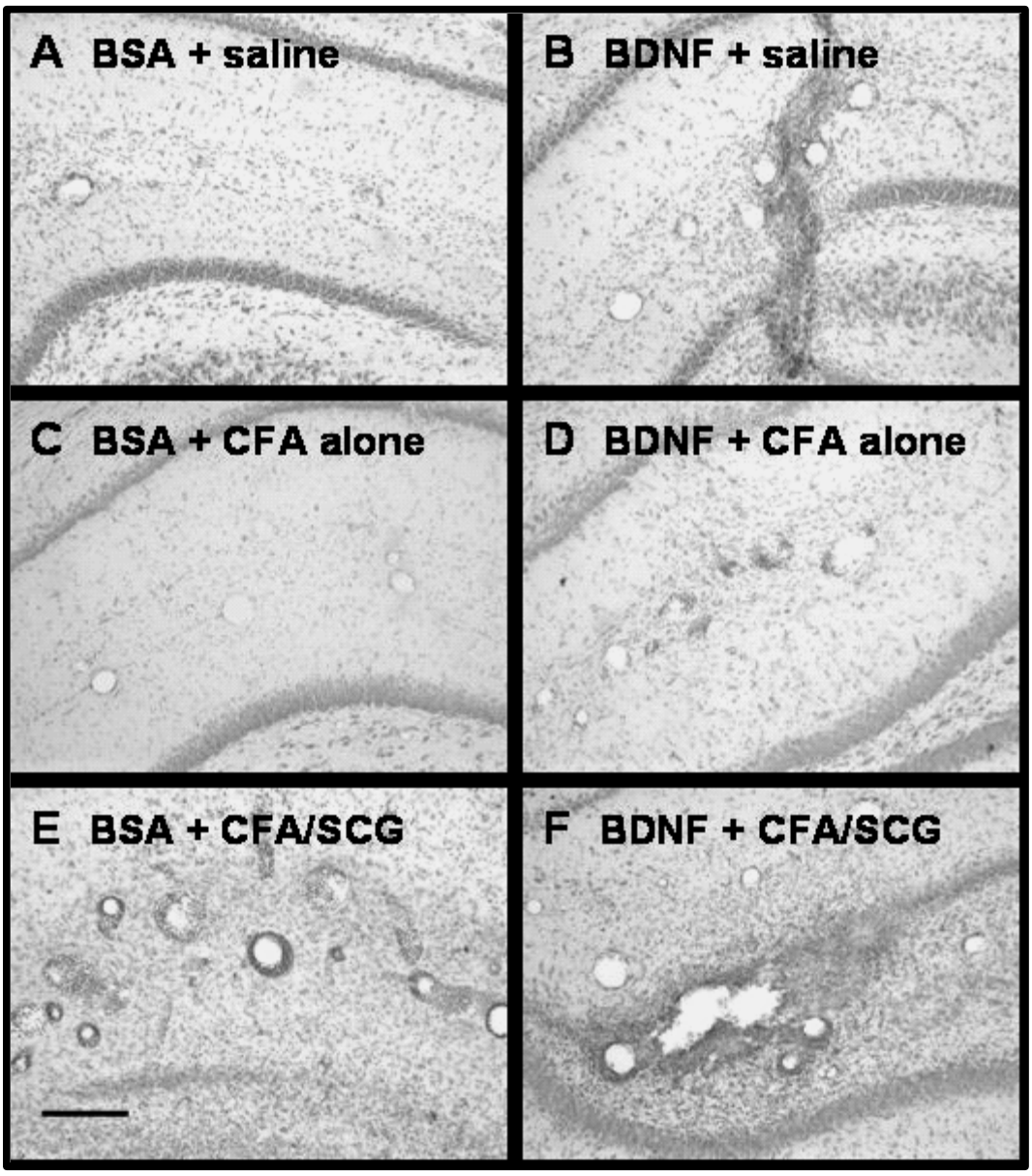

A BSA + saline
B BDNF + saline
C BSA + CFA alone
D BDNF + CFA alone
E BSA + CFA/SCG
F BDNF + CFA/SCG

Figure 34

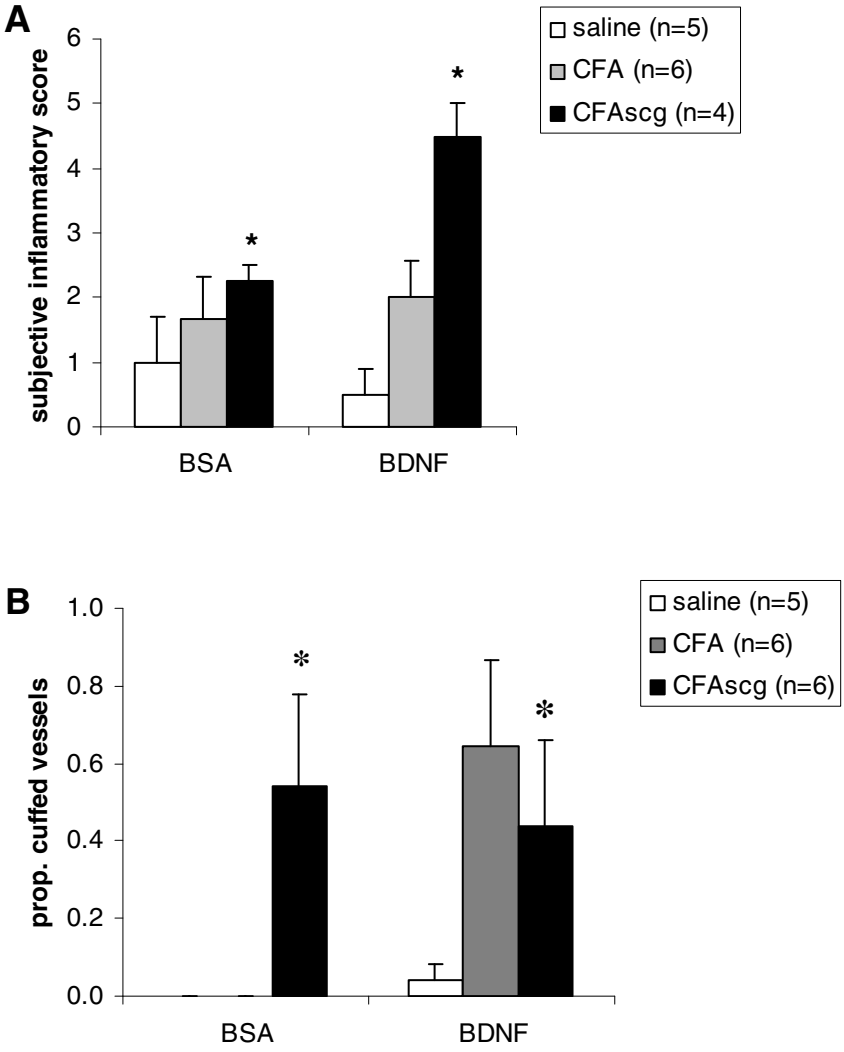

A
subjective inflammatory score
0
1
2
3
4
5
6
*
*
BSA
BDNF
saline (n=5)
CFA (n=6)
CFAscg (n=4)
B
prop. cuffed vessels
0.0
0.2
0.4
0.6
0.8
1.0
*
*
BSA
BDNF
saline (n=5)
CFA (n=6)
CFAscg (n=6)

Figure 35

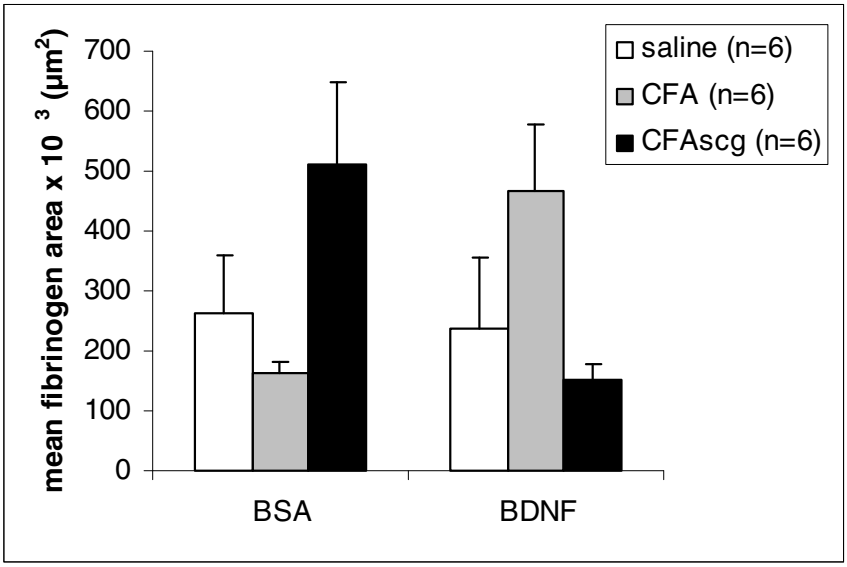
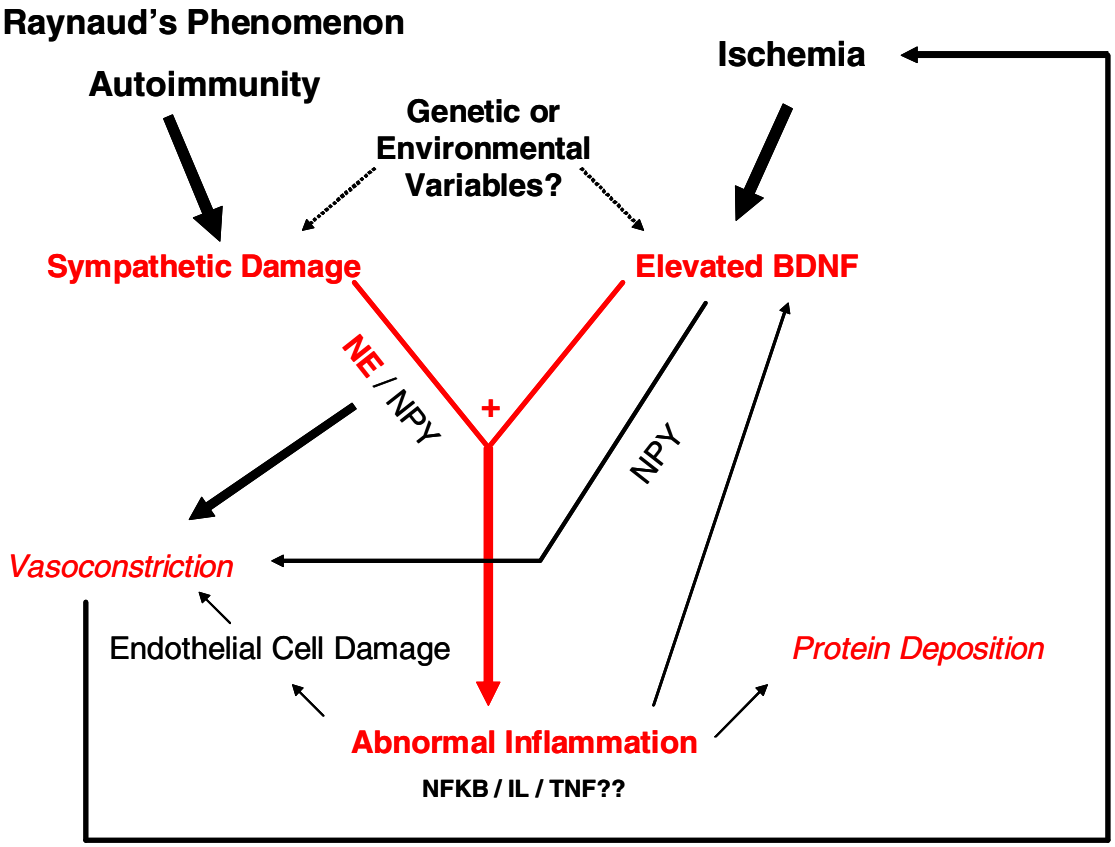

mean fibrinogen area x 10 3 (µm2)
700
600
500
400
300
200
100
0
BSA
BDNF
saline (n=6)
CFA (n=6)
CFAscg (n=6)

Figure 36


Raynaud's Phenomenon
Autoimmunity
Genetic or
Environmental
Variables?
Ischemia
Sympathetic Damage
Elevated BDNF
NE / NPY
+
NPY
Vasoconstriction
Endothelial Cell Damage
Protein Deposition
Abnormal Inflammation
NFKB / IL / TNF??

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